

**Differentiation and maturation of
pluripotent stem cell-derived
cardiomyocytes**

Ljudmila Kolker

Imperial College London
National Heart & Lung Institute

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Abstract

Human pluripotent stem cell-derived cardiomyocytes (hPSC-CMs) may serve as tools in pharmacology for drug screening and safety evaluations and in future cardiac regenerative therapies. However, the use of hPSC-CMs is hampered by their immature foetal-like structural and functional characteristics. This project aimed to evaluate two pro-maturation stimuli to enhance the maturation of hPSC-CMs: administration of triiodothyronine (T3), a hypertrophic stimulus known to be crucial for cardiac development, and overexpression of Bin1(-EGFP), which may be implicated in the formation of t-tubules (TTs). T3-signalling may be suboptimal during *in vitro* culture and TTs, a hallmark of mature ventricular cardiomyocytes, are absent in hPSC-CMs.

To investigate the effect of the pro-maturation stimuli initially cardiomyocytes were derived from the human embryonic stem cell line Shef3 via spontaneous or directed differentiation methods but due to low differentiation efficiency all maturation studies were conducted on iCell-CMs, a commercially produced line of human induced pluripotent stem cell-derived cardiomyocytes. To assess their initial state of maturity and subsequently to evaluate the impact of the pro-maturation stimuli on iCell-CMs the molecular, structural and electrophysiological properties of the cells were extensively characterised using qRT-PCR, immunostaining, bright field and transmission electron microscopy, micro electrode array and the whole-cell patch-clamping technique.

Characterisation of iCell-CMs revealed an overall immature phenotype: iCell-CMs were smaller than adult cardiomyocytes; exhibited relatively low expression of key ion channels (Kir2.1 and Kv4.3), major Ca^{2+} handling components (e.g. RyR2, SERCA2a and CSQ2) and TT-associated proteins (e.g. Bin1 and caveolin 3); showed poor ultrastructural organisation and lacked TTs. However, the sarcoplasmic reticulum (SR) Ca^{2+} contributed to cell contraction similarly to adult cardiomyocytes. Administration of T3 increased the expression of the hypertrophic marker ANP and enhanced the maturation of iCell-CMs as measured by a shift in the expression levels of SERCA2a, Kv4.3 and Cav3.1 closer to the levels of adult heart controls. Overexpression of Bin1(-EGFP) increased the expression of CSQ2, Kir2.1 and Kv4.3 and induced formation of TT-like sarcolemmal invaginations. However, the tubular structures were not aligned to myofibrils and may have been connected to the SR at only few foci. Localisation of L-type Ca^{2+} channels on the induced tubules could not be definitely confirmed but the increased Ca^{2+} current density in transfected cells suggested that the channels may have been recruited to the tubules. Also, overall the expression levels of most analysed genes in T3-treated or Bin1-EGFP-transfected cells remained markedly below adult heart controls including RyR2, CSQ2, Kir.2.1, Kv4.3 and caveolin 3.

In conclusion, both T3-treatment and overexpression of Bin1(-EGFP) promoted the maturation of iCell-CMs to some measure but did not induce a fully adult-like phenotype. Thus the ultimate maturation strategy for hPSC-CMs still remains to be found.

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Declaration of originality

I certify that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been appropriately referenced.

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Abbreviations

αMHC	alpha myosin heavy chain
3-D	three-dimensional
AH	adult human heart
ANP	atrial natriuretic peptide
AP	action potential
βMHC	beta myosin heavy chain
bFGF	basic fibroblast growth factor
Bin1	bridging integrator 1 (muscle specific)
BMDCs	bone marrow-derived adult stem and progenitor cells
BMP4	bone morphogenic protein 4
bpm	beats per minute
Ca^{2+}	calcium
cAMP	3'-5'-cyclic adenosine monophosphate
Cav1.2	alpha 1S pore-forming subunit of voltage-dependent L-type calcium channel
Cav3	caveolin 3
Cav3.1	alpha 1G subunit of voltage-dependent T-type calcium channel
CDI	Cellular Dynamics International
CMO	CellMask Orange
CICR	calcium-induced calcium release
CSCs	cardiac stem/progenitor cells
CSQ2	calsequestrin 2
cTNT	cardiac troponin T type 2
Cx43	connexin 43
D1	deiodinase 1
D2	deiodinase 2
D3	deiodinase 3
EB	embryoid body
ECC	excitation-contraction coupling
ECM	extracellular matrix
EGFP	enhanced green fluorescent protein
FCS	foetal calf serum
FH	foetal human heart
Fig.	figure

GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GATA4	Cardiac-specific zinc-finger transcription factor, early cardiac marker
h	hour
hESC-CMs	human embryonic stem cell-derived cardiomyocytes
hESCs	human embryonic stem cells
hiPSCs	human induced pluripotent stem cells
hPSCs	human pluripotent stem cells
iCell-CMs	iCell cardiomyocytes
IF	immunofluorescence microscopy
IMEFs	mouse embryonic fibroblasts mitotically-inactivated with mitomycin C
IP3R2	Inositol 1,4,5-trisphosphate receptor type 2
iPSC-CMs	induced pluripotent stem cell-derived cardiomyocytes
iPSCs	induced pluripotent stem cells
JP2	junctional protein 2
Kir2.1	inward rectifier potassium channel 2
Kv4.3	voltage-gated potassium channel subunit Kv4.3
M	mitochondria
MBD	Myc-binding domain
MEA	micro electrode array
MEF-conditioned medium	mouse embryonic fibroblast conditioned medium
MEFs	mouse embryonic fibroblasts
MHC	myosin heavy chain
MI	myocardial infarction
min	minutes
ml	millilitres
MLC2V	myosin light chain 2v
Myf	myofibrils
Myfil	myofilaments
N	nucleus
NCX1	sodium/calcium exchanger
ng	nanogram
Nkx2.5	homeobox-containing transcription factor
P	passage
PCR	polymerase chain reaction
PI	phosphoinositide-binding domain

PLN	phospholamban
PSCs	Pluripotent stem cells
qRT-PCR	real-time quantitative reverse transcription polymerase chain reaction
RT	room temperature
RyR2	ryanodine receptor 2, cardiac-type
s	seconds
SERCA2a	sarco/endoplasmic reticulum calcium ATPase
SH3	Src homology 3
Shf3-CMs	Shf3-derived cardiomyocytes
SR	sarcoplasmic reticulum
t	time
T3	triiodothyronine
T4	tetraiodothyronine
Tcap	titin-cap, telethonin
TEM	transmission electron microscopy
TGFβ	transforming growth factor beta
THRα	thyroid hormone receptor alpha
THRβ	thyroid hormone receptor beta
TRITC	tetramethylrhodamine-isothiocyanate
TTs	transverse tubules, t-tubules

Chapter 1: General introduction

1.1. **Introduction to human pluripotent stem cells:** **human embryonic and human induced pluripotent stem** **cells**

Human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), two pluripotent stem cell types (PSCs), are of great interest in the biomedical field. Both cell types can be propagated in culture almost indefinitely and give rise to any cell type in the body (except placenta tissue) including cardiomyocytes (Thomson, 1998; Takahashi et al., 2007).

Cardiomyocytes derived from hESCs and hiPSCs (hESC-CMs and hiPSC-CMs) are advocated as valuable tools for a variety of applications. They could represent *in vitro* cardiac model systems for disease studies, drug discovery and drug safety evaluation and can also be used as cell transplants in cardiac regenerative medicine (Harding et al., 2007; Robinton and Daley, 2012). However, the use of hESCs and their derivatives is ethically controversial because the derivation of hESCs from early human *ex-utero* embryos requires the destruction of the embryos. Although new derivation methods allow preservation of the embryo integrity (Klimanskaya et al., 2006) many of the well-characterised hESC lines [e.g. H1, H7 and H9 (Thomson, 1998)], which are used in stem cell research today, were isolated from embryos, which were subsequently destroyed.

hiPSCs do not pose the ethical issues as hESCs. They are generated by conversion of somatic cells into pluripotent cells via the expression of four “stemness” factors Oct4, Sox2, cMyc and Klf (Takahashi et al., 2007). Furthermore, the iPSC technology allows the generation of disease- and patient-specific hiPSC-CMs, which opens opportunities for “personalised” regenerative medicine and modelling of cardiac genetic diseases (Robinton and Daley, 2012).

A variety of methods for the *in vitro* differentiation of hPSCs into functional cardiomyocytes has been already established for various stem cell lines. However, compared to adult cardiomyocytes both hESC-CMs and hiPSC-CMs show primarily an immature phenotype

with regard to their structural and functional features (Snir et al., 2003; Germanguz et al., 2011). There are concerns that the immaturity of hPSC-CMs may affect their therapeutic potential and applications in research and pharmacology. Thus there is a focus in the cardiac stem cell research field on thorough characterisation of the structural and electrophysiological properties of hPSC-CMs and the development of strategies to promote their maturation.

1.1.1. Definition of pluripotent stem cells

Pluripotent stem cells are defined by two features: 1) they are able to proliferate sustainably in culture and 2) they are capable of differentiating into cells of all three embryonic germ layers (ectoderm, mesoderm and endoderm) except the extra-embryonic trophectoderm (gives rise to the placenta) (Thomson, 1998; Evans and Kaufman, 1981; Chambers and Smith, 2004).

PSCs typically express the transcriptional factors OCT4, SOX2 and Nanog, which are thought to be the master regulators of pluripotency. They cooperatively activate the expression of genes which are necessary to maintain the pluripotent state, and repress genes which initiate differentiation. These factors also autoregulate their own promoters promoting their own expression and sustaining the pluripotent state (Chambers and Smith, 2004; Niwa, 2007).

A functional gold standard to assess the pluripotency of stem cells is to evaluate their capacity for teratoma formation *in vivo*. When injected into immunodeficient mice truly pluripotent stem cells usually spontaneously form tumor-like masses containing tissues from each of the three germ layers (Przyborski, 2005; Gutierrez-Aranda et al., 2010).

hESCs and hiPSCs are characterised as pluripotent. In contrast, many of the adult stem cell types such as the bone-marrow-derived haemopoietic stem cells and the endogenous cardiac stem/progenitor cells are referred to as multipotent. That means they can differentiate into multiple but lineage restricted cell types.

1.1.2. Characterisation of hESCs

Embryonic stem cells are isolated from the inner cells mass of early stage embryos (blastocysts). The first isolation of mouse ESCs was reported in 1981 (Evans and Kaufman, 1981; Martin, 1981) and the first isolation of human ESCs from embryos produced by *in vitro* fertilisation almost twenty years later in 1998 (Thomson, 1998).

Characteristic features of all hESCs include a high nucleus to cytoplasm morphology, high level of telomerase activity, which prevents telomere shortening in these highly proliferative cells, a high expression of cell surface markers such as the stage-specific embryonic antigen SSEA4, the glycoproteins TRA-1-60 and 81, and the transcription factors Oct4, Nanog and Sox2 (Thomson, 1998; Reubinoff et al., 2000). However, comparison of various hESC lines exhibited that there can be significant variations in the gene expression profiles (Skottman et al., 2005) and differentiation propensities between the lines (Osafune et al., 2008; Allegrucci and Young, 2007).

1.1.3. Characterisation of hiPSCs

In 2006 Takahashi and Yamanaka (Takahashi and Yamanaka, 2006) reported the successful generation of the so-called induced pluripotent stem cells (iPSCs) from adult mouse fibroblast cells through the ectopic expression of only four transcription factors (“stemness factors”), Oct3/4, Sox2, KLf4 and c-Myc. The generated iPSCs display the same key features as ESCs: self-renewal and the ability to form cells of all three embryonic germ layers (see above). Thus it was shown that it is possible to convert (“reprogramme”) fully differentiated somatic cells into pluripotent stem cells by overexpression of stemness genes. Shortly after the derivation of mouse iPSCs a similar method was used to generate the first human iPSCs (Takahashi et al., 2007).

Later studies showed that not exclusively the expression of Oct3/4, Sox2, KLf4 and c-Myc but also the introduction of alternative combinations using only three or even two transcription factors (e.g. Oct4 and Sox2 with addition of the histone deacetylase inhibitor valproic acid) can reprogramme somatic cells into PSCs (Huangfu et al., 2008; Ichida et al., 2009).

In the first wave of reprogramming experiments iPSCs were generated using retroviruses to express the stemness factors. However, the use of retroviruses, which integrate permanently into the host genome, would highly limit the use of hiPSCs in clinical application because of the risk of insertional mutations and tumour formation due to residual transgene expression (Patel and Yang, 2010). Since the first derivation of iPSCs a variety of alternative reprogramming methods has been developed, which diminish or even abolish the risk of integration. These include the use of non-integrating adenoviral and plasmid vectors, excisable transposons and DNA-free approaches such as transfection of somatic cells with microRNAs, which are implicated in the control of pluripotency-related genes (Patel and Yang, 2010; Miyoshi et al., 2011).

1.1.4. Comparison of hESCs with hiPSCs

Comparison of hiPSCs with hESCs reveals similarity in morphology, cell surface marker expression and the ability of teratoma formation *in vivo* (Thomson, 1998). However, studies analysing the gene expression or DNA methylation pattern revealed that hiPSCs and hESCs lines are similar but not identical since numerous genetic (Chin et al., 2009) and epigenetic differences (Lister et al., 2011) could be identified. These differences may partially be attributed to aberrant methylation during the reprogramming process and partially to incomplete reprogramming. For example, it is reported that iPSCs retain some type of somatic (Lister et al., 2011) or epigenetic memory (Kim et al., 2010) displaying residual gene expression (Ghosh et al., 2010) or DNA methylation pattern (Kim et al., 2010; Polo et al., 2010; Lister et al., 2011) characteristic to the cell of origin. This can affect gene expression and the differentiation propensity of hiPSCs towards a specific lineage.

In light of the significant variability between hESCs and hiPSCs as well as between hESC and hiPSC lines themselves Bock and colleagues generated genome-wide reference “scorecards” to assist researchers to evaluate the quality and to predict the differentiation potential of individual hESC and hiPSC lines (Bock et al., 2011).

1.1.5. *In vitro* culture of hPSCs

The initial and most widely used method to culture hPSCs is the maintenance on mitotically-arrested feeder layers, typically mouse embryonic fibroblasts (MEFs), in medium containing serum or serum-replacement (Thomson, 1998; Reubinoff et al., 2000). MEFs serve as a basal layer and promote the survival and undifferentiated growth of hPSCs by secreting numerous soluble factors (Diecke et al., 2008; Villa-Diaz et al., 2008). These include adhesion molecules, extracellular matrix (ECM) components (e.g. type IV collagen, fibronectin and laminin) and various growth factors such as basic fibroblast growth factor (bFGF), transforming growth factor beta 1 and 2 (TGF β 1 and TGF β 2), activins and bone morphogenic proteins (BMPs) (Diecke et al., 2008; Villa-Diaz et al., 2008).

bFGF and members of the TGF β family such as activin A and TGF β 1 (activate TGF β /NodalActivin signalling) were shown to promote self-renewal and maintenance of the pluripotent state of hPSCs (Eiselleova et al., 2009; James et al., 2005; Vallier et al., 2005; Beattie et al., 2005). However, members of the TGF β family have a paradoxical role and can maintain the pluripotent state and induce differentiation, respectively (Schuldiner et al., 2000; Laflamme et al., 2007). Their action likely depends on certain conditions such as the presence and concentration of different interaction partners.

However, the culture on feeders and the use of animal-derived components such as serum and serum-replacement are associated with several disadvantages and would limit the use of hPSCs in clinical applications: a) the factors produced by MEFs are not fully defined; b) the performance of MEFs vary batch to batch, c) there are difficulties of scaling up and d) there is a risk of cross-species pathogen contaminations.

Some of these limitations have been overcome by the development of new feeder-independent culture conditions. Xu and colleagues introduced a culture system, in which hPSCs are maintained on Matrigel (a gelatinous protein matrix derived from mouse tumour cells) in MEF-conditioned medium (containing serum-replacement) (Xu et al., 2001). This feeder-free culture method facilitates large-scale production of hPSCs but still requires feeders and animal-derived serum-replacement for the production of MEF-conditioned

medium. Also, although Matrigel is readily available to use, it is not strictly chemically defined, exhibits lot to lot variations and may also introduce xenogeneic contaminations.

Recently, a number of chemically defined, serum- and xeno-free (feeder-free) media were introduced which were shown to support self-renew of hPSCs in conjunction with Matrigel as substrate [e.g. mTeSR, NutriStem and StemPro (Ludwig et al., 2006a; Wu et al., 2012; Wang et al., 2007)]. These media comprise a bovine albumin source and specific cytokines and growth factors such as bFGF, activin A and TGF β 1, which were identified to support undifferentiated proliferation of hPSCs *in vitro*.

A further step to completely xeno-free and fully defined culture conditions is the recent development of alternative synthetic substrates, which show little batch-to-batch variation, are defined, stable and were shown to be suitable for prolonged culture of undifferentiated hPSCs (Brafman et al., 2010; Mei et al., 2010; Melkounian et al., 2010).

1.2. Applications of hPSCs

1.2.1. Application of pluripotent and adult stem cells in regenerative medicine

Myocardial infarction (MI; heart attack) and chronic heart disease causing heart failure is associated with a severe loss in cardiomyocytes, which the normal regenerative capacity of the human heart cannot compensate. Although there is growing evidence that the heart has the potential to regenerate itself via endogenous, multipotent cardiac stem/progenitor cells (CSCs) (Torella et al., 2007) the regenerative potential of the heart is very limited. A study analysing the integration of carbon 14 (generated during nuclear weapon testing in the 1960s) into the DNA of cardiomyocytes revealed that the normal human heart of a 25 year old regenerates at a rate of only 1 % per year (Bergmann et al., 2009). Heart transplantation is often the last resort for end stage heart disease patients but is hampered by the severe shortage of donor organs (Gepstein, 2002).

Recently, cell transplantation (cell-therapy) has been proposed as a potential approach for the regeneration of the damaged heart. Due to their self-renew ability and great potential to differentiate into cardiomyocytes hESCs and hiPSCs are considered as candidates for cardiac cell-therapy. Other candidate cell types are skeletal myoblasts, various bone marrow-derived adult stem and progenitor cells (BMDCs; haematopoietic and mononuclear cells, endothelial progenitor cells and mesenchymal stem cells) and heart-resident CSCs (Segers and Lee, 2008).

An overview about the suitability of the different candidate cell types for cardiac cell-therapy and recent experiences of the use of various cell types in animal models and clinical studies is given below.

1.2.2. Bone marrow-derived cells

Numerous clinical trials have already evaluated the capacity of different BMDC populations for cardiac cell-therapy for patients either with acute myocardial infarction or chronic ischemic heart disease and gave mixed results. Some studies showed no effect whereas others demonstrated that transplantation of BMDCs had a modest beneficial effect on the damaged heart as manifested by improvement in tissue perfusion, tissue viability and/or cardiac left ventricular function (Abdel-Latif et al., 2007; Janssens, 2010).

It is widely believed that the beneficial effects of BMDCs on the diseased myocardium are predominantly due to paracrine effects rather than direct differentiation of the transplanted cells into cardiac myocytes. BMDCs can secrete a variety of growth factors and cytokines, which may promote myocyte survival and angiogenesis, decrease the postinfarction inflammation and fibrosis and possibly also induce endogenous regeneration by activation of CSCs (Gnecchi et al., 2008).

The cardiomyogenic potential of BMDCs is generally very limited *in vivo* (Badorff et al., 2003; Murry et al., 2004; Segers and Lee, 2008). However, a subset of BMDCs such as the multipotent MSCs was shown to differentiate into cardiomyocytes under specific conditions *in vitro* and *in vivo* (Caplan and Dennis, 2006).

1.2.3. Heart-resident cardiac stem/progenitor cells

Heart-resident CSCs can differentiate into cardiomyocytes and other cell types found in the myocardium including endothelial and vascular smooth muscle cells but do not form teratomas in immunodeficient mice (Chong et al., 2011; Bollini et al., 2011). Various pools of CSCs have been identified in the adult heart and classified based on their properties and stem cell surface marker expression: e.g. c-kit⁺, Sca-1⁺, Islet 1⁺ and SSEA-1⁺ and cardiospheres (Bollini et al., 2011). Cardiospheres are clusters, which arise spontaneously from *in vitro* cultures of biopsied heart tissue and contain proliferative cardiac stem cells expressing stem and endothelial progenitor markers (e.g. c-kit, Sca-1 CD31, and CD34) (Messina et al., 2004; Bollini et al., 2011).

Therapeutic approaches using CSCs for cell-therapy focus either on transplantation of autologous CSCs, which were isolated from patient-specific biopsies and expanded *ex vivo* beforehand or *in situ* stimulation of heart resident CSCs via growth factor administration or paracrine factors secreted by CSC-transplants.

Initial two-year results of a pioneering clinical trial with autologous CSCs (c-kit⁺ CSCs) are encouraging [Cardiac Stem cell Infusion in Patients with Ischemic Cardiomyopathy (SCIPIO); (Bolli et al., 2011)]. The administration of autologous CSCs to patients with LV dysfunction caused by MI resulted in substantial and sustained improvement of LV function which was accompanied by a reduction in infarct size and an increase in LV viable mass (Bolli et al., 2011; Chugh et al., 2012). However, because only a relatively low number of CSCs were administered, the beneficial effects on the myocardium are probably associated with indirect paracrine effects of the CSCs, which may promote survival and recovery of resident cardiac cells, rather than direct participation of the injected CSCs to the production of myocytes (Ellison et al., 2011; Ellison et al., 2012).

Furthermore, pre-clinical studies focusing on stimulation of endogenous CSC proliferation *in situ* revealed that the intracoronary injection of allogeneic CSCs into pigs after myocardial infarction (a clinically relevant MI model) lead to a significant activation of endogenous CSCs via paracrine stimulation. This resulted in enhanced myocardial cell survival, increased new

cardiomyocyte and capillary formation and improved cardiac function (Ellison et al., 2012). These results are promising and demonstrate feasibility and safety of CSCs cell-therapy for the diseased heart.

In addition, it was shown in the pig model that endogenous CSCs can also be stimulated by paracrine growth factors such as insulin-like growth factor (IGF-1) and hepatocyte growth factor (HGF) (Ellison et al., 2011). These growth factors are known to be secreted by transplanted cells and to be involved in their regenerative paracrine effect. However, the newly formed myocytes after growth factor administration show slow and heterogeneous maturation so that despite the regeneration of the cardiomyocyte numbers there is no prominent increase in cardiac function.

1.2.4. hESCs

hESCs can be expanded *in vitro* and have great potential to differentiate into cardiomyocytes. A variety of differentiation methods to generate hESC-CMs *in vitro* is described in the following section (see section 1.3.2.).

So far, the potential of hESC-CMs as therapeutic cell transplants has been evaluated in different studies using mainly rodent models of myocardial infarction. Most of these studies revealed that transplanted hESC-CMs can survive for at least three months in infarcted hearts and can significantly improve cardiac function and structure at least in the short-term (Laflamme et al., 2007; van Laake et al., 2007; Cai et al., 2007; Caspi et al., 2007a; Singla et al., 2007). However, whether the reconstitutive effects of transplanted hESC-CMs are associated with direct contribution of the engrafted hESC-CMs to heart contractility is not completely clear. On the one hand it was observed that hESC-CMs engrafted in the injured heart of mice couple predominately with each other rather than with the host tissue and are isolated from the host myocardium by fibrotic tissue, which could impair electrophysiological coupling (van Laake et al., 2007; van Laake et al., 2010). Furthermore, studies by van Laake and colleagues showed that no significant difference could be observed between hESC-CMs transplant and control groups in the long-term in models of acute myocardial infarction (van Laake et al., 2007; van Laake et al., 2008). Thus the investigators concluded that the short-

term beneficial effects of hESC-CMs may be driven by a paracrine mechanism. On the other hand several studies provided evidence for electromechanical integration of the grafted hESC-CMs by demonstrating gap junction formation between the transplanted cells and the host myocardium (Caspi et al., 2007a; Kehat et al., 2004). Direct evidence for electromechanical coupling was provided recently by Shiba et al., who generated hESC-CMs which stably express a fluorescent calcium sensor (Shiba et al., 2012). By monitoring epicardial fluorescent transients *in vivo* the research group demonstrated that hESC-CMs engrafted into injured myocardium of immunosuppressed guinea pigs, which show a slower heart rate than mice and rats [200–250 beats per minute (bpm)], could not only beat synchronically with the host tissue but also protect it from induced arrhythmias. However, the authors noted that graft integration into the host tissue was incomplete and needs to be improved. The poor graft integration may be due to biological differences between the species. The heart rate of guinea pigs (200-250 bpm) is still approximately two to four times higher than the spontaneous beating rate of hESC-CMs (50-150 bpm).

Human clinical trials with hESC-CMs for myocardial cell-therapy are hampered by various issues. Apart from the ethical debate these include the risk of teratomas formation by undifferentiated hESCs and the need for life-long immunosuppression to prevent graft rejection by the host immune system. However, advances have been made to develop strategies for hESC-CMs purification [e.g. Percoll gradient separation (Xu et al., 2002) and genetic selection of hESC-CMs (Huber et al., 2007)] to avoid teratomas formation and immune rejection may be avoided by the generation of HLA(human leukocyte antigen)-matched hESC-lines.

1.2.5. hiPSCs

hiPSCs are generally similar to hESCs. They can be expanded and differentiated into cardiomyocytes *in vitro*. Also, comparison of hESC-CMs and hiPSC-CMs showed that these cells are overall similar in their structural and functional properties (see section 1.5.1.).

The use of hiPSCs provides several advantages over hESCs (Alcon et al., 2012). hiPSCs are devoid of the ethical issues of hESCs since they can be derived for example from skin

biopsies, which are easily obtainable; patient-specific autologous hiPSCs can be produced, which could be immune-tolerated. However, the immunogenicity of autologous hiPSCs-CMs should be evaluated before implantation because it was reported that even autologous hiPSC-derivates can provoke an immune response (Zhao et al., 2011). Also, it has to be considered that autologous cells may show the same genetic abnormalities (e.g. genetic arrhythmia) as the donor patient thus autologous cells may not always be suitable for cell-therapy. Furthermore, it was reported that some hiPSC lines can display genetic and epigenetic differences compared to hESCs (see section 1.1.4.) and it is not known so far how this may affect the function of cardiomyocytes derived from these cells. Also, for both hESCs and hiPSCs there are concerns about the immaturity of their cardiac derivates, which generally show foetal-like structural and functional properties.

1.2.6. The use of hPSCs in drug development and disease modelling

For drug screening and cardiotoxicity assays the pharmaceutical industry currently relies mainly on animal primary cardiac cells, whole animal models and immortalised human cell lines, which for example overexpress specific ion channels [e.g. CHO overexpressing HERG (Meyer et al., 2004)] (Rajamohan et al., 2012). The use of human primary cardiac tissue for drug development is problematic because the access to healthy or disease-specific cardiac material is limited and primary cardiomyocytes are not expandable, structurally and functionally not stable in long-term cultures (more than 48 h) and their genetic manipulation is inefficient (Harding et al., 2007). However, the use of immortal cell lines and animal models bears several disadvantages. These models cannot always accurately predict the efficacy and toxicity of a compound on the human heart because they cannot fully represent the complex human biology and there are substantial differences in gene expression and physiology between animals and humans [e.g. heart rate in mouse approximately 500 bpm versus 60 in human] (Rajamohan et al., 2012).

In the past years various drugs including the anti-inflammatory drug Vioxx and the anti-cancer drug doxorubicin had to be withdrawn from the market due to side effects on the human heart, which were not detected in animal models (Zeevi-Levin et al., 2012). Besides

being a health risk for patients the withdrawn drugs are also associated with a substantial financial loss for the pharmaceutical companies since the costs for the development of a drug (over an average period of 13 years) can reach 900 million US dollars (Kola and Landis, 2004). The pharmaceutical industry and the cardiac field in general needs new cardiac (disease) model systems, which are relevant to the human phenotype, and can reliably predict compound effectiveness and cardiotoxic side effects.

hPSCs can be expanded almost indefinitely and differentiated into cardiomyocytes with structural and functional characteristics of their adult counterparts. The potential of hPSC-CMs to serve as *in vitro* (disease) model systems for drug screening and cardiotoxicity assays is currently investigated in detail.

The response of hPSC-CMs to pharmacological agents has been already validated for several drugs, the effect of which is known in humans (Rajamohan et al., 2012). The results are promising revealing that for a variety of tested compounds (e.g. antiarrhythmic agent quinidine and arrhythmias-associated cisapride) the drug response of hPSC-CMs showed good association with clinically observed results (Braam et al., 2010; Zeevi-Levin et al., 2012; Liang et al., 2013). Many reports have also demonstrated that hPSC-CMs show ionotropic responsiveness to β -adrenergic stimulation (e.g. with isoproterenol) (Kehat et al., 2001; Xu et al., 2002; Yokoo et al., 2009; Germanguz et al., 2011).

Also, several studies have already demonstrated the use of hiPSC-CMs as cardiac disease models (Moretti et al., 2010; Itzhaki et al., 2011b; Carvajal-Vergara et al., 2010; Sun et al., 2012; Lan et al., 2013). For example various research groups generated hiPSC-CMs derived from patients with long QT and LEOPARD syndrome (an acronym formed from its main characteristics: lentigines, electrocardiographic abnormalities, ocular hypertelorism, pulmonary valve stenosis, abnormal genitalia, retardation of growth and deafness) and showed that these *in vitro* models could recapitulate the major disease phenotype such as prolonged action potential duration and cardiomyocyte hypertrophy, respectively (Moretti et al., 2010; Itzhaki et al., 2011b; Carvajal-Vergara et al., 2010). In addition, these studies elucidated, which signalling pathways may be implicated in the disease phenotype, or identified compounds, which could aggravate or ameliorate the condition.

The findings of the studies using hPSC-CMs for disease modelling and drug screening are encouraging. However, the use of hPSC-based models for drug discovery and safety evaluation by the pharmaceutical industry still faces various challenges. These include the generally immature phenotype of hPSC-CMs [e.g. low density of IK1 potassium current (Jonsson et al., 2012)], which raises concerns whether they can respond to all pharmacological compounds in the same way as their adult counterparts; the cost-effective scale-up of hPSC-CM production; the integration of hPSC-CMs into automated high throughput analysis platforms (Zeevi-Levin et al., 2012).

1.3. Differentiation of hPSCs to cardiomyocytes

1.3.1. Molecular specification during hPSC cardiomyocyte differentiation

hPSC differentiation is thought to recapitulate embryonic development therefore many of the developed cardiac differentiation protocols are based on our knowledge of molecular mechanisms, which govern the formation of the three germ layers during gastrulation and further cardiogenesis in the developing embryo (Murry and Keller, 2008). Similar to processes during embryonic cardiogenesis hPSCs are thought to differentiate into cardiomyocytes by sequential transition over several progenitor cell stages in four major steps (Fig. 1.1): 1) mesoderm formation; 2) mesoderm specification; 3) cardiac specification; and 4) cardiomyocyte formation (Burridge et al., 2012).

In various studies with chick, amphibian and mouse embryos a number of signalling pathways and factors, which drive cardiogenesis *in vivo*, have been identified. The induction of mesoderm (step 1) seems to be cooperatively regulated by members of the TGF- β superfamily such as bone morphogenetic protein 4 (BMP4) and Nodal/actinin A, wingless/Int(Wnt)/ β -catenin proteins and fibroblast growth factors (FGF) (Gadue et al., 2006; Lindsley et al., 2006; Naito et al., 2006). *In vivo*, all of these factors and their inhibitors are secreted by the endoderm layer and their expression is temporarily and spatially tightly regulated. Mesoderm formation is characterised by the downregulation of

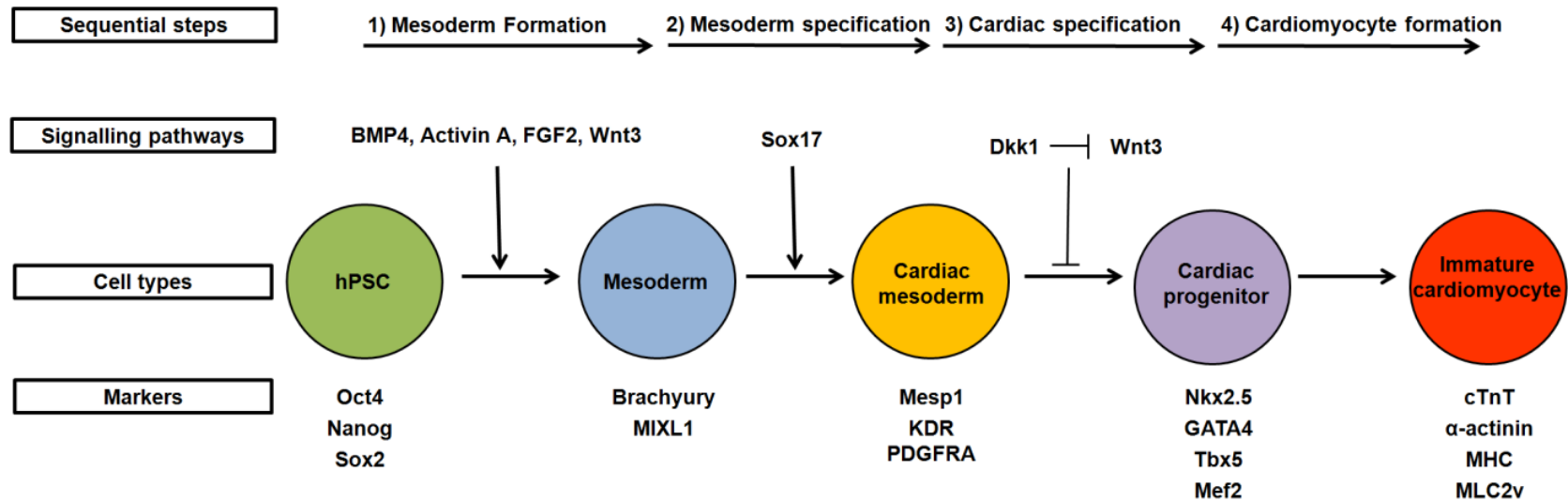


Fig. 1.1. Diagram of cardiac differentiation of hPSCs via sequential progenitor stages.

Immature cardiomyocytes are formed from hPSCs in four sequential steps: 1) mesoderm formation; 2) mesoderm specification; 3) cardiac specification; 4) cardiomyocyte formation. Some of the identified signalling pathways, which mediate the transition of hPSCs through the major progenitor stages (mesoderm, cardiac mesoderm and cardiac progenitor) towards immature cardiomyocytes, are displayed. Also shown are markers associated with each of the cell types of the differentiation process. Adapted from Vidarsson and co-workers (Vidarsson et al., 2010).

pluripotent markers such as Oct4, nanog and Sox2 and induced expression of mesodermal markers such as Brachyury and MXL1.

Once mesoderm is induced stage-specific inhibition of canonical Wnt/ β -catenin signalling is necessary for specification of cardiac mesoderm (step 2) (Naito et al., 2006). For this step the transcription factor Sox17 was reported to be important. Sox17 appears to mediate the inactivation of the canonical Wnt/ β -catenin pathway by inhibiting β -catenin transcriptional function (Liu et al., 2007b). The cells in the cardiac mesodermal stage typically express cell surface marker such as the receptor tyrosine kinase KDR, PDGFRA and the transcription factor Mesp1, which has been described as a master regulator for cardiac progenitor specification (step 3) (Bondue et al., 2008; Murry and Keller, 2008). Mesp1 can promote cardiac differentiation via Dkk-1-mediated inhibition of Wnt-signalling and may directly activate the expression of cardiovascular transcription factors such as Nkx2-5 and Gata4 by binding to their promoter regions (David et al., 2008; Bondue and Blanpain, 2010).

The committed cardiac progenitors express multiple factors including Nkx2.5, Gata4, Tbx5 and Mef2. These transcriptional factors play a key role in cardiomyocyte differentiation (step 4) and can in cooperation with other proteins activate cardiac structural genes such as actinin, troponin (cTnT), myosin heavy chain (MHC) and myosin light chain (MLC2v) (Sepulveda et al., 2002; Molkentin, 2000).

1.3.2. Cardiac differentiation methods

A number of cardiac differentiation approaches have been developed and improved in the past decade. The initial and most commonly used strategy to generate cardiomyocytes *in vitro* is the differentiation of hPSCs via embryoid body (EB) formation (Itskovitz-Eldor et al., 2000). In this method differentiation is induced by culturing hPSCs in suspension in three dimensional cell aggregates (termed EBs) with foetal calf serum (FCS). FCS and the aggregation process promote the spontaneous differentiation of the cells into all three germ layers including cardiomyocytes. Since the EB formation is not cardiac specific the proportion of cardiomyocytes within EBs is typically less than 5 % even though the proportion of contracting EBs can be high (Harding et al., 2007).

Another cardiac differentiation approach comprises the co-culture of hPSCs with endoderm-like cells, typically a mouse visceral endoderm-like cell line (END-2), which promotes the formation of cardiomyocytes presumably by secreting endoderm-like signals (Mummery et al., 2003). This method has been reported to yield more than 10 % of cardiomyocyte in a differentiated cell population. Addition of ascorbic acid or the small molecular inhibitor of the p38 mitogen-activated protein kinase (p38 MAPK) was shown to enhance cardiogenesis (Passier et al., 2005; Graichen et al., 2008).

In order to increase the reproducibility and yield of cardiomyocytes various research groups have for example modified the EB-based differentiation method. The recent protocols focus on the production of EBs of uniform and defined size (Ng et al., 2005; Ungrin et al., 2008) and replacement of FCS with defined medium supplemented with a growth factor cocktail containing activin A, BMP4, FGF, Dkk1 and other factors, which were described to guide cardiac differentiation *in vivo* (see above) (Yang et al., 2008).

Similarly, a serum-free differentiation approach described by Laflamme and colleagues (Laflamme et al., 2007) relies on guided cardiac differentiation with growth factors but without EB formation. In this method hPSCs are first cultured at high-densities in feeder-free conditions on Matrigel and then differentiated into cardiomyocytes by sequential treatment with activin A and BMP4. High levels of activin A have been shown to have endoderm and mesoderm inducing activity and BMP4 is important for the specification of mesoderm to the cardiac-muscle lineage (Kubo et al., 2004). This guided differentiation technique using hPSC monolayers is described to be more efficient than the classical EB formation method yielding more than 30 % cardiomyocytes (derived from the H7 hESC line).

The protocol for guided cardiac differentiation on cell monolayers has been recently improved by several research groups. For example, differentiation efficiency was shown to be enhanced by addition of Wnt (Wnt3a) in an early and the Wnt-inhibitor Dkk1 in a later stage of differentiation (Paige et al., 2010). In another approach hPSCs are treated sequentially with a combination of growth factors [Activin A, BMP4, and bFGF], which are administrated in an extracellular matrix overlay (Matrigel sandwich approach) to support soluble growth factor signalling (Zhang et al., 2012). This protocol produces cardiomyocytes at a purity of 40-90 %.

1.4. Characterisation of adult cardiomyocytes

1.4.1. Structural features of adult cardiomyocytes

The heart is a highly organised tissue and contains various cell types including cardiomyocytes (ventricular, atrial and nodal), fibroblasts, endothelial cells and vascular smooth muscle cells (Walker and Spinale, 1999). Although, cardiomyocytes represent only 30-40 % of the total cardiac cells population, they account for 80-90 % of the heart volume. The cardiomyocytes are the cells which contract to enable the myocardium to pump blood. However, cardiomyocytes located in the heart nodal areas (coordinate heart contraction), the atria (pump blood to the ventricles) and ventricles (pump blood to the body) are morphologically, molecularly, and functionally distinct. They differ significantly in both their electrophysiological and contractile properties. A distinctive feature of nodal myocytes is the spontaneous generation of action potentials, which spread through the atria and ventricles and lead to contraction of atrial and ventricular cardiomyocytes. Comparison of atrial and ventricular muscle showed that ventricular myofibrils generate more active tension than atrial myofibrils, whereas atrial muscle contracts and relaxes more quickly (Sprung et al., 2000; Piroddi et al., 2007). The latter is believed to be related to the difference in expression of myosin heavy chain (α - and β MHC) and myosin light chain (atrial and ventricular myosin essential light chains, ALC-1 and VLC-1) isoforms, which have an impact on the cross-bridge kinetics (Hoh et al., 1978; Piroddi et al., 2007; Morano et al., 1996). A common feature of adult nodal, atrial and ventricular cardiomyocytes is that they do not divide. They are terminally differentiated and permanently withdrawn from the cell cycle (Torella et al., 2007).

Structural analysis of cardiac muscle cells showed that cardiomyocytes display an elongated, rod-shaped morphology and that the bulk of each cell is composed of highly aligned bundles of myofibrils (contractile fibres; Fig. 1.2), which are made up of chains of sarcomeres, the basic contractile units of striated muscle cells. A sarcomere primarily contains thin and thick filaments, which can slide along each other during muscle contraction and relaxation. The thin filaments are formed by actins and their associated proteins such as troponins and tropomyosin and thick filaments are formed by myosin proteins. The borders of sarcomeres

are confined by the Z-lines, to which the thin and thick filaments are connected (Walker and Spinale, 1999). In the light microscope the regions with thin and thick filaments of sarcomeres appear as alternating bright (I-band) and dark (A-band) bands, which give the cells their banding pattern characteristic for striated muscles.

Apart from myofibrils the characteristic structural features of cardiomyocytes include a high amount of mitochondria, which supply the energy for contraction, the sarcoplasmic reticulum (SR) membrane network and a transverse tubule (T-tubule or TT) system.

The sarcoplasmic reticulum is the main intracellular store for Ca^{2+} ions required for contraction. It forms a web-like system around each myofibril and is in close opposition to the TT system. TTs are deep invaginations of the cell plasma membrane (sarcolemma), which run transversely at the Z-line level of sarcomeres of all myofibrils, but can also have longitudinal elements (Brette and Orchard, 2003). TTs can be found mainly in ventricular cardiomyocytes being absent or only rudimentarily developed in atrial and nodal cardiomyocytes. Together with the SR TTs are central structures for mature excitation-contraction coupling (ECC) in adult cardiomyocytes. TT invaginations allow uniform propagation of action potentials throughout the cell depth and synchronous contraction of all myofibrils.

Another distinctive feature of cardiomyocytes are intercalated discs, the connections between adjacent cardiomyocytes. These specialised regions of the sarcolemma comprise fascia adherens (anchoring junctions), macula adherens (expanded desmosomes) and gap junctions, which together allow mechanical connection and rapid conduction of action potentials between myocytes (Walker and Spinale, 1999).

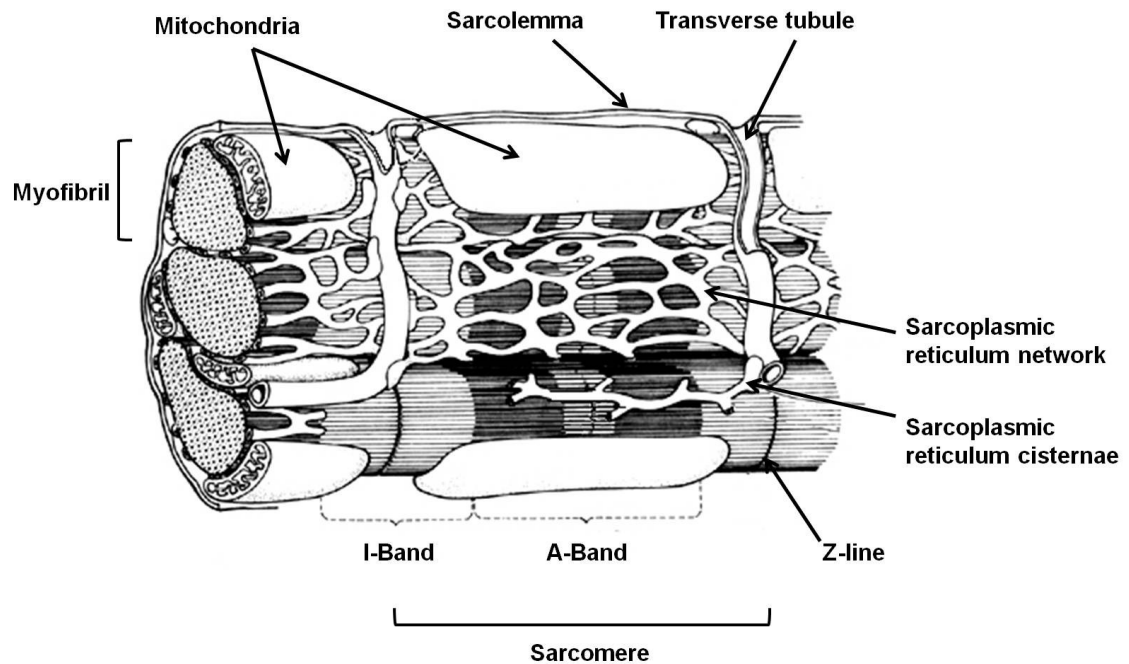


Fig. 1.2. Cross section of a cardiomyocyte.

The image shows bundles of aligned myofibrils, which are composed of arrays of sarcomeres, the contractile units within the myocytes. Each myofibril is surrounded by the sarcoplasmic reticulum network, the cardiac Ca^{2+} store. Transverse tubules occur at the Z-lines of each sarcomere. The imaged sarcomeres show the characteristic A-band and I-bands corresponding to regions containing thick and thin filaments, respectively. The image also shows the cell plasma membrane (sarcolemma) and various mitochondria, which are in close proximity to myofibrils. Adapted from Katz (Katz, 2001).

1.4.2. Excitation-contraction coupling in adult cardiomyocytes

During a cardiac action potential a small amount of Ca^{2+} enters the cell through voltage-sensitive L-type Ca^{2+} -channels, which occur highly concentrated in the TT membrane, and triggers a bulk Ca^{2+} release from the SR via the cardiac ryanodine receptors (RyR2), the SR Ca^{2+} release channels. This process, known as calcium-induced calcium release (CICR), occurs at functional units termed couplons, where the SR RyR are localised in close adjacency (less than 20 nm) to the TT-resident L-type Ca^{2+} channels. The SR Ca^{2+} flooding into the cytosol initiates the contraction of myofibrils. For relaxation Ca^{2+} is transported out of the cytosol by reuptake of Ca^{2+} into the SR by the cardiac SR Ca^{2+} -ATPase pump SERCA2a and ejection of Ca^{2+} from the cell by the sodium-calcium exchanger (NCX) and the sarcolemmal Ca^{2+} -ATPase (Bers, 2002).

1.4.3. Proteins involved in T-tubule formation and maintenance

TTs in cardiac cells are dynamic but labile structures. They are absent in neonatal cardiomyocytes of smaller mammals but develop rapidly after birth (Ziman et al., 2010; Sedarat et al., 2000). TTs decrease progressively, when primary cells are placed in culture, and a loss of TTs has also been reported in cardiomyocytes from failing hearts (Brette and Orchard, 2003; Ibrahim et al., 2011).

The mechanisms responsible for TT formation are widely unknown. However, it is assumed that TTs form initially at the cell edge as invaginations of the sarcolemma and then penetrate inward. Various proteins have been implicated in TT formation or their structural organisation.

Caveolin 3 is a muscle-specific member of the caveolin family, which are structural proteins of caveolae, the specialized lipid rafts in the plasma membrane (Parton et al., 1997). It may be involved in the organisation and maintenance of the TT system in cardiomyocytes since mice lacking caveolin 3 develop TTs with abnormal morphology (Parton et al., 1997; Carozzi et al., 2000; Galbiati et al., 2001).

The titin-binding protein titin-cap (Tcap; also called telethonin) is described to be part of a

stretch-sensing complex at the Z-disc, which can trigger downstream pathways in response to mechanical stretch (Knoll et al., 2002). Its role in TT formation in cardiomyocytes has not been studied in detail so far. However, a recent study demonstrated that disruption of T-cap in zebrafish leads to a muscular dystrophy-like phenotype with impaired TT development (Zhang et al., 2009b).

Junctophilins 2 (JP2), an isoform abundantly expressed in the heart, functions as a structural bridging protein between the SR and the plasma membrane, thus ensuring that the intracellular and the surface ion channels RyR2 and L-type Ca^{2+} channels are in close juxtaposition, which is required for the CICR mechanism in mature cardiomyocytes (Pozzan et al., 1994; Takeshima et al., 2000). Loss of JP2 in mice resulted in a significant disruption of normal TT structure and impaired CICR causing heart failure (van Oort et al., 2011).

Finally, the muscle-specific bridging integrator 1 (Bin1; also called M-amphiphysin 2) has been shown to induce tubulogenesis in skeletal muscle and was suggested to play an important role in induction of TT formation (Lee et al., 2002).

The muscle-specific Bin1 is a splice variant of the Bin1 protein. Bin1 has a dozen of ubiquitous and tissue-specific isoforms, which were implicated in various different cellular processes including endocytosis, intracellular trafficking and regulation of cell growth (Elliott et al., 1999; Leprince et al., 2003; Ren et al., 2006).

Structurally, the muscle-specific Bin1 isoform (isoform 8) is similar to other Bin1 splice variants and contains several domains (Fig. 1.3): the amino-terminal N-BAR (Bin/amphiphysin/Rvs) domain comprising an amphipathic helix, which is thought to initiate membrane deformation, and the BAR domain, which forms a banana-like structure and can sense and stabilise membrane curvature (Itoh and De Camilli, 2006); a Myc binding domain (MBD) (Sakamuro et al., 1996); and a carboxy-terminal Src homology 3 (SH3) domain, which can bind numerous proteins including dynamin (David et al., 1996), which is involved in the fission of clathrin-coated endocytic vesicles (Kohji Takei et al., 1995). Furthermore, the central region of the muscle-specific Bin1 isoform contains a unique phosphoinositide-binding domain (PI), which was shown to be important for targeting of Bin1 to the sarcolemma



Fig. 1.3. Domain diagram of the muscle-specific Bin1.

The muscle-specific Bin1 isoform 8 contains an N-terminal amphipathic helix (N) adjacent to the BIN1/amphiphysin/Rvs167 (BAR) domain, a phosphoinositide-binding (PI) domain, a Myc-binding domain (MBD) and a Src homology 3 (SH3) domain, which can bind various proteins including dynamin. Adapted from Nicot and colleagues (Nicot et al., 2007).

(Lee et al., 2002). In contrast to other Bin1 isoforms it does not contain clathrin-binding sites.

The muscle-specific Bin1 is highly expressed in striated muscle and is concentrated at TTs (Butler et al., 1997; Wechsler-Reya et al., 1997). A common feature of all BAR domain containing proteins is their ability to induce membrane curvature (Ren et al., 2006). The muscle-specific Bin1 may be involved in the formation or organisation of TT biogenesis. It was shown to induce membrane tubule formation in non-muscle and in skeletal muscle cells (Takei et al., 1999; Farsad et al., 2001; Razzaq et al., 2001). Also, disruption of the Bin1 gene in *Drosophila* results in a severely disorganised TT system (Razzaq et al., 2001).

Whether Bin1 is involved in TT formation in cardiac cells is not clear yet. However, deletion of the Bin1 gene in mice caused severe ventricular cardiomyopathy and was perinatal lethal (Muller et al., 2003). Moreover, recently it was shown that Bin1 can interact with microtubule and recruit L-type Ca^{2+} channels to TTs (Hong et al., 2010). Collectively these reports suggest that Bin1 may play a role in TT biogenesis and localisation of L-type Ca^{2+} channels to TTs in cardiomyocytes.

Henceforth, the muscle-specific isoform of Bin1 (isoform 8) will be referred to as Bin1.

1.4.4. The role of triiodothyronine in cardiomyocyte maturation *in vivo*

Triiodothyronine (T3) is known to have a variety of effects in the cardiovascular system and particularly on the development and function of cardiomyocytes (Klein and Ojamaa, 2001; Pantos et al., 2008). T3 is the active cellular form of the thyroid hormone. However, the thyroid gland initially secretes the pro-hormone tetraiodothyronine (T4) and T3 is generated via removal of an iodine residue from T4 by the deiodinase enzymes D1 and D2.

Many of the actions of T3 in cardiomyocytes (Fig. 1.4) are mediated via the classic genomic pathway by binding of T3 to its nuclear-localised receptors, thyroid hormone receptor alpha and beta ($\text{THR}\alpha$ and $\text{THR}\beta$), which then can bind to response elements in T3-targeted genes. T3-responsive genes encode various structural and regulatory cardiac proteins including α - and β MHC (Morkin, 1993), β -adrenergic receptor and Ca^{2+} handling proteins

such as SERCA2a and phospholamban (Cernohorsky et al., 1998; Ojamaa et al., 2000). In addition, it was demonstrated that T3 can modulate the expression and performance of a number of ion transporters and channels via transcriptional and post-transcriptional regulation. These include the plasma-membrane ion transporters Na^+/K^+ -ATPase and $\text{Na}^+/\text{Ca}^{2+}$ -exchanger (NCX) and the voltage-gated potassium channels such as Kv1.5, Kv4.2 and Kv4.3 (Ojamaa et al., 1999; Kahaly and Dillmann, 2005). In the absence of T3 the unliganded thyroid hormone receptors can repress the expression of genes, which are positively regulated by T3 (Klein and Ojamaa, 2001). During early foetal life T3 levels are low mainly due to the high expression levels of the enzyme deiodinase 3 (D3; inactivates T3) in the placenta and foetal tissue. Thus thyroid hormone receptors in the foetal tissue are mainly present in the unliganded state blocking the expression of a number of genes (Pantos et al., 2008).

In addition to direct transcriptional regulation via its intracellular receptors T3 can also affect cellular function through different non-genomic signalling pathways including mitogen-activated protein kinases (MAPK) and phosphoinositide 3-kinase (PI3K) signalling cascades (Lin et al., 1999; Storey et al., 2002). Also, cytosol-localised TR α seems to play an important role in non-genomic signalling pathways. Recently it was demonstrated that T3 can induce physiological hypertrophy in cardiomyocytes by activating the PI3K/Akt-mTOR signalling pathway through direct interaction of PI3K with cytosolic TR α (Kenessey and Ojamaa, 2006). Furthermore T3 was shown to mediate changes in cell geometry promoting a more elongated cell form, which involves ERK signalling (Pantos et al., 2007). In contrast to the effects of phenylephrine the T3-induced hypertrophic growth is not accompanied by cardiac fibrosis or activation of a foetal gene programme (Ziegelhoffer-Mihalovicova et al., 2003).

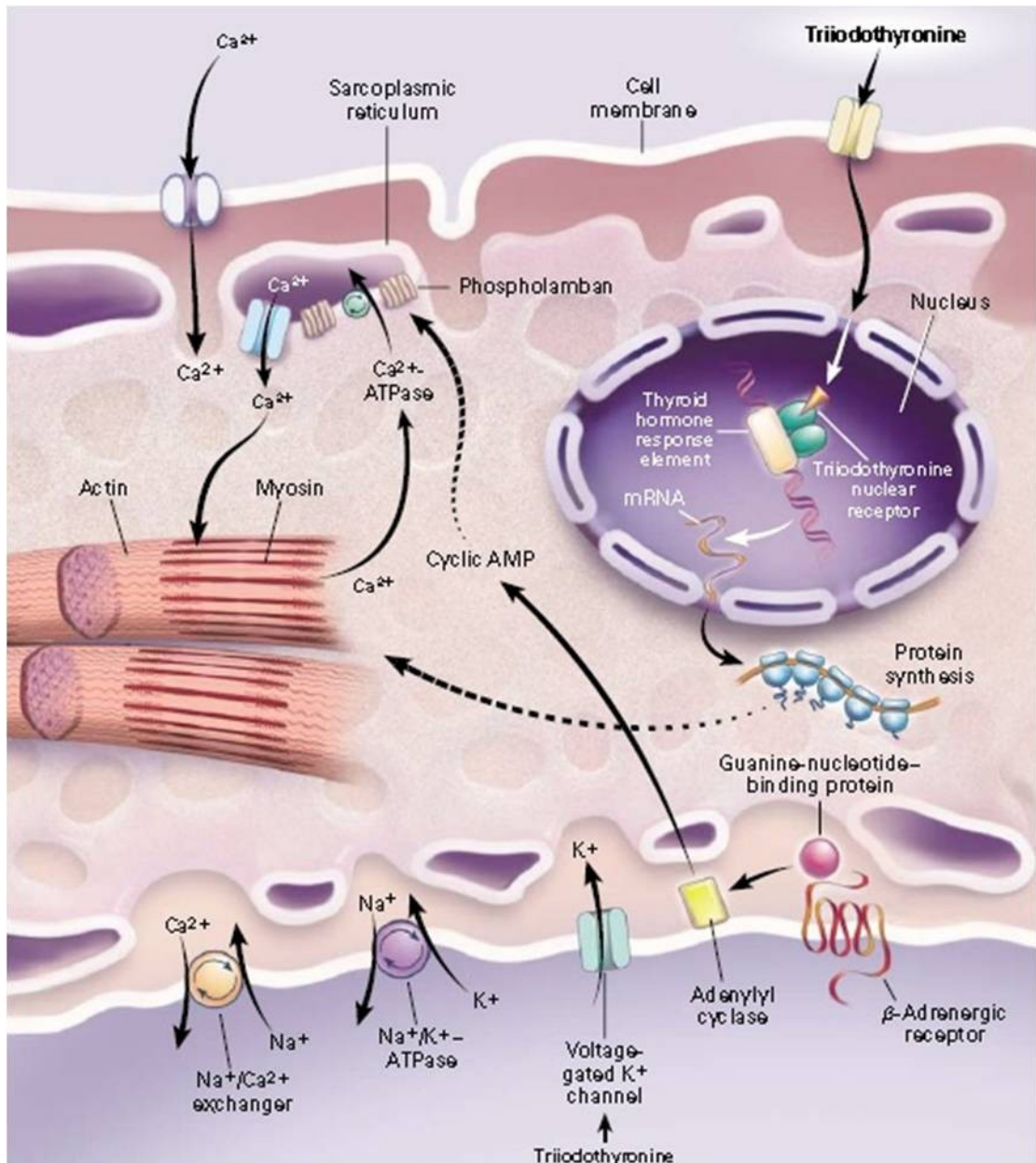


Fig. 1.4. Effect of triiodothyronine on cardiomyocytes.

Triiodothyronine (T3) exerts both genomic and non-genomic effects on cardiomyocytes. Genomic mechanisms involve T3 binding to nuclear T3 receptors, which can bind to T3 hormone response elements of several cardiac genes and regulate transcription of these genes, including Ca²⁺-ATPase (SERCA) and phospholamban in the sarcoplasmic reticulum, myosin, β-adrenergic receptors, adenylyl cyclase, guanine-nucleotide-binding proteins, Na⁺/Ca²⁺-exchanger, Na⁺/K⁺-ATPase and voltage-gated K⁺-channels. Non-genomic mechanisms include direct modulation of membrane ion channels (e.g. Na⁺, K⁺ and Ca²⁺ ion channels). Dashed arrows indicate pathways with multiple steps.

1.5. Characterisation of human pluripotent stem cell-derived cardiomyocytes

1.5.1. Comparison of hESC-CMs and hiPSC-CMs

Both hESC-CMs and hiPSC-CMs display key cardiac molecular, structural and electrophysiological characteristics and show generally similar structural and functional features. Also, various studies have demonstrated that they are highly comparable in terms of global transcriptional signatures, sarcomeric organisation, electrophysiological characteristics and calcium handling properties (Gupta et al., 2010; Gherghiceanu et al., 2011). Comparison with adult cardiomyocytes showed, however, that the structural and functional properties of hPSC-CMs are largely immature resembling a foetal-like stage of development but structural and functional maturation during long-term *in vitro* culture has been described (Snir et al., 2003; Sartiani et al., 2007; Lundy et al., 2013).

When characterising hPSC-CMs, the cells are often categorised according to their time in culture in days post induction of differentiation (dpi): early stage (10-40 dpi), intermediate stage (41-60 dpi) and late stage (more than 60 dpi) (Földes et al., 2011). However, this division is not uniform and different research groups may refer to slightly different time periods. Also, comparison of the maturation state of hPSC-CMs is often difficult due to the variation in phenotype between the different hESC-CM and hiPSC-CM populations (Boheler et al., 2011; Robertson et al., 2013). The heterogeneity of distinct hPSC-CM populations may be partially due to intrinsic properties of the original hPSC lines, different differentiation methods or different culture conditions.

1.5.2. Gene expression profile and structural properties of hPSC-CMs

Global gene expression analysis showed that from the early stage hPSC-CMs express key proteins characteristic for the cardiac phenotype. They express multiple myofilament proteins such as α -myosin heavy chain (α MHC), α -actinin and troponin, major calcium handling proteins including RyR, SERCA2a and PLN, ion channels including L-type Ca^{2+} channels and

other cardiac markers such as GATA4 and atrial natriuretic peptide (ANP). Overall the gene expression profile of hPSC-CMs resemble foetal cardiomyocytes (Beqqali et al., 2006; Cao et al., 2008). However, various studies demonstrated that the expression levels of many cardiac genes [e.g. α - β MHC, SERCA2a and connexin 43 (Földes et al., 2011; Lundy et al., 2013)] approach the adult gene expression levels with further *in vitro* culture time.

Similarly, (ultra)structural studies demonstrated a largely immature structural phenotype in early stage hPSC-CMs. These cells are typically small and round [5-10 μ m in diameter (Snir et al., 2003; Gherghiceanu et al., 2011)] and have myofibrils, which are unaligned, randomly distributed within the cell and show a variable degree of sarcomeric organisation (Mummery et al., 2003; Snir et al., 2003; Germanguz et al., 2011). However, after prolonged *in vitro* culture the morphology of hPSC-CMs changed from small round cells to larger more elongated cells resembling the rod-shaped adult cardiomyocytes. Myofibrillar alignment and sarcomeric organisation gradually increased with recognisable A, I and Z-bands in late stage hPSC-CMs (Snir et al., 2003; Lundy et al., 2013). Yet TTs could be detected neither in early nor in late stage hPSC-CMs (Snir et al., 2003; Lundy et al., 2013).

1.5.3. Electrophysiological properties of hPSC-CMs

hPSC-CMs display contractile activity (contract spontaneously and synchronously) and cardiac electrophysiological properties with atrial-, ventricular- and pacemaker-like action potential (AP) morphologies from early stages (He et al., 2003; Zhang et al., 2009a). However, compared to adult cardiomyocyte most AP characteristics in early stage hPSC-CMs are immature but improve with extended culture period. Late stage hPSC-CMs display faster AP upstroke velocity (represents a measure of the depolarisation rate) and their relatively positive resting membrane potential becomes more negative (Lundy et al., 2013). However, even late stage hPSC-CMs show spontaneous contraction, which is typical for embryonic cardiomyocytes but is normally not found in adult atrial or ventricular cardiomyocytes (DeHaan and Gottlieb, 1968; Lundy et al., 2013).

Furthermore, it was shown that hPSC-CMs express various ion channels such as cardiac-specific L-type calcium channel Cav1.2 (α 1C subunit), the sodium channel Nav1.5, the

inward rectifier Kir2.1 [though at very low levels (Otsuji et al., 2010; Jonsson et al., 2012)], the transient outward potassium channel Kv4.3, and the delayed rectifier potassium channel IKr (HERG) (Satin et al., 2004; Sartiani et al., 2007; Yokoo et al., 2009).

Also, hPSC-CMs exhibit major ionic currents, though often at relatively small levels, including L-type Ca^{2+} current ICa,L (mediated by Cav1.2), which triggers excitation-contraction coupling, transient outward potassium currents Ito1 (mediated by Kv4.3), which contributes to the repolarisation of AP, inward rectifier potassium current IK1 (encoded by Kir2.1), which plays an important role in stabilising cardiac resting membrane potential, and a large sodium current INa (Mummery et al., 2003; Satin et al., 2004; Sartiani et al., 2007).

The density of ICa,L , IK1 and Ito1 was shown to increase during *in vitro* culture (Sartiani et al., 2007). However, compared to adult cardiomyocytes the amplitude of the IK1 current and the expression of Kir2.1 remains very low even at late stage hPSC-CMs (Otsuji et al., 2010).

1.5.4. Excitation-contraction coupling in hPSC-CMs

Similar to adult cardiomyocytes hPSC-CMs show Ca^{2+} transients and express L-type Ca^{2+} channels and key SR proteins such as RyR2 (though usually dramatically lower than in adult cardiomyocytes) and SERCA2a (Mummery et al., 2003; Germanguz et al., 2011; Itzhaki et al., 2011a). However, at least for hESC-CMs there are conflicting reports in the literature about the mechanism of excitation-contraction coupling (ECC) and the development of functional SR Ca^{2+} stores due to variable responses of different hESC-CM populations to caffeine (induces SR Ca^{2+} -release by opening RyR2), ryanodine (inhibits RyR2) and thapsigargin (inhibits SERCA2a). Dolnikov and colleagues (Dolnikov et al., 2006) for example reported that neither caffeine, nor ryanodine or thapsigargin affected Ca^{2+} transients in hESC-CMs (7 or 55 days old) and noted further that the cells did not express calsequestrin, the major SR Ca^{2+} binding protein and regulator of RyRs. Thus they concluded that SR stores are not functional in hESC-CMs and that contraction did not depend on SR Ca^{2+} release but largely on transsarcolemmal Ca^{2+} influx. In contrast various other studies demonstrated that functional SR stores, which are sensitive to caffeine, ryanodine and thapsigargin respectively, can be found in hESC-CMs even at early developmental stages

(Liu et al., 2007a; Satin et al., 2008) and that the SR Ca^{2+} release is a major contributor to overall intracellular Ca^{2+} transients (Satin et al., 2008; Zhu et al., 2009). A study by Zhu and colleagues (Zhu et al., 2009) also showed that Ca^{2+} influx via L-type Ca^{2+} channels was necessary for ECC and that SR Ca^{2+} release in hESC-CMs is activated by a tight local mechanism similar to mature ventricular cardiomyocytes. Similarly, it was shown for hiPSC-CMs that functional RyR2- and SERCA2a-mediated SR stores are present in the cells and that the whole cell Ca^{2+} transients were dependent on both Ca^{2+} influx via L-type Ca^{2+} channels and Ca^{2+} release from SR stores (Germanguz et al., 2011; Itzhaki et al., 2011a).

Overall, the Ca^{2+} handling properties of hPSC-CMs improve during prolonged *in vitro* culture as evident by increased cytosolic Ca^{2+} transients, enhanced transient kinetics and increased SR Ca^{2+} content (Liu et al., 2007a; Satin et al., 2008; Lundy et al., 2013). However, the absence of a TT system and an underdeveloped SR contribute to non-uniform Ca^{2+} dynamics (Lieu et al., 2009).

1.6. Maturation of hPSC-CMs

Various reports demonstrated that hPSC-CMs are capable to mature structurally and functionally during prolonged *in vitro* culture (Satin et al., 2008; Sartiani et al., 2007; Lundy et al., 2013). However, prolonged cell culture is expensive and can be laborious. Depending on the purity of the hPSC-CM population the cells may need regular replating on a fresh matrix and excision of overgrowing non-cardiac cells may be necessary. Furthermore, for various features hPSC-CMs do not reach the adult phenotype completely even after long culture periods. For example, the cell size of elongated late stage hPSC-CMs (25 μm x 10 μm) does not reach the typical size of human adult cardiomyocytes (20 μm x 120 μm) (Snir et al., 2003; del Monte et al., 1995), the cells lack TTs (Snir et al., 2003; Lundy et al., 2013), show relatively immature AP characteristics (Robertson et al., 2013) and at least for hESC-CMs the expression of Kir2.1 and the corresponding IK1 current was shown to be significantly smaller than in adult cardiomyocytes (Otsuji et al., 2010).

The inability of hPSC-CMs to reach the same degree of maturity as adult cardiomyocytes might have different explanations (Snir et al., 2003): intrinsic genetic properties of the original hPSC line; *in vitro* culturing conditions; lack of *in vivo* signals for hypertrophic growth; lack of hemodynamic workload, which was shown to be important for appropriate cell alignment and ultrastructural development (Bishop et al., 1990).

1.6.1. Strategies to promote hPSC-CM maturation

In recent years in addition to prolonged *in vitro* culture various approaches, which apply exogenous stimuli, have been evaluated to facilitate cardiac maturation of human or mouse PSC-CMs (mPSC-CMs): temporal three-dimensional (3-D) culturing conditions (Otsuji et al., 2010); administration of T3 [so far reported only for mPSC-CMs; (Lee et al., 2010)]; mechanical stress (Tulloch et al., 2011; Földes et al., 2011); culture on a structured substrate (Rao et al., 2012); forced expression of calsequestrin (Liu et al., 2009); forced expression of Kir2.1 (Liao et al., 2013; Lieu et al., 2013). These approaches mimic *in vivo* physiological cues, which are missing during *in vitro* culture, or apply genetic manipulation to introduce a specific factor, which is absent in hPSC-CMs. The impact of some of these pro-maturation approaches on hPSC-CMs is described below.

Regular replating and temporal culturing of hPSC-CMs in suspension in three-dimensional aggregates was demonstrated to enhance the maturation of the cells by increasing the expression of various genes (e.g. ion α MHC, HERG1b, and HCN1) and enhancing ionic channel function (e.g. HERG channels). This approach by Otsuji and colleagues (Otsuji et al., 2010) showed that cell culturing conditions can influence the maturation process of hPSC-CMs and implied that suspension culture, which allows free contractility of the cells, can promote functional maturation of hPSC-CMs by increasing expression of several genes, inhibited during adhesive culturing.

Administration of T3 during the differentiation of mESCs into cardiomyocytes was shown to enhance cardiogenesis and maturation of generated mESC-CMs with regard to electrophysiological and Ca^{2+} handling properties (Lee et al., 2010). T3-treatment was shown to increase various cardiac genes including Nkx2.5, MLC2v, α - and β MHC and the SR Ca^{2+}

handling proteins SERCA2a and RyR2. The increase in SERCA2a and RyR2 expression was accompanied with enhanced Ca^{2+} handling as manifested by higher SR Ca^{2+} content and improved Ca^{2+} dynamics (increased cytosolic Ca^{2+} transient and improved Ca^{2+} kinetics). T3-treated mESC-CMs also showed improved AP characteristics.

Mechanical stretch of hPSC-CMs was shown to improve sarcomere alignment, enhance the expression of contractile proteins and increase cell size (Tulloch et al., 2011; Földes et al., 2011). Mechanical stretch is known to be a hypertrophic stimulus, which induces hypertrophic cell growth and reexpression of a set of foetal genes including ANP and βMHC . This effect is similar to the hypertrophic effects of phenylephrine (Frank et al., 2008) and seems to rely on active p38-MAPK-signalling (Földes et al., 2011).

Also, we recently showed that the culture of hiPSC-CMs (iCell cardiomyocytes, iCell-CMs; Cellular Dynamics International) in a structured substrate (microgrooved polydimethylsiloxane scaffolds) promoted cellular alignment and sarcomeric organisation and improved Ca^{2+} cycling properties. This was evident by shorter time to peak Ca^{2+} transient amplitudes and a more organised release of Ca^{2+} from SR stores in response to treatment with caffeine. However, the changes were not accompanied with increased expression of SR calcium handling components or other cardiac genes (Rao et al., 2012).

Transgenic overexpression of calsequestrin, the Ca^{2+} binding protein and regulator of RyR2 in the SR, mainly enhanced SR function by increasing SR Ca^{2+} load and improving RyR2-mediated SR Ca^{2+} release. This resulted in larger Ca^{2+} transients and enhanced Ca^{2+} transient kinetics. However, it did not alter the expression of other Ca^{2+} handling proteins such as RyR2 and Cav1.2, triadin and junctin and did not promote the formation of TTs (Liu et al., 2009).

Forced expression of the inward rectifier potassium channel Kir2.1 in mouse and human PSC-CMs enhanced the electrical properties of the cells as evident by a more negative resting membrane potential and the appearance of a robust IK1 current (Liao et al., 2013; Lieu et al., 2013).

In summary, each of the described pro-maturation strategies so far has enhanced the maturity of hPSC-CMs. However, usually only a limited set of properties was improved. Also, it was not reported that any of the stimuli induced the formation of TTs.

1.7. Aims of the study

This project aimed to explore pro-maturation strategies to facilitate the structural and functional maturation of early-stage hESC-CMs and hiPSC-CMs towards an adult phenotype. The hypothesis was that the maturation of hPSC-CMs can be enhanced via a) stimulation with the thyroid hormone triiodothyronine (T3), which induces hypertrophy and maturation *in vivo* but is suboptimal during *in vitro* cell culture and b) overexpression of Bin1, which potentially induces TT structures, which are absent in hPSC-CMs.

To test the hypothesis the specific aims were:

- To derive cardiomyocytes from the Shef3 hESC line by establishing a feeder-free culture system and a robust cardiac differentiation protocol for the Shef3 hESCs. This to our knowledge has not been reported before.
- To assess the impact of the pro-maturation strategies on Shef3-CMs and a commercially available hiPSC-CM line [iCell-CMs, Cellular Dynamics International, (Madison, USA)] by characterising the gene expression profile and the structural and electrophysiological properties of non-stimulated and stimulated hPSC-CMs and comparison with adult cardiomyocytes.

Chapter 2: Materials and methods

2.1. hESC culture

Cell culture reagents were purchased from Invitrogen (Paisley, U.K.) unless otherwise specified. Cell culture was carried out at 37 °C in a humidified atmosphere containing 5 % CO₂.

2.1.1. Feeder-based hESC culture on mouse embryonic fibroblasts

The hESC line Shef3 (Draper et al., 2004) with normal male karyotype was obtained from the UK Stem Cell Bank (UKSCB; South Mimms, U.K.).

In order to maintain Shef3s in an undifferentiated state hESCs colonies were cultured on monolayers of mitotically inactivated mouse embryonic fibroblasts (iMEFs) typically in 6-well culture plates in hESC medium (2.5 ml/well), containing knock-out DMEM, 20 % knock-out serum-replacement (KO SR), 2 mM GlutaMAX, 10 mM non-essential amino acids and 0.1M β-mercaptoethanol, and supplemented with 4 ng/ml basic fibroblast growth factor (bFGF). hESCs were fed every other day by complete medium change. Undifferentiated hESC colonies were sub-cultured after six to seven days by manually-dissecting individual colonies under a dissecting microscope. Cell supernatant was discarded and replaced with fresh hESC medium, undifferentiated areas of a hESC colony were dissected into sections (approximately 400 μm by 400 μm) via a 10 μm pipette tip and lifted from the feeders carefully by scraping with the same tool. Finally, the floating sections were transferred onto a fresh iMEF layer (typically at a split ratio of 1:5 or 1:6). Typically, hESCs were not used beyond passage 100. Shef3 hESCs were tested for karyotypic abnormalities. For karyotype analysis living hESC cultures were sent to The Doctors Laboratory (London, U.K.).

2.1.1.1. Mouse embryonic fibroblast culture

Mouse embryonic fibroblasts (MEFs), isolated from mouse embryos of the MF-1 strain at 13.5 days of gestation (P0), were provided by UKSCB. MEFs were typically cultured and expanded up to passage 3 before being mitotically inactivated with mitomycin C (see 2.1.1.2.)

to be used as supportive feeder layers in feeder-based hESC culture or for the preparation of mouse embryonic fibroblast conditioned medium for the feeder-free hESC culture on Matrigel (see 2.1.2.). MEFs were cultivated as a monolayer in T75 or T175 tissue culture flasks pre-coated with gelatin (0.2 %) and maintained in MEF medium, containing DMEM, 10 % foetal calf serum (FCS) and 2 mM GlutaMAX. MEFs were passaged when cells were at 70-80 % confluency. The cell supernatant was discarded, the cells were washed once with phosphate buffered saline (PBS, without calcium and magnesium) and incubated with the cell dissociation enzyme TrypLE Express (3-5 min, at 37 °C; Invitrogen). The flask was tapped gently to ensure complete cell detachment and two volume of MEF medium containing FCS was added to the flask to inactivate the enzyme. The cell suspension was transferred into a sterile tube and pelleted by centrifugation (5 min at 300xg), cell supernatant was removed, the cells resuspended in the required volume of fresh MEF medium and aliquoted into new pre-gelatinised tissue culture flasks at a split ratio of 1:5.

2.1.1.2. Mitomycin C inactivation of MEFs

In order to maintain a defined number of MEFs in feeder-based hESC culture and during the preparation of MEF-conditioned medium, MEFs were mitotically arrested using mitomycin C at passage 3.

The cell supernatant was discarded and the cells were incubated for 2 h at 37 °C with MEF medium containing mitomycin C (10 µg/ml). After inactivation the cell medium was removed, the cells were washed twice with PBS and either used immediately for preparation of MEF-conditioned medium or cryopreserved and stored in liquid nitrogen for later applications.

2.1.1.3. Cryopreservation of inactivated MEFs

Mitomycin C inactivated MEFs were detached from culture vessels using TrypLE Express (3-5 min, at 37 °C) and pelleted by centrifugation (5 min, 400xg). Cell supernatant was removed, the cells were resuspended in 90 % FCS (v/v), containing 10 % dimethyl sulfoxide

(v/v), and aliquoted into 1 ml cryovials at a density of 1×10^6 cells/ml. Finally the vials were placed at -80°C overnight and then transferred into liquid nitrogen for long term storage.

2.1.1.4. Cell count using a haemocytometer

In experiments, in which a definite cell number was required, cells were counted using a disposable haemocytometer (Labtech International, Ringmer, U.K.). The single cell suspension to be counted was well mixed before an aliquot (typically 10 μl -100 μl) was removed and mixed with a trypan blue solution (Sigma-Aldrich, Dorset, U.K.) with the dilution factor depending on the expected concentration of the cell sample (typically 1:10). 10 μl of the cell suspension/trypan blue mix was carefully pipetted into a chamber of the haemocytometer and viable cells were counted in $4 \times 1 \text{ mm}^2$ squares under a light microscope. Viable cells appear phase bright whereas non-viable cells, the membrane of which is porous and permeable for the trypan blue stain, appear blue. The total number of viable cells per ml was determined by multiplying the average number of viable cells per 1 mm^2 by the dilution factor and 1×10^4 .

2.1.1.5. Plating of MEFs for the feeder-based hESC culture

In order to prepare feeder layers to support the maintenance of hESC in an undifferentiated state iMEFs were seeded in MEF medium into 6-well plates pre-coated with gelatin (0.2 %) at a density of 2×10^4 cells/ cm^2 and incubated overnight at 37°C for cell attachment. One day before passaging of hESCs on the feeders, MEF medium was replaced by hESC medium to optimise the attachment of hESC colonies to the feeders.

2.1.2. Feeder-free hESC culture on Matrigel

The Shes3 hESC line was cultured on MEFs for more than six passages before being transferred to a feeder-free culture on Matrigel. hESCs on Matrigel were cultured either in MEF-conditioned medium supplemented with further 8 ng/ml bFGF or mTeSR1 medium (Stemcell Technologies, Grenoble, France). mTeSR1 medium was prepared by combining

mTeSR1 Basal Medium (400 ml) with mTeSR1 5X Supplement (100 ml) as indicated by the manufacturer.

2.1.2.1. Preparation of MEF-conditioned medium for the feeder-free hESC culture

For the preparation of MEF-conditioned medium MEFs (P0) were expanded up to passage 3 and mitotically inactivated with mitomycin C (see section 2.1.1.2.). Subsequently, iMEFs were attached overnight onto pre-gelatinised (0.2 %) T175 flasks in MEF medium at 80000 cells/cm². MEF medium was then replaced with hESC medium (114 ml/T175 flask) supplemented with 4 ng/ml bFGF and incubated with iMEFs for 24 h (conditioned). The MEF-conditioned medium was harvested and replaced by fresh hESC medium daily. The iMEFs were used for the production of conditioned medium for up to 7 days. The harvested medium was vacuum filtered through a 0.2 µm membrane (Stericup filter units; Fisher Scientific, Loughborough, U.K.) and stored at -80 °C.

2.1.2.2. Matrigel specification

Matrigel (hESC-qualified Matrix; BD Biosciences, Oxford, U.K.) is a solubilised basement membrane isolated from the Engelbreth-Holm-Swarm tumour. It polymerizes at room temperature (RT) forming a membrane, which is similar to the mammalian cellular basement matrix. Its major components are laminin and other extracellular matrix proteins such as collagen IV, entactin and heparan sulfate proteoglycan (Kleinman et al., 1986). In addition, it was reported to contain growth factors, collagenases, plasminogen activators and other undefined components (Vukicevic et al., 1992, McGuire and Seeds, 1989).

2.1.2.3. Preparation of Matrigel-coated plates

Matrigel was thawed overnight on ice (1-2 h), aliquoted into lot-specific volumes indicated by the manufacturer and stored at -80 °C. An aliquot was thawed on ice (1-2 h) and diluted with cold DMEM/F-12 medium [25 ml (Stemcell Technologies)]. Diluted Matrigel was added

immediately to 6-well culture plates (1 ml/well) and left to polymerise at RT for at least one hour prior to use. Coated plates could be stored at 4 °C up to seven days. If stored at 4°C, plates were moved to RT one hour prior to use. Matrigel was removed immediately before use and MEF-conditioned medium, mTeSR1 medium, respectively (2-3 ml) was added to the 6-well plate.

2.1.2.4. Transfer of hESCs on Matrigel

Shef3s, cultured on iMEFs for six to seven days, were detached from the feeders by manual dissection as described in 2.1.1. and transferred on Matrigel-coated culture plates either into MEF-conditioned medium (with 8 ng/ml bFGF) or mTeSR1 medium.

2.1.2.5. Culture of hESC on Matrigel

Shef3 cells on Matrigel were fed daily (except of first day after passaging) by complete medium change. Undifferentiated colonies were sub-cultured after 6-7 days of growth. For cultures grown in MEF-conditioned medium (with 8 ng/ml bFGF) cell medium was removed and the cells were incubated for 3-5 min with collagenase IV (200 U/ml, 1 ml/well), which mediates the dissociation of the cells from the Matrigel matrix. Next, cells were washed once with PBS, coated with MEF-conditioned medium (with 8 ng/ml bFGF) and passaged by manual dissection (see 2.1.1.) onto fresh Matrigel plates. Shef3s cultured in mTeSR1 were passaged on to fresh Matrigel plates coated with mTeSR1 medium as described above except that no enzyme treatment was required.

2.2. Cardiac differentiation of hESCs

2.2.1. Differentiation via conventional EB formation

hESC colonies at 5-6 days of culture on iMEFs in hESC medium or on Matrigel in mTeSR1 medium were dispersed into small clumps by manually dissecting individual colonies into

sections (approximately 800 μm by 800 μm) and uplifting from the culture surface using a 10 μm pipette tip. Culture medium with the floating cell clumps was then filtered through a 40 μm cell strainer (BD Biosciences, Oxford, U.K.) to remove single cells and cell debris, the hESC clumps remaining on the filter were transferred to 6-well low adherence plates (Corning, Corning, USA) and grown in suspension for four days in standard differentiation medium, containing 20 % FCS (γ -irradiated, Australian origin; Biosera, Ringmer, U.K.), 2 mM GlutaMAX, 10 mM non-essential amino acids and 0.1M β -mercaptoethanol. During the first day in suspension the cell clumps were treated with Y-27632 (Calbiochem, Nottingham, U.K.), a selective inhibitor of p-160-Rho-associated coiled-coil kinase (ROCK inhibitor), to decrease dissociation-induced apoptosis. After four days of suspension culture the formed EBs were plated onto 0.5 % gelatin-coated plates with typically 10-20 EBs/well of a 6-well plate. The medium was replaced every day during EB formation in suspension and changed every two to three days when EBs were plated on gelatin. Adherent EBs gave rise to beating clusters from day six onwards. The numbers of contracting EBs were counted visually using a conventional light microscope.

2.2.2. Differentiation via the non-EB-based activin A/BMP4 method

Manually dissected hESCs colonies were seeded onto Matrigel-coated surfaces (24-well plates) at densities of 200×10^3 cells/cm² and were cultured for a further six days under feeder-free conditions in MEF-conditioned medium (1 ml/well, with 8 ng/ml bFGF) or in mTeSR1 medium. In order to obtain accurate cell seeding densities hESC colonies in one or two wells to be used for differentiation were trypsinised into single cells and counted. The results of the cell count were used to estimate cell number in the other wells, which were passaged for differentiation by manual dissection. For induction of differentiation MEF-conditioned medium was replaced with RPMI-B27 (0.5 ml/well) containing activin A (100 ng/ml). After 24 h medium was replaced by RPMI-B27 (1 ml/well) containing BMP4 (10 ng/ml). After four continuous days of culture with BMP4 the medium was replaced by RPMI-B27 alone (1 ml/well), feeding cells every 2-3 days. Spontaneously contracting hESC-clusters were typically observed 12 days after induction of differentiation.

2.2.3. Cardiac differentiation of AggreWell-formed EBs

2.2.3.1. EB formation in AggreWells

AggreWell plates represent a standardised approach to produce EBs of uniform size and shape, which aims to make differentiation experiments involving EB formation more reproducible. In this project AggreWell 400 plates were used. These plates contain eight wells with silicon wafer based surfaces, which are micropatterned forming approximately 1200 microwells per well. A microwell is approximately 400 μm in diameter. EB formation was performed as described in the AggreWell protocol by Stemcell Technologies. Briefly, Shef3 hESCs (previously cultured in mTeSR1) were dissociated with TrypLE Express into single cells and 1.2×10^6 cells and 2.4×10^6 cells were added per well to the AggreWell plate (Fig. 2.1) in 2 ml medium in order to generate EBs containing 1000 cells/EB and 2000 cells/EB, respectively. The AggreWell plates were then centrifuged (100xg for 3 min) to distribute the cells in the microwells and cultured for a minimum of 24 h to allow aggregation of hESCs and formation of EBs within each microwell. All EBs were formed with ROCK inhibitor in serum-free and chemically defined STEMdiff APEL medium (APEL medium; albumin, polyvinyl alcohol, essential lipids; Stemcell Technologies) (Ng et al., 2008).

2.2.3.2. Differentiation of AggreWell-formed EBs with serum

EBs were formed in AggreWells as described above in APEL medium. For cardiac differentiation with serum 24 h or 48 h after formation EBs were harvested from the AggreWell plates. EBs were dislodged from AggreWells by firmly pipetting medium in a well up and down 2-3 times using a micropipettor with a 1 ml pipette tip. Subsequently the EB suspension was filtered through a 40 μm cell strainer (BD Biosciences) and the aggregates remaining on the filter were washed five times with PBS. Finally the EBs were placed into 6-well low adherence plates (Corning) in standard differentiation medium and differentiated as described for conventional EB formation in section 2.2.1.

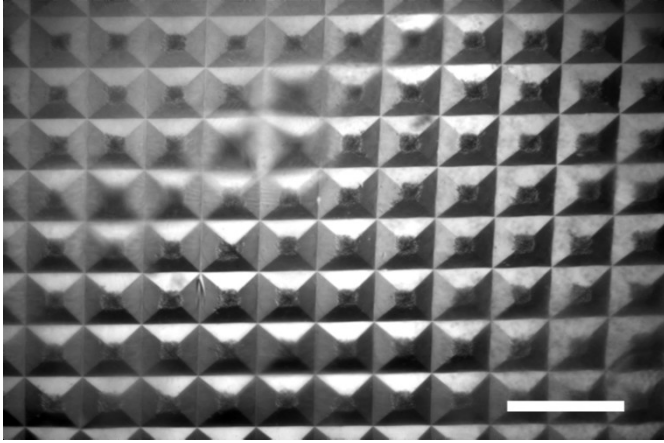


Fig. 2.1. EB formation in microwells of an AggreWell 400 plate.

A single cell suspension of Shef3 hESCs was seeded into an AggreWell plate in serum-free APEL medium supplemented with ROCK inhibitor and centrifuged for 3 min at 100xg to capture the cells in the microwells. Scale bar 800 μm .

2.2.3.3. Growth factor-directed cardiac differentiation of AggreWell-formed EBs in APEL medium

In this differentiation experiment serum-free APEL medium supplemented with combinations of growth factors was used to direct the differentiation of AggreWell-formed EBs to cardiomyocytes. Shef3 hESCs used for EB formation were previously cultured on Matrigel in mTeSR1 medium. Differentiation was performed as described in the protocol published by Stemcell Technologies (Stemcell Technologies, 2012b) and based on the report by Yang and colleagues (Yang et al., 2008). The entire differentiation procedure of 16 days was accomplished in the AggreWell plates. The differentiation protocol included the following steps:

- 1) On day 0 EBs of 1000 or 2000 hESCs each were prepared in AggreWell 400 plates in APEL medium in the presence of ROCK inhibitor and BMP4 (0.5 ng/ml).
- 2) On day 1 the medium was replaced with APEL medium with addition of BMP4 (10 ng/ml), activin A (10 ng/ml) and bFGF (5 ng/ml).
- 3) On day 4 the medium was replaced with APEL medium supplemented with VEGF (10 ng/ml) and Dkk1 (150 ng/ml) and cells were incubated for four days.
- 4) On day 8 the medium was changed to APEL medium with addition of VEGF (10 ng/ml), Dkk1 (150 ng/ml) and bFGF (10 ng/ml).
- 5) On day 12 the medium was replaced with fresh APEL medium supplemented with the same growth factor combination as on day 8 and cultured without any further medium change for additional 4 days.

The AggreWell plates were monitored daily for the emergence of beating clusters though the peak of beating was reported to be on day 16.

2.2.4. Culture of Shef3-EBs

Shef3-EBs, which contained spontaneously contracting clusters, were cultured on gelatin-coated plates in differentiation medium. The medium was changes every other day. Beating

clusters were manually microdissected and re-plated on fresh pre-gelatinised plates every two weeks to avoid overgrowth by non-cardiac cells.

2.3. Culture of hiPSC-CMs (iCell-CMs)

iCell-CMs were purchased from Cellular Dynamics International (CDI; Madison, USA) (Cellular Dynamics International, 2012a). These cells are cardiomyocytes terminally differentiated from hiPSCs, which in turn were formed presumably from a commercially available human foetal lung fibroblast line (correspondence with CDI). iCell-CMs are shipped as cryopreserved single-cell suspensions in 1 ml cryogenic vials (approximately 1.5×10^6 platable cells). The shipped cells are approximately 30 days old (correspondence with CDI). When received, the frozen cryovials with iCell-CMs were transferred immediately into liquid nitrogen.

The culture of iCell-CMs is described in the user's guide by CDI (Cellular Dynamics International, 2012b). As recommended by the guide iCell-CMs were cultured at 37 °C in a humidified atmosphere containing 7 % CO₂. Briefly, the cryopreserved cells were thawed and plated on pre-gelatinised (0.5 %) cell culture plates, typically 1 vial into one 6-well plate, in serum-containing iCell-CMs Plating Medium (CDI) and incubated for 48 h at 37 °C. The plating medium was then replaced with serum- and antibiotic-free iCell-CMs Maintenance Medium (CDI), which was used for further iCell-CM culture. The iCell-CMs Maintenance Medium was changed every other day.

2.4. Isolation and culture of adult rat cardiomyocytes

2.4.1. Isolation of adult rat cardiomyocytes

Adult rat ventricular cardiomyocytes were isolated from male Sprague-Dawley rats (8-12 weeks old) by Dr. Peter O'Gara. Briefly, rats were heparinised (1000 U/kg), killed by cervical dislocation and the heart was rapidly removed and immersed in ice-cold Krebs-Henseleit

(KH) solution (119 mM NaCl, 4.7 mM KCl, 0.94 mM MgSO₄, 1.2 mM KH₂PO₄, 25 mM NaHCO₃, 11.5 mM glucose, 1 mM CaCl₂; equilibrated to pH 7.4 with 95 % O₂/5 % CO₂). The heart was then perfused with KH solution via the Langendorff perfusion method for 5 min after a steady flow was established. A further perfusion step was performed for 5 min with a low calcium solution [120 mM NaCl, 5.4 mM KCl, 5 mM MgSO₄, 5 mM pyruvate, 20 mM glucose, 20 mM taurine, 10 mM HEPES and 5 mM nitrilotriacetic acid (NTA) with a pH of 6.95 and calcium added to achieve a final concentration of 12-14 mmol/l; equilibrated with 100 % O₂]. The heart was then perfused for 10 min with enzyme solution [composition as low calcium solution but without NTA and with addition of 200 µM Ca²⁺, collagenase (1 mg/ml) and hyaluronidase (0.6 mg/ml)]. The ventricles were then cut into 5-6 strips and shaken at 35 °C under 100 % O₂ for 5 min with the same enzyme solution as above. Subsequently the tissue strips were filtered and incubated once more with the enzyme solution for further 10-30 min until all tissue was digested. The cell suspension was then centrifuged at 400xg for 1 min at RT, the supernatant was discarded and the cell pellet resuspended in the low calcium solution but without NTA and with added Ca²⁺ (200 µmol/l) or in M199 culture medium [M199 DMEM, 1 % P/S (100 U/ml penicillin, 100 µg/ml streptomycin), 0.2 % BSA, 100 mmol/l ascorbate, 5 mmol/l creatine, 5 mmol/l taurine and 2 mmol/l carnitine].

All chemicals and culture medium components were purchased from Sigma-Aldrich.

2.4.2. Short-term culture of adult rat cardiomyocytes

Freshly-isolated adult rat cardiomyocytes were cultured short-term (up to 2 h) on culture surfaces pre-coated with laminin (14 mg/ml; Cultrex 3D Culture Matrix Laminin; R&D Systems) in M199 culture medium at 37 °C with 4 % CO₂. Typically, approximately 1 million cells (a mixture of living and dead cells) were resuspended in 10 ml M199 culture medium in a 15 ml tube. The cells were mixed by carefully inverting the tube and 150-200 µl of cell suspension per cm² were transferred on laminin-coated culture dishes. The cells were left to attach at 37 °C for 1 h, were then washed once with M199 culture medium to remove unattached dead cells, covered with fresh M199 culture medium and processed for transmission electron microscopy or immunofluorescence studies within 1 h.

2.4.3. Coating of cell culture surfaces with laminin

Cell culture surfaces were coated with laminin (14 mg/m; Cultrex 3D Culture Matrix Laminin; R&D Systems) by applying 100 μ l of undiluted laminin per cm^2 on the culture surface, removing the laminin after 1 min and leaving the substrate to air dry for 20 min at RT.

2.5. Real-time quantitative reverse transcription polymerase chain reaction

2.5.1. Principles of standard polymerase chain reaction

The polymerase chain reaction (PCR) allows amplification of a target sequence in a DNA strand *in vitro* using primers specific for the target region (Avison, 2007). Usually, a PCR consists of a series of 20-40 cycles with each cycle typically comprising three discrete temperature steps:

- 1) Denaturation: The PCR reaction mix is heated to a temperature usually above 90 °C. This causes the hydrogen bonds between complementary bases in double-stranded DNA to break and results in separation of double-stranded DNA into two single strands.
- 2) Primer annealing: The temperature is lowered to 40–65 °C to allow primers to bind (anneal) to their complementary target sequence. The annealing temperature is typically approximately 3-5 °C below the primer melting temperature.
- 3) Elongation: The temperature is raised to approximately 72 °C to allow optimal activity of the Taq polymerase (a thermostable DNA polymerase enzyme), which binds to the primer-template hybrid and begins to replicate the DNA strand. The DNA polymerase synthesises a new DNA strand, which is complementary to the DNA template, by promoting the binding of complementary deoxyribonucleoside triphosphates (dNTPs) to the template in 5' to 3' direction.

2.5.1.1. Limitations of standard PCR

The amplification product of a standard PCR reaction is usually detected in the final phase of the PCR process for example by gel electrophoresis. However, the end-point detection generally delivers only qualitative but no quantitative data because in the end stage of a PCR reaction the DNA amplification does not occur at an exponential rate (as in initial phases) due to substrate and reagent limitation and self-annealing of the accumulating product among others.

2.5.2. Principles of real-time quantitative reverse transcription polymerase chain reaction

Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) allows continuous detection of the concentration of the generated PCR product during each cycle of a PCR run (Avison, 2007). The term “reverse transcription” indicates that the real-time quantitative PCR is combined with a reverse transcription step. That is, in order to analyse gene expression of tissues the total RNA (messenger RNA and non-coding RNA) is reverse transcribed into complementary DNA (cDNA), which is considerably more stable than RNA.

To quantify an amplified PCR product during qRT-PCR there are generally two approaches:

- The use of fluorescent dyes such as SYBR Green I and EvaGreen, which can bind to double-stranded DNA. The intensity of the dye fluorescence increases proportional to the amount of DNA generated during the PCR process.
- The application of fluorophore-containing DNA probes such as TaqMan probes, Molecular Beacons and Scorpion Primers, which are sequence specific. So that the fluorescence intensity is proportional to the amount of amplified product with a particular sequence.

2.5.2.1. Quantification of PCR product via SYBR Green I

SYBR Green I is the most commonly used reporter dye for double-stranded DNA reported for real-time PCR. The dye can bind to the minor groove of the DNA double helix. When unbound SYBR Green I displays only little fluorescence. However, its fluorescence increases significantly (approximately 1000-fold) upon binding to double-stranded DNA. SYBR Green I fluorescence is usually measured in the end of the elongation step of each PCR cycle when new DNA strands are synthesised by the DNA polymerase so that double-stranded DNA molecules occur (Fig. 2.2). Since with each elongation step the amount of DNA template doubles the fluorescence of SYBR Green I also increases approximately twice.

2.5.2.2. Quantification of PCR product via fluorescent probes

The probes such as Taq Man employed to monitor the DNA amplification during real-time PCR are usually designed to bind to a region of a specific PCR product. The probes also usually contain a fluorophore and a quencher. When the target is absent the quencher prevents the fluorophore from emitting light. However, when the target sequence becomes available and the probe binds to it the fluorophore and the quencher separate, which results in increased fluorescence. The intensity of fluorescence increases with each PCR cycle in proportion to the increased amount of target DNA sequence, which can bind the fluorescent probes.

Compared to SYBR Green I the probe technology is more specific because it measures only the PCR product with a specific sequence whereas SYBR Green I binds any double stranded DNA molecules. However, fluorophore-containing probes can be relatively expensive.

2.5.3. qRT-PCR experiments

In this project qRT-PCR was performed using SYBR Green I-based amplicon detection because this technique was already well established in the laboratory at NIBSC and less expensive than the use of fluorophore-containing probes.

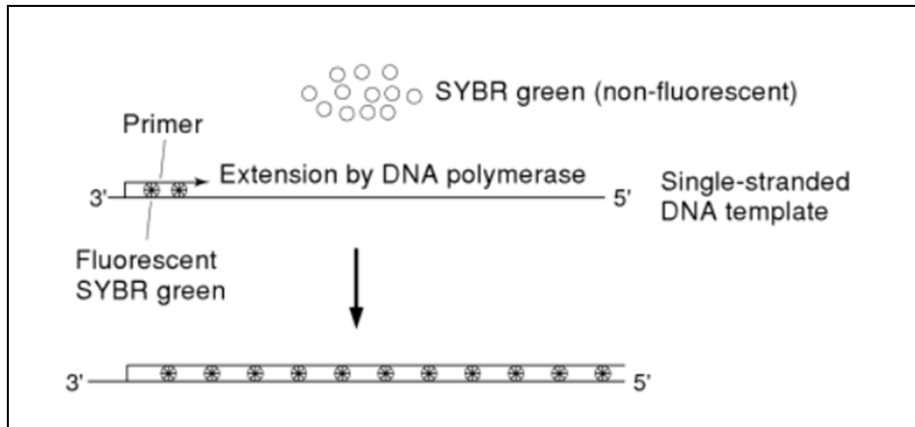


Fig. 2.2. The use of SYBR Green I in real-time PCR.

Throughout the elongation step an increasing amount of SYBR Green I molecules bind to the newly synthesised double-stranded DNA so that the fluorescent intensity of SYBR Green I increases at the end of the elongation step of each cycle. Adapted from Avison (Avison, 2007).

For the analysis of gene expression via qRT-PCR the total RNA of experimental samples was isolated (section 2.5.3.1.) and reverse transcribed into cDNA (section 2.5.3.3.). Total RNA from human adult and foetal heart tissue was purchased from Agilent (Wokingham, U.K.). All gene expression values of experimental samples obtained by qRT-PCR were quantified relative to a standard curve, which was prepared for each gene of interest including the house keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Each standard curve was generated from seven gene standards serially diluted 10-fold (gene standard preparation in section 2.5.3.5). All results were further normalised to GAPDH expression. In addition, the human adult heart control (section 2.5.3.2) was used as calibrator thus the normalised gene expression values of experimental samples were divided by the normalised corresponding gene expression values of the human adult heart control.

Also, after each qRT-PCR run a DNA melt-curve analysis was performed to confirm the identity of PCR products (because SYBR Green I binds to double-stranded DNA non-specifically). For the generation of melt-curve profiles the PCR products are heated slowly from 72 °C to 95 °C and the fluorescence of SYBR Green I is measured and plotted as a function of temperature. A sharp drop in fluorescence can be observed when the DNA reaches its melting point, which is defined as the temperature at which 50 % of the double-stranded DNA molecules dissociate into single strands and is generally unique for each PCR product type.

2.5.3.1. Isolation of total RNA

Total RNA from cell samples was isolated using RNeasy Mini Kit from Qiagen (Crawley, U.K.) following the protocol in the RNeasy Mini Handbook. The Qiagen technique is based on the RNA-selective binding properties of a silica membrane, which is attached to a spin column. Briefly, total RNA was isolated by following procedure:

- 1) Cells grown in feeder-free conditions on Matrigel were lysed by adding the highly denaturing guanidine-thiocyanate-containing RLT buffer directly to the cell-culture dish (typically 700 µl/well of 6-well plate). The RLT buffer also inactivates RNases to maintain RNA integrity.

- 2) The cell lysate was vortexed and passed 5 times through a 1 ml gauge needle (0.9 mm) for homogenisation.
- 3) The homogenised lysate was mixed with ethanol (70 %) to precipitate the RNA and centrifuged through the RNA-binding silica membrane in RNeasy spin columns. The column was then washed once by centrifugation with buffer RW1.
- 4) The precipitated RNA sample was treated on the column for 15 min at RT with DNase I to digest any genomic DNA.
- 5) The membrane was washed in a series of washes by centrifugation with the buffers RW1 and RPE to remove contaminants and the RNA finally eluted by centrifugation with RNase-free water (30 μ l).

All centrifugation steps were performed at 8000xg. All RNA eluates were quantified using a NanoDrop spectrophotometer (NanoDrop ND1000; ThermoScientific, NanoDrop products, Wilmington, USA) and reverse transcribed into cDNA immediately or transferred to -80 °C for long term storage to prevent RNA degradation.

2.5.3.2. Human adult and foetal heart controls

Total RNA from human adult heart (1.0 μ g/ μ l) and foetal heart (0.8 μ g/ μ l) tissue were obtained from Agilent. Total RNA from human adult heart was derived from a single, 63-year-old female donor whereas total RNA from human foetal heart was derived from a pool of ten female donors at 19 weeks (x4), 20 weeks (x5) and 21 weeks of gestation. No further information about the RNA source was available.

2.5.3.3. cDNA synthesis via RNA reverse transcription

Total RNA was reverse transcribed into cDNA using the reverse transcriptase derived from the Molony Murine Leukaemia Virus (MMLV-RT). All reagents for the reverse transcription reaction were purchased from Promega (Madison, USA). For cDNA synthesis up to 1 μ g total RNA was mixed with 1 μ l Oligo (dT)15 primers (0.5 μ g/ μ l), 1 μ l random primers (0.5 μ g/ μ l) and nuclease free water to a total volume of 12 μ l. The mixture was heated for 5 min to 70 °C

to melt any secondary structures within the template, then cooled for 5 min on ice and combined with the reverse transcription reaction mix to a final volume of 20 μ l (see Table 2.1.).

The combined reverse transcription reaction mix was incubated for 50 min at 42 °C to activate the MMLV-RT enzyme and allow cDNA synthesis before incubating for 15 min at 70 °C to inactivate the enzyme. cDNA was stored at -20 °C.

Reagent	Volume (1x) in μ l
Reaction buffer (5x)	4
dNTP mix (10 mM)	1
RNAse inhibitor (40 U/ μ l)	2
MMLV-RT (200 U/ μ l)	1

Table 2.1. Reverse transcription reaction mix for cDNA synthesis.

2.5.3.4. PCR and qRT-PCR primers

Forward and reverse oligonucleotide primers complementary to a gene sequence of interest were designed online via Roche Universal ProbeLibrary Assay Design Center (<http://qpcr.probefinder.com/organism.jsp>) and chemically synthesised by Integrated DNA Technologies (IDT; Leuven, Belgium). The lyophilised IDT primers were reconstituted in molecular biology grade water at a stock concentration of 100 μ M. All primers used in standard PCR were also used in qRT-PCR and are detailed in Table 2.2.

2.5.3.5. Preparation of gene standards via standard PCR

The initial undiluted gene standards were generated by standard PCR amplification of gene sequences of interest using cDNA of the adult human heart control as template because it was assumed that this control sample expressed all relevant cardiac genes of interest. The PCR reaction mix was set up by diluting the cDNA of the human adult heart control 1:5 with DNase-free water and preparation of a 50 μ l PCR mix (see Table 2.3.) including a target-specific primer pair (see Table 2.2.). Subsequently the PCR mix was loaded into a PCR

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')	Product size (bp)	T (°C)
GAPDH	gtcagtgggtggacctgacct	ccctgttgctgtagccaaat	251	60
MYH6 (α MHC)	ctcaagctcatggccactct	gcctccttgctttaccact	75	61
MYH7 (β MHC)	acaccctgactaaggccaaa	tccagggatccttcagat	64	60
MYL2 (MLC2v)	acatcatcacccacggagaa g aga	attggaacatggcctctgga t gga	247	62
TNNT2 (cTnT)	ttcaccaaagatctgctcctcg ct	ttattactggtgtggagtggg tgtgg	166	63
NKX2-5 (Nkx2.5)	acctcaacagctccctgactct	ataatcgccgccacaaact ct cc	155	62
GATA4 (Gata4)	ggaagcccaagaacctgaat	gttgctggagttgctggaa	98	61
NPPA (ANP)	caggatggacaggattggag	tcctccctggctgttatcttc	77	61
CACNA1C (Cav1.2)	tgacatcgaggagaaaaact	acattagacttgactgcggc	129	60
CACNA1 (Cav3.1)	tgctcttcaatttgctggtc	tcttcccgtttgctgatttc	70	60
SLC8A1 (NCX1)	ggttgggactaacagctgga	ccacattcatcgctcatc	84	63
KCNJ2 (Kir2.1)	gtgtccgaggtcaacagctt	ggttgtctgggtctcaatgg	60	62
KCND3 (Kv4.3)	cttgtgatgatcccctgtt	gctcatcaataaactcgtgtt tc	67	60
RYR2 (RyR2)	ctgcgccattctatagtgg	agtgaagaccgggagggtg	60	60
ITPR2 (IP3R2)	cctactccaaaactgcacagg	gtctgacattgatatcccat ct	77	60
ATP2A2 (SERCA2a)	aacgtcggggaagttgtct	gaatcaaagcctcgggaa at	64	61
PLN	tgatgatcacagctgccaag	tgagcgagtgaggtattgga	147	60
CASQ2 (CSQ2)	gagtttgatggcgagtttgc	ttgctgctgatgatctccac	86	62
BIN1 (Bin1)	acgggagcaacaccttca	gccgcgaaaacagtttactt	63	60
CAV3 (Cav3)	gaggcccagatcgtaag	tcacgtcttcaaaatccacct	106	61
JPH2 (JP2)	ctgaccgtcgcttaccaga	cctggcactgcaaaaagac	70	62
TCAP (Tcap)	ggcagaatggaaggatctga	tctcatgtctctgggtgtcct	83	60
THRA (THR α)	ggctgtgctgctaattgtcaa	aggtagcctcctgactcttc	78	60
THRB (THR β)	gggcactggtaatttggtca	cagaaggaaatcgagat cc	61	60
DIO3 (D3)	caacggacaattgactgaact t	cttggttgaggcgcttg	76	60

Table 2.2. Primers used in PCR and qRT-PCR. T: primer annealing temperature.

thermal cycler (Applied Biosystems 2720 Thermal Cycler; Applied Biosystems, Carlsbad, USA). A standard PCR programme was performed, which included the following steps:

- 1) Heat activation of the GoTaq Hot Start Polymerase at 94 °C for 2 min.
- 2) 40 cycles of denaturation at 94 °C for 45 seconds, primer annealing at 57 °C (annealing temperature) and elongation (polymerisation of single-stranded DNA) at 72 °C for 1 min.
- 3) Annealing at 72 °C for 2 min (formation of double-stranded DNA from single-stranded products).
- 4) Cooling to 4 °C and hold for short term storage of the reaction mix.

To confirm that the DNA sequence of interest was amplified 20 µl of each PCR sample were loaded on an agarose gel and detected via gel electrophoresis (section 2.5.3.6.). If the resulting DNA bands showed the expected product size the remaining 30 µl of the PCR reaction were purified via the QIAquick PCR Purification Kit (Qiagen; section 2.5.3.7.).

Reagent	Volume (1x) in µl
5x Reaction buffer	10
dNTP mix (10 mM)	2
MgCl ₂ (25 mM)	3
Forward primer (10 µM)	2.5
Reverse primer (10 µM)	2.5
cDNA/H ₂ O mix	1.5
GoTaq polymerase	0.5
PCR-grade H ₂ O	28
Total volume	50 µl

Table 2.3. Reagents for 50 µl standard PCR mix.

2.5.3.6. Gel Electrophoresis

2.5.3.6.1. Preparation of agarose gels

Agarose gels (1.8 %) were prepared by dissolving 0.9 g agarose (UltraPure agarose, Invitroge) in 50 ml of 1X Tris borate EDTA buffer (TBE) buffer (see Appendix) and heating the

mixture in a microwave for approximately 2 min until all agarose had dissolved. Subsequently the agarose solution was cooled and supplemented with 5 µl SYBR SAFE DNA gel stain (10000x; Fisher). The mixture was poured into a gel tray and allowed to set.

2.5.3.6.2. Electrophoresis

Each cDNA sample was mixed with 6 µl of 3x loading buffer (Promega) and loaded onto an agarose gel in an electrophoresis tank filled with 1x TBE buffer. A PCR DNA ladder was also added to each gel to allow the identification of PCR products by size. Electrophoresis was conducted at 70 V for approximately 45 min. Afterwards the DNA was visualised under UV light using a transilluminator. Images were taken using a gel documentation imaging system (InGenius LHR; Syngene, San Diego, USA).

2.5.3.7. Purification of PCR product

cDNA samples (gene standards) amplified via standard PCR were purified via spin-column purification using the QIAquick PCR Purification kit (Qiagen) to remove primers, nucleotides, enzymes, mineral oil, salts, and other impurities, which may interfere with downstream qRT-PCR assays. The purification procedure was carried out as described in the handbook of the kit. Briefly, the following steps were performed:

- 1) 5 volumes of Buffer PB were added to 1 volume of the PCR sample and mixed. Buffer PB contains high-salt concentrations, which are required to bind DNA to the silica-based membrane in the QIAquick spin-column.
- 2) The sample was transferred into a QIAquick spin-column and centrifuged for 30-60 s to bind the cDNA to the silica-based membrane.
- 3) The flow-through was discarded and the sample washed by adding 0.75 ml Buffer PE to the QIAquick column and centrifuging for 30–60 s. Buffer PE contains ethanol, which removes salts from the sample.
- 4) The flow-through was discarded and the column centrifuged once more for an additional 1 min to remove all residual ethanol from Buffer PE.

- 5) The QIAquick column was placed in a clean 1.5 ml microcentrifuge tube and the cDNA was eluted by adding 30 μ l DNase-free H₂O (pH 7.0–8.5) to the QIAquick membrane and centrifuging the column for 1 min.

All centrifugation steps were carried out at 17900xg at RT. The purified cDNA samples were stored at -20 °C.

2.5.3.8. qRT-PCR assay

qRT-PCR reactions were carried out using the SensiMix SYBR No-ROX Kit (Bioline, QT650-05), which is a 2x mastermix containing SYBR Green I dye, dNTPs, heat-activated Taq DNA Polymerase, stabilisers and enhancers. A qRT-PCR reaction was prepared for each gene (including the housekeeper GAPDH) in a total volume of 20 μ l (Table 2.4.) using approximately 150 ng of cDNA of a sample of interest (cell sample or adult and foetal heart control) as a template. The samples of interest were run together with the corresponding seven gene standard dilutions and a negative control, in which the template cDNA was replaced with H₂O.

All qRT-PCR reactions were prepared using the CAS-1200 automated PCR set-up robot (Corbett Research/Qiagen, Crawley, U.K.) and carried out on the Rotor-Gene™ 6000 real-time PCR cycler (Corbett Research/Qiagen). The reaction conditions were as follows: 10 min at 95 °C followed by 40 repeated cycles of 5 s at 95 °C, 15 s at 57 °C (primer annealing temperature) and 10 s at 72 °C. The fluorescence was acquired at the end of the elongation step.

Reagent	1x
2x SensiMix SYBR No-ROX	10 μ l
dH ₂ O	6 μ l
10 μ M Forward Primer	1 μ l
10 μ M Reverse Primer	1 μ l
cDNA /dH ₂ O	2 μ l
Total	20 μl

Table 2.4. Typical qRT-PCR reaction mix.

2.6. Immunofluorescence studies

For immunofluorescence analysis of fixed samples cells were cultured in plastic cell culture dishes on sterilised glass cover slips (10–20 mm diameter, grade 0; Agar Scientific, Stansted, U.K.). For immunofluorescence imaging of live cells the samples were cultured on glass-bottom dishes (MatTek Corporation, Ashland, USA).

Bright field and fluorescence images were captured using an inverted fluorescent microscope (Olympus IX71; Olympus, Japan) or a confocal laser microscope (Leica TCS SP2; Leica, Vienna, Austria). The contrast and brightness of acquired images were adjusted using the software Image J and Imaris x64 7.5.0 (Bitplane, Zurich, Switzerland).

2.6.1. Immunofluorescence analysis of fixed cell samples

2.6.1.1. Cell fixation

Cell culture medium was removed and the cells were washed once with PBS. Subsequently, the samples were incubated for 12 min at 37 °C in paraformaldehyde (4 % in PBS; Agar Scientific, Stansted, U.K.). After fixation the samples were washed three times with PBS and immunolabelled.

2.6.1.2. Immunostaining of fixed cell samples

All fixed cell samples were immunolabelled according to the following protocol:

- 1) Fixed cell samples were permeabilised and blocked simultaneously for 30 min at RT in blocking buffer (PBS, 3 % bovine serum albumin, 0.2 % Triton X-100).
- 2) Samples were incubated for 1 h at RT with primary antibodies (Table 2.5.) diluted in blocking buffer. Afterwards the cells were rinsed four times for 5 min with blocking buffer.

- 3) Cell samples were incubated for 1 h at RT in the dark with a fluorescein-conjugated secondary antibody (Table 2.6.) diluted in blocking buffer. Subsequently, the samples were rinsed three times at 5 min with blocking buffer and two times in PBS.
- 4) Cell nuclei were stained for 1 min at RT with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen). Then the cells were washed two times in PBS.
- 5) Cell samples grown on glass cover slips were mounted with mounting medium (ProLong Gold antifade reagent; Invitrogen) onto a microscope slide, left to air dry at RT overnight and then sealed with clear nail varnish.

Control samples were prepared as described above but without primary antibodies.

2.6.2. Immunofluorescence analysis of live cells

2.6.2.1. Cell plasma membrane staining with di-8-ANEPPS

Di-8-ANEPPS (Invitrogen) is a member of the ANEP (Amino/Naphthyl/Ethenyl Pyridinium) class of voltage-sensitive membrane dyes. These dyes respond to alterations of electrical potential in their environment by changes in their electronic structure, which alter the fluorescence intensity (typically 2-10 % per 100 mV) and the spectral profile of the dyes (Loew et al., 1992; Invitrogen, 2006). When bound to phospholipid membranes, di-8-ANEPPS show excitation and emission maxima of approximately 467 nm and 631 nm, respectively.

The plasma membrane of cardiomyocytes was labelled with Di-8-ANEPPS according to the protocol provided by Invitrogen. Briefly, cells were incubated for 15 min at RT in the dark with cell culture medium supplemented with di-8-ANEPPS (10 μ M) and pluronic F-127 (0.05 %; Invitrogen), a polyol which facilitates the solubilisation of water-insoluble dyes. Afterwards, the cells were washed once for 30 min with PBS (with Ca^{2+} and Mg^{2+}) to allow time for de-esterification and then imaged immediately in PBS (with Ca^{2+} and Mg^{2+}) via confocal microscopy.

Antibody	Isotype	Host	Conc.	Working dilution	Manufacturer
Oct-3/4 (C-10; sc-5279)	IgG	Mouse	200 µg/ml	1:50	Santa Cruz Biotechnology, Santa Cruz, USA
SSEA-4 (MC813; ab16287)	IgG	Mouse	1 mg/ml	1:50	Abcam, Cambridge, U.K.
Tra-1-60 (ab16288)	IgM	Mouse	2 mg/ml	1:50	Abcam
α-Actinin (EA-53; A7811)	IgG	Mouse	Ascites	1:100	Sigma-Aldrich
Caveolin 3 (ab30750)	IgG	Rabbit	0.3 mg/ml	1:60	Abcam
Bin1 (Clone 99D; B9428)	IgG	Mouse	Ascites	1:50	Sigma-Aldrich
Telethonin (G11; sc-25327)	IgG	Mouse	200 µg/ml	1:100	Santa Cruz
RyR (34C; ab2868)	IgG	Mouse	1 mg/ml	1:500	Abcam
SERCA2a (2A7-A1; ab2861)	IgG	Mouse	2.1 mg/ml	1:400	Abcam
Cav 1.2 (Cardiac type α1c; ACC-013)	IgG	Rabbit	0.8 mg/ml	1:100	Alomone Labs, Jerusalem, Israel
Phospholamban (A1; A010-14)	IgG	Mouse	1 mg/ml	1:200	Badrilla, Leeds, U.K.
Junctophilin-2 (C-term; 40-5300)	IgG	Rabbit	0.25 mg/ml	1:125	Invitrogen
Connexin 43 (AB1727)	IgG	Rabbit	0.5 mg/ml	1:50	Millipore
Myosin heavy chain (3-48; NB300-284)	IgG	Mouse	Ascites	1:500	Novus Biologicals, Littleton, USA

Table 2.5. List of primary antibodies.

Conc.: stock concentration.

Antibody	Excitation/ Emission maxima (nm)	Host	Conc.	Working dilution
Alexa Fluor 488 anti-mouse IgG	494/517	goat	2 mg/ml	1:800
Alexa Fluor 488 anti-rabbit IgG	494/517	goat	2 mg/ml	1:800
Alexa Fluor 594 anti-mouse IgG	590/617	goat	2 mg/ml	1:800
Alexa Fluor 594 anti-rabbit IgG	590/617	goat	2 mg/ml	1:800
Alexa Fluor 594 anti-rabbit IgM	590/617	goat	2 mg/ml	1:800
Alexa Fluor 647 anti-mouse IgG	650/668	goat	2 mg/ml	1:800
Alexa Fluor 647 anti-rabbit IgG	650/668	goat	2 mg/ml	1:800
Alexa Fluor 555 anti-mouse IgG	556/573	goat	2 mg/ml	1:800
Alexa Fluor 555 anti-rabbit IgG	556/573	goat	2 mg/ml	1:800

Table 2.6. List of secondary antibodies.

All secondary antibodies were purchased from Invitrogen. Conc.: stock concentration.

2.6.2.2. Cell plasma membrane staining with a fluorescent wheat germ agglutinin conjugate

Wheat germ agglutinin (WGA) is a lectin, which selectively binds to specific sugar residues, such as sialic acid and *N*-acetylglucosaminyl residues, present in the glycocalyx of cell membranes, thus it can be used for labelling of cell plasma membranes in non-permeabilised cells (Wright, 1984).

For staining of cell plasma membranes non-permeabilised cells were rinsed once with Hank's balanced salt solution (HBSS; Invitrogen) and incubated for 10 min at 37 °C with WGA-Alexa Fluor 488 (5 µg/ml; Invitrogen) or for 30 min at 37 °C with WGA-Tetramethylrhodamine-isothiocyanate (TRITC) (100 µg/ml; Sigma-Aldrich) diluted in HBSS. Subsequently, the cells were washed three times with HBSS and analysed immediately via fluorescence microscopy. WGA-Alexa Fluor 488 displays fluorescence excitation/emission maxima of 496/524 nm and WGA-TRITC of 555/580 nm.

2.6.2.3. Cell plasma membrane staining with CellMask Orange

CellMask Orange (CMO; Invitrogen) stains plasma membranes in live cells. It shows slower internalisation times (45-60 min) compared to WGA conjugates and has fluorescence excitation and emission maxima at 554 and 567 nm, respectively. For staining, culture medium was removed and samples were incubated with CellMask Orange (2.5 µg/ml) in PBS (with Ca^{2+} and Mg^{2+}) for 5 min at 37 °C. Samples were then rinsed three times with PBS (with Ca^{2+} and Mg^{2+}) and imaged immediately in PBS.

2.7. Transmission electron microscopy

2.7.1. Principle of transmission electron microscopy

Observation of structures with a microscope is defined by the Abbe diffraction limit. Ernst Abbe found in 1873 that light with wavelength λ , travelling in a medium with refractive index n and converging to a spot with angle θ will make a spot with diameter

$$d = \frac{\lambda}{2(n \sin \theta)}$$

The denominator $n \sin \theta$ is called the numerical aperture (NA) and with modern light microscope optics can reach about 1.4, giving an Abbe limit of approximately $d = \lambda/2$. Thus, taking the example of green light at around 500 nm the Abbe limit would be 250 nm. This is large when compared to most nanostructures in biological cells which have sizes on the order of sub-nm.

In transmission electron microscopy (TEM) a beam of electrons instead of light is used to illuminate the specimen. Due to the smaller wavelength [in the order of 0.025 Å (0.0025 nm) in a 200 kV TEM] of electrons it is possible to resolve much smaller objects (in the range of nanometres) than with a light microscope.

In a transmission electron microscope (Fig. 2.3) electrons are produced from an electron source such as tungsten filament or a lanthanum hexaboride crystal (LaB_6), which is heated up with current. The emitted electrons are pulled and accelerated into the column, under high vacuum, of the microscope by a high voltage at an anode plate. A condenser (a series of electromagnetic lenses and apertures) focuses the electrons into a thin beam before they pass through the specimen. Depending on the density of the specimen some of the electrons are deflected (change in phase) when they pass through the sample. After passing the specimen the partially scattered electron beam is focused by objective lenses forming an image, which is then expanded by the projector lenses onto a fluorescent screen and can be digitally recorded via a CCD camera.

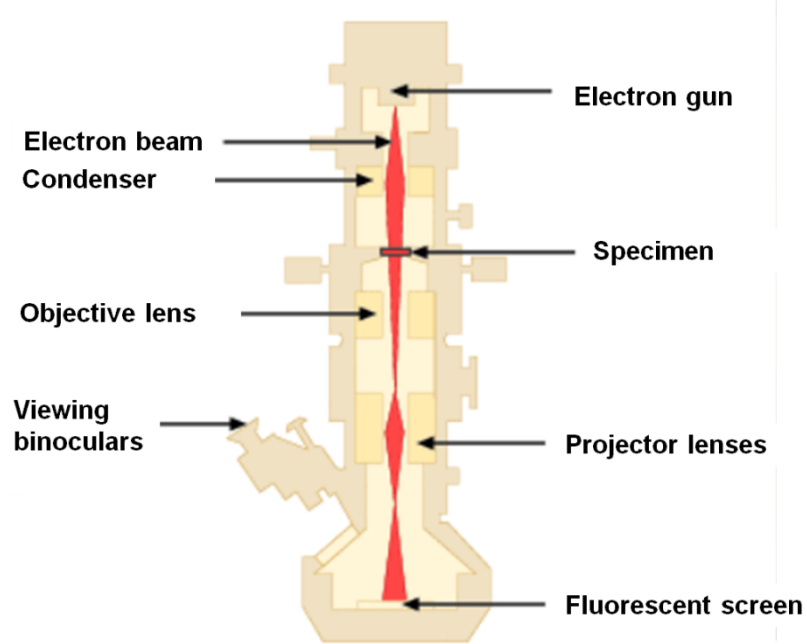


Fig. 2.3. Schematic image of a transmission electron microscope.
Adapted from learning.covcollege.ac.uk.

The contrast of TEM images depends principally on the degree of electron deflection (phase contrast) caused by the density of the specimen. Image contrast can be enhanced by staining of samples with compounds containing electron-dense heavy metals (e.g. osmium, lead, uranium or gold), which can absorb or scatter a part of the electron beam. In this study the samples were treated with osmium tetroxide (OsO_4), uranyl acetate and potassium ferrocyanide prior to TEM imaging. Osmium is primarily known as a lipid fixative because osmium oxidises unsaturated double-bonds, which are prominent in lipids (Dykstra and Reuss, 2003b), but it also stains membranes. Uranyl stains are known to interact with anionic components and to bind to phosphate groups in nucleic acids and phospholipids (Dykstra and Reuss, 2003a). Uranyl acetate is also used as both a fixative and staining compound. Post-fixation of cells in a mixture of OsO_4 and potassium ferrocyanide has been shown to enhance the staining of membrane structures such as the cell plasma membrane and endoplasmic reticulum. The exact mechanism of the staining is not known but the presence of ferrocyanide-reduced osmium intermediates seems to be essential (Hayat, 2000).

2.7.2. Fixation and standard room temperature resin embedding of cell suspensions

2.7.2.1. Trypsinisation of iCell-CMs

For obtaining cell suspensions iCell-CMs cultured in 6-well plates were treated with 0.25 % trypsin-EDTA (Invitrogen) by following procedure:

- 1) Culture medium was removed from each well and the cells were washed once with 2 ml/well PBS.
- 2) 1 ml 0.25 % trypsin-EDTA was added to each well and the plate was incubated at 37 °C for 1-2 min or until cell began to lift off the plate.
- 3) 3 ml iCell-CMs Maintenance Medium was added to each well to quench trypsin and the medium was pipetted gently up and down 1-2 times to ensure that all cells detached from the culture dish.

- 4) The cell suspension was transferred into a 15 ml centrifuge tube and pelleted with a desktop centrifuge for 5 min at 180xg.
- 5) The supernatant was removed and the cells were resuspended in 1-2 ml iCell-CMs Maintenance Medium.

2.7.2.2. Fixation of cells in suspension

Cell suspensions of freshly isolated rat adult ventricular cardiomyocytes or iCell-CMs were fixed in 2.5 % glutaraldehyde in 0.1 M sodium cacodylate buffer (glut-fixative; see Appendix) at 4 °C for 24 h. The fixative was added directly to the cell suspension medium at a ratio of 1:1. Fixed samples were stored at 4 °C.

2.7.2.3. Automated sample processing and standard room temperature resin embedding

For resin embedding approximately 1 million cells in suspension were used per sample. Before embedding in resin all cell samples were fixed in glut-fixative and embedded in 1.5 % low gelling temperature agarose in 0.1 M sodium cacodylate buffer (pH 7.4). For embedding in agarose the fixed samples were centrifuged in a 1.5 ml eppendorf tube with 0.5 ml glut-fixative and 0.5 ml agarose for 5 min at 17900xg. Subsequently, the agarose was allowed to set on ice and the agarose-embedded samples were cut into approximately 1 mm cubes. The cubes were then loaded into the EM TP automated tissue processor (Leica microsystems, Vienna, Austria), where the samples were post-fixed in osmium tetroxide, dehydrated in graded ethanol and infiltrated with an epoxy resin based on Epon 812 (TAAB, Aldermaston, U.K.). The processing steps performed during automated tissue processing are detailed in Table 2.7. Finally, after automated processing the samples were embedded in labelled capsules with freshly prepared 100 % resin and polymerised at 60 °C for 48 h.

	Processing step	Time (min)	Temperature (°C)
1	Wash in 0.1 M sodium cacodylate buffer	5	22
2	Post-fix in 1 % OsO ₄ and 1 % potassium ferrocyanide in 0.2 M sodium cacodylate buffer	120	22
3	Rinse in 0.2 M sodium cacodylate buffer	3 times, 5 min each	22
4	1 % tannic acid in 0.2 M sodium cacodylate buffer	60	22
5	1 % sodium sulphate in 0.2 M sodium cacodylate buffer	5	22
6	1 % sodium sulphate in 0.2 M sodium cacodylate buffer	10	22
7	Stain with 2 % aqueous uranyl acetate	180	22
8	Wash in H ₂ O	5	22
9	Dehydrate in 70 % ethanol	20	20
10	Dehydrate in 96 % ethanol	30	20
11	Dehydrate in 100 % ethanol	2 times, 30 min each	20
12	Dehydrate in propylene oxide	2 times, 30 min each	20
13	Resin infiltration: propylene oxide/epoxy resin mixture (1:1)	180	20
14	Resin infiltration: propylene oxide/epoxy resin mixture (1:2)	240	25
15	Resin infiltration: 100 % resin	945	25
16	Resin infiltration: 100 % resin	945	30

Table 2.7. Sample processing steps in the EM TP automated tissue processor.

2.7.3. Fixation and room temperature resin embedding of cell monolayers attached to sapphire discs

2.7.3.1. Plating of cells on sapphire discs

For TEM analysis of cell monolayers adult rat cardiomyocytes and iCell-CMs were plated on clear sapphire discs (3 mm in diameter; Leica) pre-coated with laminin (14 mg/m; Cultrex 3D Culture Matrix Laminin; R&D Systems) or gelatin, respectively. Freshly isolated rat cardiomyocytes attached to laminin-coated sapphire discs within 1 h and were fixed

immediately after attachment in 4 % formaldehyde and 0.05 % glutaraldehyde in 0.2 M HEPES (FA-fixative, see Appendix). In contrast, iCell-CMs were cultured on the sapphire discs at least for three days before being fixed in FA-fixative.

2.7.3.2. Manual sample processing and room temperature resin embedding

Cells plated on sapphire discs and fixed in FA-fixative were further processed and embedded in resin manually following a similar protocol as described for automated sample processing and standard RT resin embedding (see Table 2.7). Cells were post-fixed in OsO_4 , dehydrated by sequential washes in ethanol and gradually infiltrated with resin (see Table 2.8. for details). However, the cells were embedded into the resin block together with the sapphire discs with the cell monolayer facing inside the block. A sapphire disc was only removed from a sample once the resin has polymerised by brief immersion of the resin block in liquid nitrogen and careful lifting of the disc with a razor blade.

2.7.4. Sectioning of resin blocks

Cell samples embedded in polymerised resin blocks were cut into ultra-thin sections (with a nominal feed of 100 nm at a cutting speed of 10 mm/s) on an UC6 ultramicrotome (Leica, U.K.), using an ultra 35 diamond knife with boat (Diatome, Biel/Bienn, Switzerland). Sections were floated onto water and stretched with chloroform in order to remove any folds and creases prior to collection with a perfect loop (EMS, Germany).

2.7.5. Immunogold labelling

For the detection of specific antigens in TEM samples cell sections collected on grids were immunogold labelled using the following protocol:

- 1) Cell sections on grids were washed three times with PBS (by floating on a PBS drop) and incubated with a drop of blocking/permeabilisation buffer (see Appendix) for 20-30 min.

	Processing step	Time
1	Post-fix in 2 % OsO ₄ in 0.1M phosphate buffer (pH 6.8) on ice	25-30 min
2	Wash in H ₂ O	3 times, 1 min each
3	Add 1% thiocarbohydrazide diluted in H ₂ O	5 min
4	Wash in H ₂ O	3 times, 1 min each
5	Add a 1:1 mixture of 2 % OsO ₄ and 3 % potassium ferrocyanide	25 min
6	Wash in H ₂ O	3 times, 1 min each
7	Add 0.5 % uranyl acetate in H ₂ O and incubate at 4°C	overnight
8	Wash in H ₂ O	6 times, 1 min each
9	Dehydrate in 50 % ethanol	10 min
10	Dehydrate in 60 % ethanol	10 min
11	Dehydrate in 70 % ethanol	10 min
12	Dehydrate in 80 % ethanol	10 min
13	Dehydrate in 90 % ethanol	10 min
14	Dehydrate in 100 % ethanol	3 times, 10 min each
15	Resin infiltration: ethanol/epoxy resin mixture (2:1)	2 h
16	Resin infiltration: ethanol/epoxy resin mixture (1:1)	overnight
17	Resin infiltration: ethanol/epoxy resin mixture (1:2)	2 h
18	Resin infiltration: 100 % epoxy resin	5 h
19	Add 100 % epoxy resin and polymerise at 60 °C	overnight.

Table 2.8. Manual sample processing and resin embedding steps.

All steps were performed manually at RT except stated otherwise.

- 2) The samples were incubated for 1 h with a drop of a primary antibody (anti-Bin1 or anti- α -actinin, see Table 2.5.) diluted in blocking/permeabilisation buffer (anti-Bin1: 1:5 and anti- α -actinin: 1:10).
- 3) Samples were washed six times with PBS and incubated for 2 h in one drop of a secondary antibody, the Fab' fragment conjugated to 10 nm gold particles (BBI International) diluted 1:50.
- 4) Cell sections on grids were washed three times with PBS and left to air dry for 15 min.

2.7.6. Silver enhancement

In order to increase the size of gold particles and improve detection of particle signals the immunogold labelled samples were enhanced using the Silver Enhancement Kit from BBI International (Cardiff, U.K.) following the procedure described in the kit manual:

- 1) The samples were washed five times in distilled water.
- 2) Three drops of initiator were mixed with three drops of enhancer and one drop of the solution was added to the sample.
- 3) The reaction was left to develop for 15 min. The progress of the reaction could be monitored by placing the sample-containing grids on a microscope slide and analysing with a conventional microscope. If reaction is successful, the labelled sites will change from invisible to dark brown to black.
- 4) The reaction was stopped by washing the samples for 1-2 min in water.

2.7.7. Analysis via transmission electron microscopy

All samples were examined in the JEM 2100 transmission electron microscope at 200 kV, with magnification ranging from x6000 to x25000. Images were recorded with a Gatan 4kx4k Ultrascan CCD camera (Gatan, Pleasanton, USA) and processed using Gatan Digital micrograph (GMS2 v2.0).

The loading of specimen into the microscope and core alignments were performed by Dr. Kirsty MacLellan-Gibson. The examination of the samples was mostly performed under Dr. MacLellan-Gibson's supervision.

2.8. Microelectrode array recordings

To investigate the electrophysiological properties of hPSC-CMs their extracellular field potential (FP) was recorded using microelectrode arrays (MEAs). Small clusters of contracting Shef3-CMs or a cell monolayer of iCell-CMs were plated on pre-gelatinised MEA probes (MED64; Alpha Med Scientific, Ibaraki Osaka, Japan) consisting of 64 planar platinum black microelectrodes (70 μm in diameter) arranged in an 8 x 8 grid with an inter-electrode distance of 150 μm (Fig. 2.4) or 300 μm . Data were recorded at 10 kHz with the software MED64 Mobius 0.2.16 (MED64; Alpha Med Scientific). All recordings were made non-superfused in cell culture medium in a chamber at 37 °C, 5 % CO₂ and 60 % humidity.

2.9. Whole cell patch-clamping measurements

2.9.1. Basic overview of the patch-clamping technique

The patch-clamping technique allows low-noise recording of currents flowing through ion channels (or even only a single channel) of the plasma membrane (Zhao et al., 2008). The principle of the technique used today is that gentle suction of the cell membrane through a fine glass pipette can establish a high-resistance seal in the gigaohm range (G Ω) so that ion currents can pass only through the pipette but not through the seal. For signal detection the pipette is connected to a system of highly-sensitive amplifiers. The configuration most commonly used in cell-culture and tissue preparations is the “whole-cell” patch. In this set up stronger suction is applied to rupture the patched portion of the cell membrane so that the pipette has access to the cell interior. The cell bath and the pipette tube are filled with electrolyte solutions, which match the cellular osmolarities. This method allows the observation of currents from all ion channels within the cell or separation of specific current

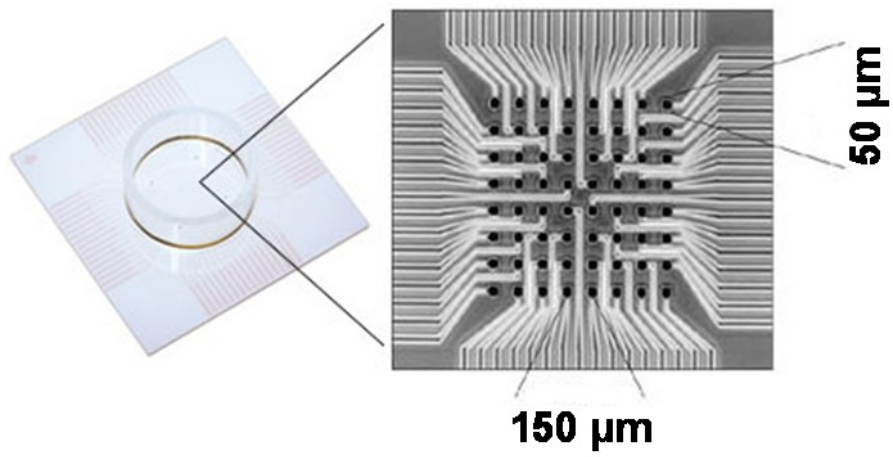


Fig. 2.4. MED64 Microelectrode array probe with an inter-electrode distance of 150 μm .

types by controlling the intra- and extra-cellular environment of the cell or by utilising specific voltage protocols. For example, to measure calcium currents the intracellular potassium can be replaced with caesium, which cannot pass through most of the potassium channels. The whole-cell patch clamping technique can be performed in two modes: a) the voltage-clamp mode, in which the membrane voltage is kept constant and the current can be measured, or b) the current-clamp mode, in which the current is controlled and alterations in the membrane potential are recorded.

2.9.2. Measurement of calcium currents

All whole-cell patch-clamping measurements were carried out by James Cartledge. For the measurements iCell-CMs were cultured on glass-bottom MatTek dishes (MatTek) at low densities (4.5×10^4 - 6.2×10^4 cell/cm²) so that Ca²⁺ current (ICa) and cell capacitance could be recorded in single isolated cells. Briefly, ICa (in pico-Amperes, pA) was measured with the patch-clamp technique in the whole cell configuration as described previously (Soppa et al., 2008) using a MultiClamp 700A patch-clamp amplifier (Axon Instruments, Union City, CA, USA). The resistance of the borosilicate glass pipette was 2-3 MΩ and the pipette-filling solution contained 115 mM caesium aspartate, 20 mM tetraethylammonium chloride, 10 mM EGTA, 10 mM HEPES, and 5 mM MgATP, adjusted to pH 7.2. The external solution contained 140 mM NaCl, 10mM glucose, 10 mM HEPES, 1 mM CaCl₂, 1 mM MgCl₂, and 6 mM CsCl, pH 7.4. Current-voltage (I-V) relationships for ICa were generated using 450-ms depolarization steps from a holding potential of -40 mV (range -40 to +40 mV, in 5mV increments) at 1 Hz. All experiments were conducted during superfusion with the external solution at 37 °C.

For specific isolation of ICa measurements were repeated in the presence of nitrendipine, a dihydropyridine inhibitor of L-type Ca²⁺ channels; the cells were perfused with 0.1 mM nitrendipine for 5 min after an initial control recording of ICa had been made in the cells without nitrendipine.

Control of the current and voltage clamp protocols, data acquisition and analysis were performed with pClamp 7 software (Axon Instruments).

2.9.3. Measurement of cell capacitance

The cell capacitance (in pico Farads; pF), which is an index for the cell membrane area, was determined using a capacitive surge technique (Fenwick et al., 1982). This consists in applying hyperpolarising pulses from -40 to -50 mV at approximately 100 Hz and fitting a monoexponential curve to the decay phase of the resulting membrane current trace. The measurement was performed on-line by the Multiclamp software (Axon Instruments) during the electrophysiological experiments.

2.10. Treatment of cells with triiodothyronine

To determine the effect of T3 on iCell-CMs the cells were incubated with 3 nM T3 for 48 h. T3 was added directly to the cell culture medium.

2.11. Transfection studies

2.11.1. Bin1-EGFP plasmid information

The Bin1-EGFP plasmid (1 µg/µl), which encodes the Bin1-EGFP (enhanced green fluorescent protein) fusion protein, was kindly donated by Dr Marta Fernandez-Fuente from The Royal Veterinary College, London, U.K. This plasmid was constructed by cloning Bin1 (muscle specific amphiphysin 2) into the backbone vector pEGFP-C1 (Clontech, Mountain View, USA; Fig. 2.5).

pEGFP-C1 codes for EGFP, which is the red shifted variant of wild-type GFP (green fluorescent protein) with excitation and emission maxima at 488 nm and 507 nm, respectively (Cormack et al., 1996). The pEGFP-C1 vector also contains the immediate early promoter of the human cytomegalovirus ($P_{CMV IE}$), which mediates the expression of EGFP in mammalian cells; a Simian Virus 40 polyadenylation signal (SV40 poly A), which ensures the proper processing of the 3' end of the EGFP mRNA; and a multiple cloning site (MCS) between the

EGFP and SV40 poly A sequence. The MCS typically contains various restriction sites allowing the insertion of a DNA fragment.

Furthermore, pEGFP-C1 codes for an SV40 origin (SV40 ori) for replication in mammalian cells, which express the SV40 T-antigen (e.g. COS-7); a neomycin resisting cassette, which is composed of the SV 40 early promoter (P_{SV40}), the kanamycin/neomycin resistance gen (Kan/Neo) and polyadenylation signals from the Herpes simplex virus thymidine kinase gene (HSV TK poly A) and permits stably transfected eukaryotic cells to be selected with the antibiotic G418; a bacterial promoter for the expression of kanamycin resistance in *E. coli*.; a pUC origin of replication (pUC ori) for propagation of the vector in *E.coli*.; and a f1 origin for single-stranded DNA production.

Bin1 was inserted into the multiple cloning site (MSC) so that Bin1 is expressed as a fusion protein to the C-terminus of EGFP.

2.11.2. Transfection of cells via Lipofectamine

All transfection experiments were performed using Lipofectamine 2000 transfection reagent (Invitrogen). Lipofectamine is a cationic lipid (containing a positively charged head group and one or two hydrocarbon chains), which can significantly increase the transfection efficiency of nucleic acids (RNA or plasmid DNA) into the cells (Chesnoy and Huang, 2000; Invitrogen, 2005). It is thought that the positive head groups of the Lipofectamine lipids can a) complex with the negatively charged nucleic acids and promote their condensation and b) interact with the negatively charged surface membrane of living cells allowing the fusion of the lipid/nucleic acid complexes with the surface membrane and delivery of the nucleic acids into the cells.

For the transfection assays iCell-CMs were seeded at densities of 1×10^5 - 1.5×10^5 cells/cm² at least 7 days prior transfection. The transfection with Lipofectamine was performed according to the protocol provided by Invitrogen with a typical DNA (μ g) to Lipofectamine (μ l) ratio of approximately 1:2.5. The plasmid DNA and Lipofectamine reagent were diluted with Opti-MEM Reduced Serum Medium (Opti-MEM; Invitrogen).

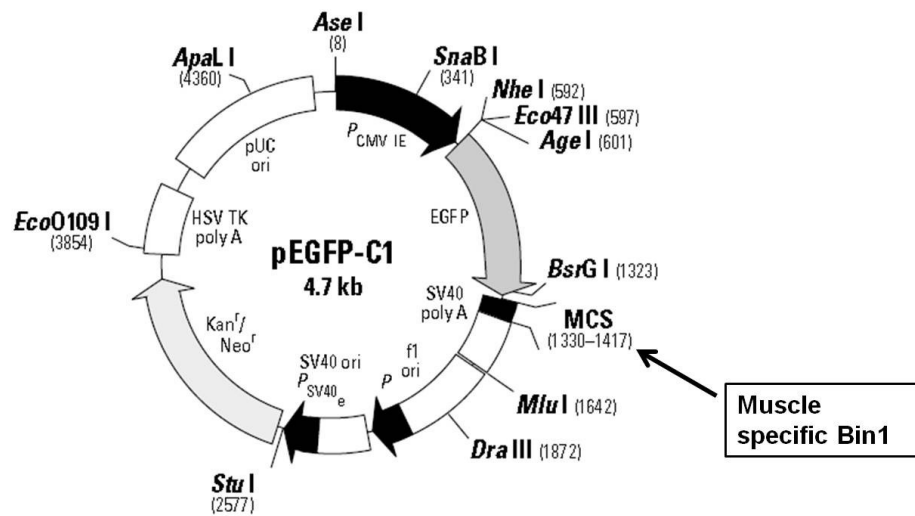


Fig. 2.5. Map of vector pEGFP-C1.

The vector encodes EGFP, the enhanced green fluorescent protein. A DNA fragment coding for the muscle-specific Bin1 was inserted into pEGFP-C1 by cloning into the MCS (multiple cloning site) downstream of the EGFP sequence. pEGFP-C1 also contains: $P_{CMV\ IE}$, the immediate early promoter of the human cytomegalovirus; SV40 poly A, Simian Virus 40 polyadenylation signal; f1 ori, f1 phage origin of replication; SV40 ori, Simian Virus 40 origin of replication; P_{SV40} , Simian Virus 40 early promoter; Kan/Neo, kanamycin/neomycin resistance gen; HSV TK poly A, polyadenylation signals from the Herpes simplex virus thymidine kinase gene; and a pUC ori, a pUC origin of replication.

The transfection experiments were typically performed in a 96- or a 24-well format using the following media volumes and reagent amounts per well:

Culture format	Volume of cell culture medium	Volume of dilution medium (Opti-MEM)	Plasmid DNA	Lipofectamine 2000
96-well	150 µl	2x25 µl	0.2 µg	0.5 µl
24-well	500 µl	2x50 µl	0.8 µg	2 µl

Table 2.9. Sample preparation for Lipofectamine transfection.

For example, for a 96-well well format the transfection samples were prepared as following for each well:

- 1) 0.2 µg plasmid DNA was diluted in 25 µl Opti-MEM and mixed gently.
- 2) 0.5 µl Lipofectamine was mixed gently, diluted in 25 µl Opti-MEM, mixed and incubated for 5 min at RT.
- 3) The diluted DNA was combined with the diluted Lipofectamine, mixed and incubated for 20 min at RT to allow complex formation between the DNA molecules and Lipofectamine.
- 4) The complex solution (total volume = 50 µl) was added to each well containing cells and cell culture medium and mixed gently by rocking the plate back and forth.
- 5) Cells were incubated at 37 °C with 4 % CO₂ for 24-48 h prior to testing for transgene expression. 4-6 h after transfection the cell medium was replaced with fresh cell culture medium.

2.12. Statistical analysis

Results were expressed as mean ± SEM. Statistical significance between means was determined using Student's t-test for unpaired samples. A p value smaller than 0.05 was considered significant. For whole-cell patch-clamping measurements “n” represents the number of single iCell-CMs and for MEA studies the number of cell clusters studies. For qRT-PCR studies “n” represents the number of qRT-PCR runs. In the figures *indicates p<0.05,

** $p < 0.01$, and *** $p < 0.001$. Statistical analysis was performed using Prism 5 software (GraphPad software, San Diego, USA).

Chapter 3: Shef3 hESC culture and cardiac differentiation

3.1. Introduction

The aim of the work in this chapter was to establish a Shef3 hESC culture and a robust cardiac differentiation protocol to generate a sufficient amount of cardiomyocytes for subsequent characterisation and maturation studies.

We chose the Shef3 line for the derivation of cardiomyocytes for various reasons: (1) this line was available and previously cultured at our laboratory at NIBSC; (2) the Shef3 line was described as being chromosomally stable over more than 180 passages (Catalina et al., 2008); (3) spontaneous beating in EB-differentiated Shef3 cultures was observed previously; (4) at the start of the project no data existed about the cardiac differentiation efficiency of the Shef3 line (as judged by beating clusters/plated EBs).

For cardiac differentiation of Shef3 hESCs initially two approaches were evaluated: 1) serum-induced differentiation from EBs (conventional EB formation) and 2) directed cardiac differentiation by sequential treatment of hESC monolayers with the growth factors activin A and BMP4 [activin A/BMP4 method; (Laflamme et al., 2007)]. Cardiac differentiation via conventional EB formation was chosen because it is a standard widely used cardiac differentiation strategy, which has been reported for many hESC lines [e.g. H1, H7, H9 and HUES7 (Xue et al., 2005; He et al., 2003; Denning et al., 2006)]. The activin A/BMP4 method was chosen because it is well-established and has been successfully used for cardiac differentiation of the H7 line at our laboratory at Imperial College. In addition, two other EB-based differentiation strategies were assessed. For both approaches EBs were initially formed by centrifugation of defined hESC numbers into microwells (AggreWell plates) in serum-free APEL medium (Stemcell Technologies). Subsequently, EBs were differentiated in a) serum-containing medium in suspension or b) in APEL medium directly in AggreWell plates with sequential addition of growth factors (activin A, BMP4, bFGF, VEGF and Dkk1) according to the stage-specific protocol developed by Stemcell Technologies (Stemcell Technologies, 2012b), which is based on the publication by Yang and co-workers (Yang et al., 2008).

In this project Shef3 hESCs were initially cultured on mitotically-inactivated MEFs, which was the routine culture method at the time in our lab at NIBSC. However, it was aimed to transfer the line to feeder-free conditions on Matrigel as this is critical for the use of the directed cardiac activin A/BMP4 differentiation method.

3.2. Methods

3.2.1. Culture of Shef3s on iMEFs

See section 2.1.1.

3.2.2. Shef3 culture on Matrigel

For the adaptation of Shef3 cells to feeder-free culture conditions on Matrigel various culture media were evaluated including MEF-conditioned medium (with addition of 8 ng/ml bFGF), which was previously prepared, and the commercially available media NutriStem (Stemgent) and mTeSR1 [Stemcell Technologies; (Ludwig et al., 2006a)], which were shown to support the growth of various other undifferentiated hESC lines on Matrigel. Shef3s were cultured and passaged as described in section 2.1.2.

3.2.3. Immunostaining

To determine whether Shef3 hESCs cultured on iMEFs or Matrigel were pluripotent the cells were immunolabelled for the pluripotency markers SSEA-4, Tra-1-60 and Oct4 (Thomson, 1998; Reubinoff et al., 2000).

3.2.4. Cardiac differentiation of Shef3s via conventional EB formation method

Shef3 colonies were cultured either on iMEFs in hESC medium or on Matrigel in mTeSR1 medium before being dissected manually into cell clumps of approximately 1 mm², cultured in

suspension in the presence of ROCK inhibitor and differentiated with serum in standard differentiation medium as described in section 2.2.1.

3.2.5. Non-EB based differentiation via activin A/BMP4 method

For the differentiation experiments, Shef3 colonies, previously grown on iMEFs in hESC medium or on Matrigel in mTeSR1 medium, respectively were passaged via manual dissection on Matrigel and cultured for 5-6 days in MEF-conditioned medium or mTeSR1 medium, respectively. Afterwards differentiation was induced according to the directed differentiation protocol with activin A/BMP4 (Laflamme et al., 2007) as outlined in section 2.2.2.

3.2.6. EB formation and differentiation via AggreWell plates

Shef3 hESCs were grown on Matrigel in mTeSR1 medium for at least five passages, before being dissociated into single cells, seeded at appropriate densities into AggreWell 400 plates and aggregated into EBs of 1000 or 2000 cells by centrifugation in the presence of ROCK inhibitor according to the protocol by Stemcell Technologies (Stemcell Technologies, 2012a). EB formation in AggreWells was performed in serum-free proprietary defined APEL medium. For cardiac differentiation the formed EBs were transferred to a low attachment plate and differentiated via the conventional EB-based differentiation protocol with serum (see section 2.2.3.2.). Alternatively, EBs were differentiated directly in the AggreWell plates in APEL medium by sequential treatment with a number of growth factor (specifically activin A, BMP4, bFGF, VEGF and Dkk1) over a course of 16 days following the directed cardiomyocyte differentiation protocol by Stemcell Technologies (Stemcell Technologies, 2012b) (see section 2.2.3.3). For this directed differentiation approach 1000 hPSCs per EB is suggested by Stemcell Technologies to be the optimal cell number for cardiac differentiation.

3.3. Results

3.3.1. Shef3 hESC culture

3.3.1.1. Culture of Shef3s on iMEFs

Vitrified colonies of Shef3 hESCs could be successfully recovered and cultured in an undifferentiated state on iMEF feeder layers in hESC culture medium. After two passages on iMEFs (dashed arrows in Fig. 3.1) by manual dissection the Shef3 colonies showed generally robust growth and many characteristics typically described for undifferentiated hESCs, including relatively uniform round cell morphology with high nuclear to cytoplasm ratio and well-defined colony boundaries (arrow in Fig. 3.1. A). Only small amounts of spontaneous differentiation occurred between two passages (6-7 days) at the edges and in the centres of some colonies, as seen by the appearance of bright multilayered colony areas (arrow in Fig. 3.1. B) or enlarged, elongated stromal-like cells (arrow in Fig. 3.1. C).

Immunofluorescence analysis revealed that most of the Shef3 cells on iMEFs expressed markers associated with pluripotency such as the nuclear expressed Oct4 (Fig. 3.2. A1-A2) and the surface antigens SSEA-4 (Fig. 3.2. B1-B2) and Tra-1-60 (Fig. 3.2. C1- C2).

3.3.1.2. Shef3 culture on Matrigel in MEF-conditioned medium

In order to establish a feeder-free Shef3 culture on Matrigel, cells grown and expanded on iMEFs were transferred by manual dissection onto Matrigel-coated plates into previously prepared MEF-conditioned medium supplemented with additional bFGF (8 ng/ml). After the first passage on Matrigel about half of the cell clumps were observed to attach. Three days after passaging the attached colonies showed large areas of undifferentiated morphology as described above. However, at the edges and occasionally the centres of virtually all colonies spontaneously differentiated fibroblast-like cells were visible. For the next passage only areas with cells showing undifferentiated morphology were excised and transferred to fresh culture

plates. However, only few cells clumps attached to the plates and cell differentiation increased dramatically (Fig. 3.1. D) preventing culture expansion generally beyond passage three.

3.3.1.3. Shef3 culture on Matrigel in NutriStem and mTeSR1 medium

Next, it was examined whether another culture medium would support the Shef3 Matrigel culture better. Undifferentiated Shef3s cultured on iMEFs were manually-dissected and transferred onto Matrigel-coated plates either into NutriStem or mTeSR1.

Shef3 cells transferred into NutriStem showed, after two passages on Matrigel, similar low attachment rates and differentiated morphology as the cultures in MEF-conditioned medium (Fig. 3.1. E). Thus, the NutriStem cultures could not be further expanded. In contrast, cell colonies in mTeSR1 displayed generally an undifferentiated and phenotypically homogeneous morphology (Fig. 3.1. F) with only few differentiated areas (arrows in Fig. 3.1. F) over more than six passages and expressed multiple antigens associated with pluripotency such as Oct4 (Fig. 3.2. D1-D2), SSEA-4 (Fig. 3.2. E1-E2) and Tra-1-60 (Fig. 3.2. F1-F2). The successfully established cultures were used in further experiments.

3.3.2. Cardiomyocyte differentiation of Shef3 hESCs

3.3.2.1. Cardiac differentiation of Shef3s via conventional EB formation method

In this section we evaluated the suitability and efficiency of the conventional EB formation method for the generation of cardiomyocytes from both the Shef3 feeder-based and the established Matrigel-based cultures in mTeSR1. For EB formation undifferentiated Shef3 colonies were manually dissected into smaller sections, cultured in suspension as cell clumps for four days in a medium containing serum and then plated on gelatin-coated plates. EBs generated via manual-dissection of colonies were heterogeneous in shape and size (Fig. 3.3. A) with aggregates of 10 μ m to 150 μ m in diameter.

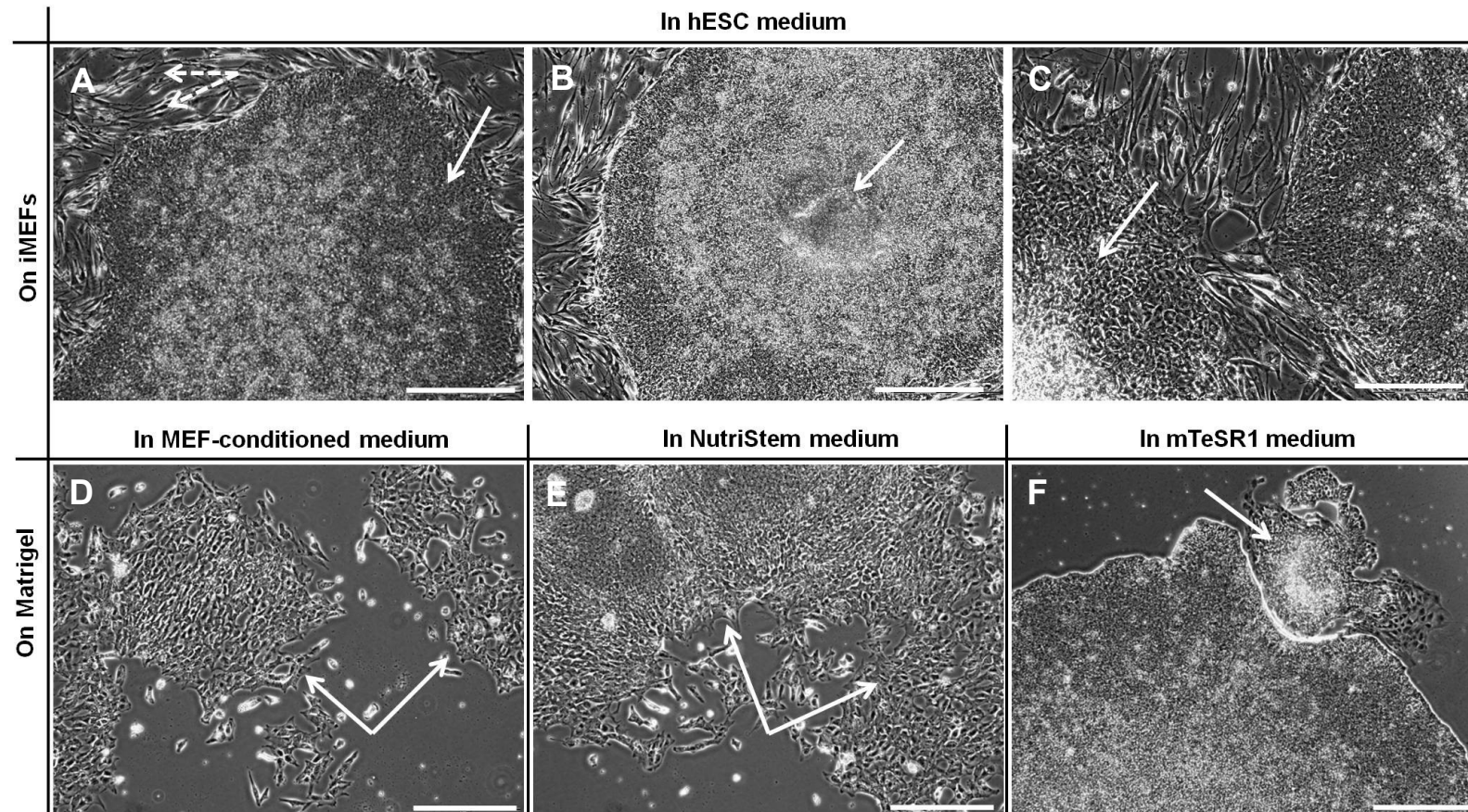


Fig. 3.1. Morphology of Shef3 colonies in different culture conditions (day 5 after passaging).

(A-C) Undifferentiated Shef3s on iMEFs in hESC medium (P44) showed compact colony structure with relatively uniform round cells (A, arrow shows Shef3 colony; dashed arrows show iMEFs). Only little spontaneous differentiation occurred in the centres and at the edges of colonies as seen by the appearance of bright multilayered colony areas (B, arrow) or enlarged stromal-like cells (C, arrow). Shef3 colonies on Matrigel (P2) cultured in MEF-conditioned medium (D) or NutriStem medium (E) revealed large areas of spontaneous differentiation with stromal-like stretched cells (D, E, arrows) at the edges and in the centres of the colonies. Shef3s on Matrigel (P2) in mTeSR1 medium (F) formed compact colonies with well defined boundaries and showed only little spontaneous differentiation (F, arrow). Scale bars 500 μ m. Bright field.

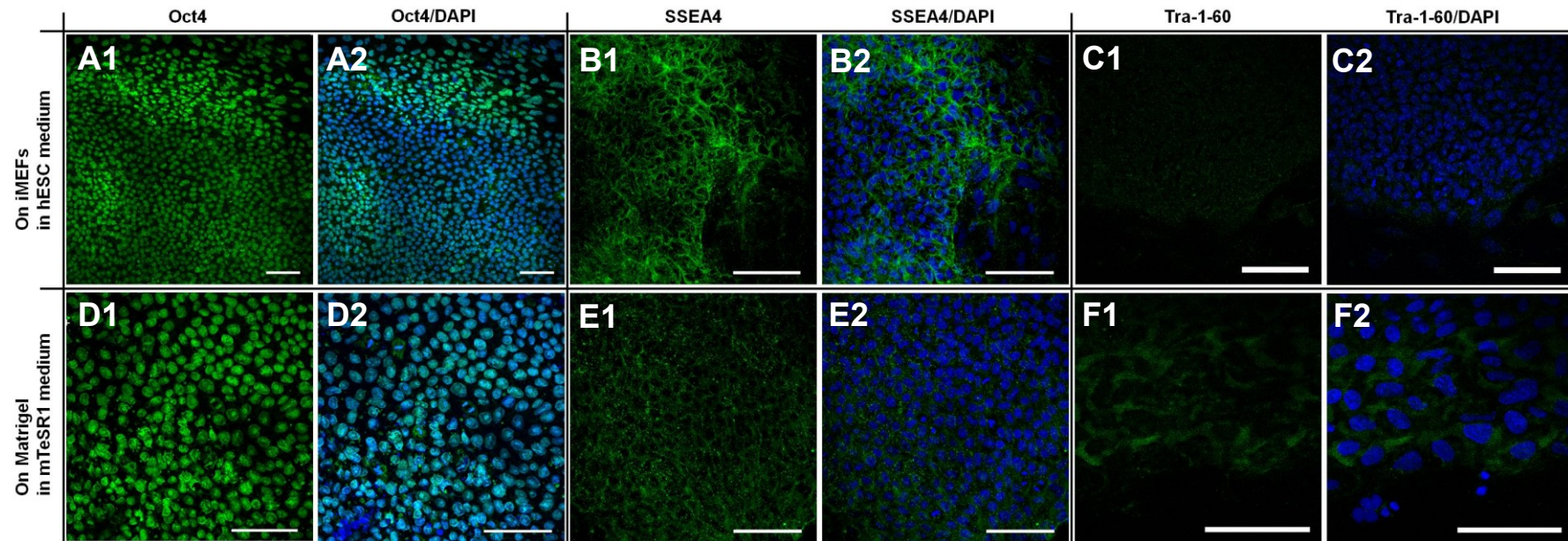


Fig. 3.2. Immunostaining of pluripotency markers expressed by Shef3 hESCs.

Shef3 hESCs, cultured on iMEFs in hESC medium (A1-C2) or on Matrigel in mTeSR1 (D1-F2), were fixed in 4 % paraformaldehyde and stained with antibodies against Oct-4 (A1-A2 and D1-D2), SSEA-4 (B1-B2 and E1-E2) or Tra-1-60 (C1-C2 and F1-F2). The secondary antibodies were Alexa Fluor 488 anti IgG (green) or Alexa Fluor 594 anti IgM (red). The nuclei were visualized via DAPI (blue). Scale bars 100 μ m.

The first spontaneously contracting cells, derived from either of the Shef3 cultures, appeared in clusters usually on day 8 after induction of differentiation and increased in numbers up to day 15. However, contracting clusters were identified in only 3-10 % of total plated EBs (Fig. 3.3. B) counted on day 15. Plated EBs could be cultured for more than three months without losing their beating capacity but we found that every three weeks it was necessary to dissect and to re-plate beating clusters since they were significantly overgrown by non-cardiac cells.

3.3.2.2. Non-EB based differentiation via activin A/BMP4 method

Due to low yields of differentiation reached by the conventional EB formation method it was investigated whether the non-EB-based directed differentiation protocol with activin A/BMP4 described by Laflamme et al. (Laflamme et al., 2007) (see 2.2.3.) was more efficient to produce cardiomyocytes from the Shef3 line. This protocol was evaluated using the Matrigel-based cultures grown in MEF-conditioned medium (short-term) or mTeSR1.

For differentiation experiments in MEF-conditioned medium Shef3s, initially grown on feeder layers, were seeded at high densities (approximately at 200×10^3 cells/cm²) on Matrigel, cultured for 4-5 days in MEF-conditioned medium and then sequentially treated with activin A and BMP4 in RPMI-B27 medium. However, due to poor adaptation of Shef3s to Matrigel culture in MEF-conditioned medium (as described in section 3.3.1.2) only half of the seeded cell clusters attached to the matrix and approximately 30 % of the attached cells differentiated spontaneously during the culture in MEF-conditioned medium. Nevertheless, by day 12 after induction of differentiation the appearance of beating clusters could be observed. However, the yield of differentiation was low with a maximum of 2 clusters per 400×10^3 cells seeded. This experiment was repeated three times.

For differentiation experiments with mTeSR1-cultures on Matrigel Shef3 cultures were used, which were passaged in mTeSR1 for at least three passages. Shef3s were seeded in high densities on Matrigel-coated plates as described above but instead of MEF-conditioned medium the cells were seeded in mTeSR1 to ensure high attachment rates. Directed differentiation was induced 5-6 days after passaging by replacing the mTeSR1 medium with

RPMI-B27 supplemented with activin A/BMP4. The differentiation experiment was repeated four times but no beating cluster could be detected at any time.

3.3.2.3. EB formation and differentiation via AggreWell plates

The aggregation of Shef3s in AggreWells resulted in EB formation in virtually all microwells after 24 h. The formed EBs were consistently spherical in shape and relatively uniform in size (Fig. 3.3. C) at approximately 100 μm (1000 hESCs/EB) or 150 μm (2000 hESCs/EB) in diameter. Furthermore, for many EBs it was visible in phase contrast that the cells within the EBs were organised in an inner core domain and an outer layer domain (arrows in Fig. 3.3. C). Cardiac differentiation was performed via two approaches: First, EBs (1000 or 2000 hESCs/EB) were transferred from AggreWells to low attachment plates 24 h or 48 h after formation and cultured for 4 days in suspension in serum-containing differentiation medium before being placed on pre-gelatinised culture plates for attachment. This approach did not produce any beating clusters. Second, without removing the EBs (1000 or 2000 hESCs/EB) from the microwells, the EBs were further cultured in APEL medium and treated sequentially over a course of 16 days with a number of growth factors including BMP4, activin A, bFGF Dkk1 and VEGF (Stemcell Technologies, 2012b). The integrity of the EBs was examined daily using a light microscope (day 0 in Fig. 3.4. A-B; day 2 in Fig. 3.4. C-D). From day 8-10 of the differentiation procedure the EBs disintegrated increasingly and appeared darker (Fig. 3.4. E-F) suggesting that the cells underwent apoptosis. By day 16 all EBs were disintegrated and no beating clusters could be observed. All AggreWell-based differentiation approaches were repeated at least three times but no spontaneously contracting EBs were observed.

3.4. Discussion

3.4.1. Shef3 hESC culture

In this project the Shef3 hESC line was successfully maintained and expanded on iMEFs for more than 30 passages by passaging the colonies weekly via manual dissection. However,

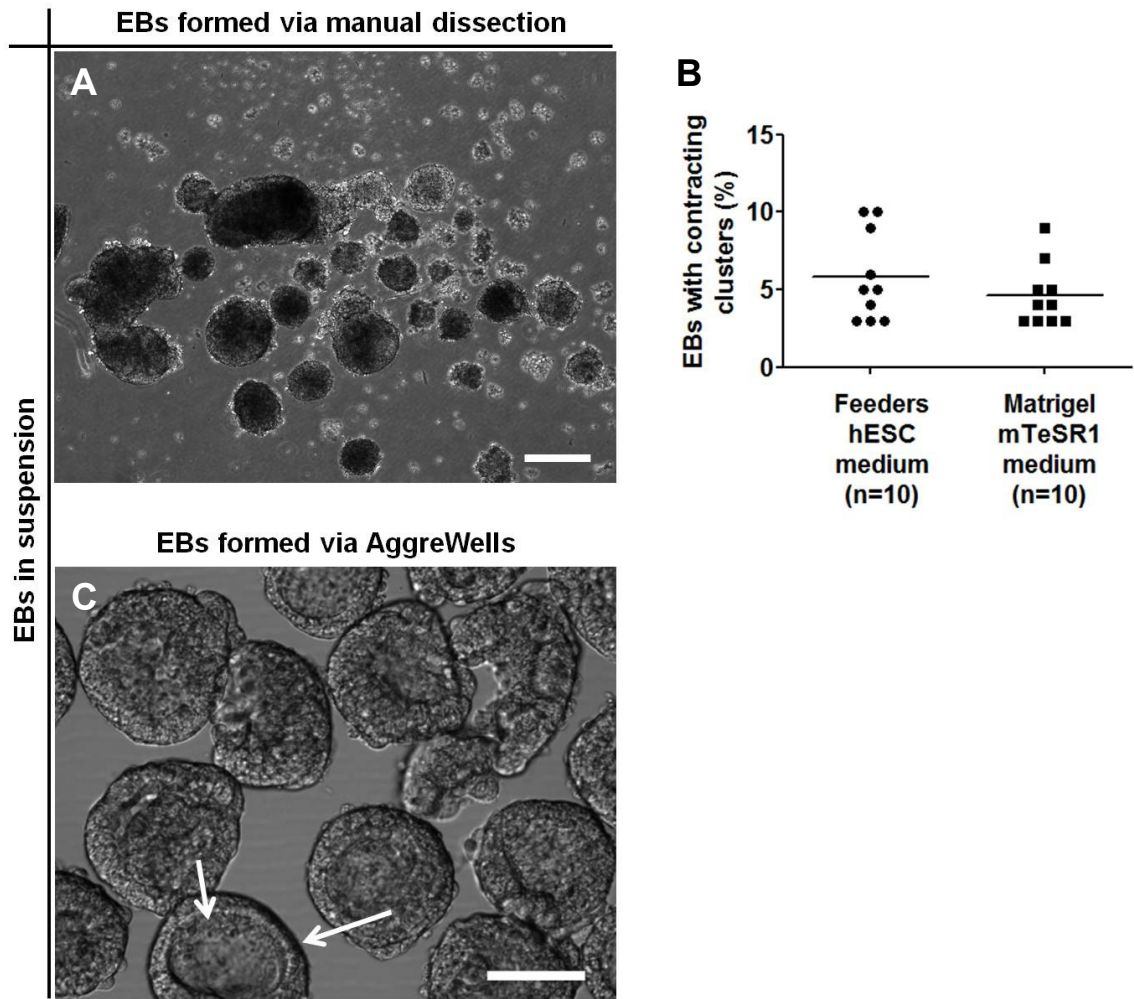


Fig. 3.3. Shef3 EBs.

(A) EBs formed via manual dissection of Shef3 colonies (grown on Matrigel in mTeSR1) differed widely in their size and shape (48 h after formation). (B) Percentage of Shef3-EBs containing spontaneously contracting clusters derived from Shef3 cultures on iMEFs in hESC medium or Shef3 cultures on Matrigel with mTeSR1 medium (counted on day 15 after induction of EB formation). n represents the number of separate differentiation experiments. (C) EBs generated via AggreWells ($400\ \mu\text{m}^2$) were predominately homogeneous in their size and shape (48 h after formation) and displayed the presence of two different domains (arrows), which were visible in phase contrast. Scale bars $100\ \mu\text{m}$. Bright field (A and C).

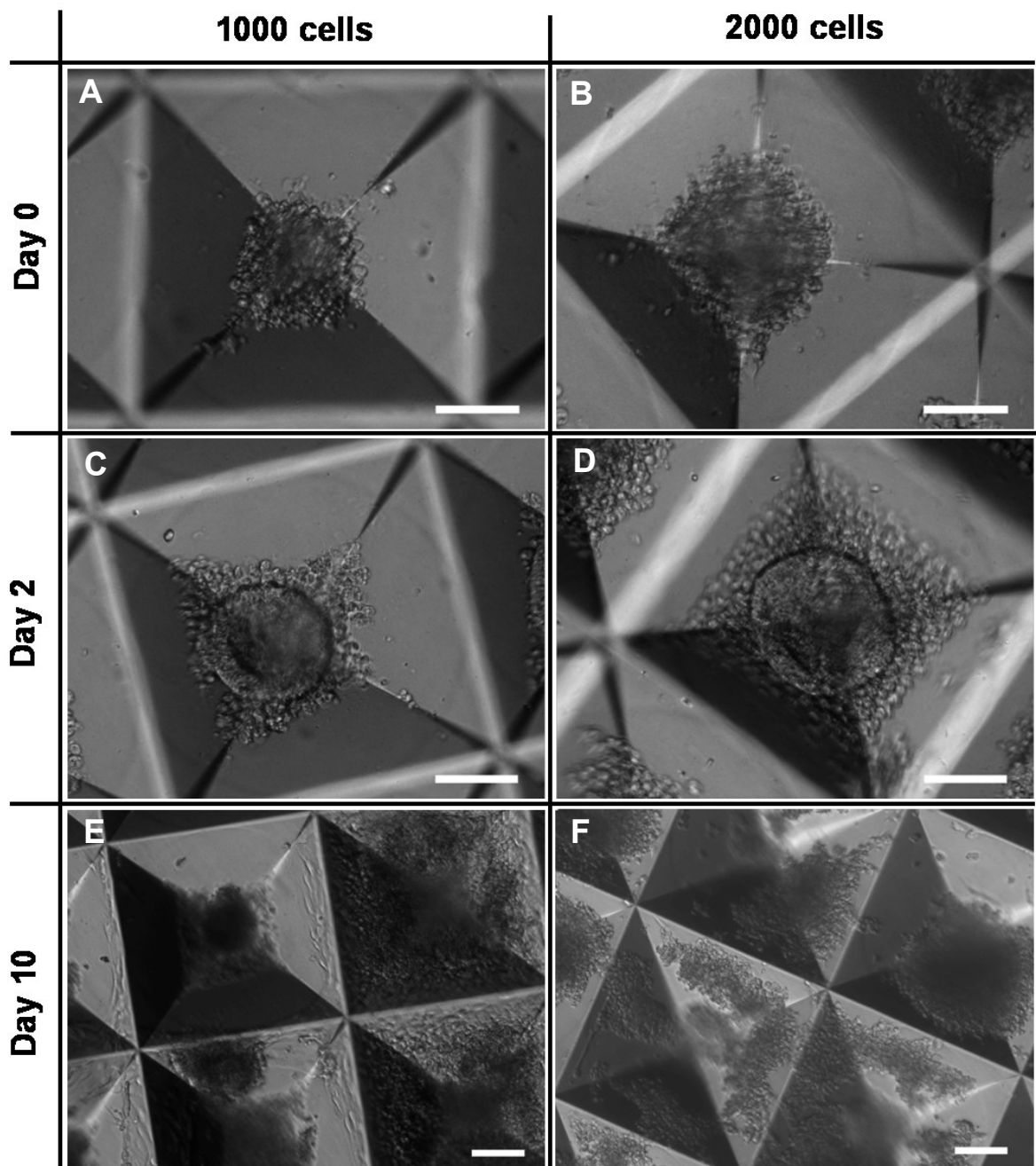


Fig. 3.4. Formation and differentiation of Shef3-EBs in AggreWell 400 with APEL medium.

EBs of 1000 (A, C and E) or 2000 Shef3 cells (B, D and F) were formed in AggreWells in APEL medium with ROCK inhibitor and treated sequentially with the growth factors BMP4, activin A, bFGF, VEGF and Dkk1 over a course of 16 days according to the cardiomyocyte differentiation protocol by Stemcell Technologies. EBs were successfully formed and cultured in the AggreWell plates for 7-9 days and monitored daily for the appearance of beating clusters. However, after day 7-9 the cells appeared dark and the EBs started to disintegrate. No beating clusters could be detected. Displayed are AggreWells with Shef3 cells at day 0 (A-B), Shef3 EBs at day 2 (C-D) and disintegrated Shef3 EBs at day 10 (E-F). Scale bars 100 μ m. Bright field.

there were difficulties in adapting the Shef3 line to a feeder-free culture on Matrigel in MEF-conditioned medium (supplemented with 8 ng/ml bFGF). This line showed poor attachment and high differentiation rate post passage on Matrigel in MEF-conditioned medium. The adaptation difficulties of Shef3 to feeder-free culture might be attributed to inherent line properties since a successful adaptation to Matrigel culture in MEF-conditioned medium (with 8 ng/ml bFGF) has been already reported for the H7 hESC line (Laflamme et al., 2007). On the other hand, Catalina et al. (Catalina et al., 2008) described in their study the establishment of a Shef3 culture on Matrigel in medium conditioned by human foreskin fibroblasts (X-ray inactivated; supplemented with 8 ng/ml bFGF). This suggests that the main problem for the poor growth of Shef3 hESCs on Matrigel was probably the MEF-conditioned medium. This medium was prepared and handled in the laboratory according to a standard protocol and the medium was conditioned on the same mitotically-inactivated MEF line, which supported undifferentiated growth of Shef3s in feeder-based culture conditions. However, the performance of MEF-conditioned medium can vary significantly batch-to-batch, this medium is not fully defined and it is not completely understood which factors, supplied by feeder cells, support undifferentiated hESCs.

Other media tested for the Matrigel culture were the fully defined, xeno-free media NutriStem (Stemgent) and mTeSR1 (Stemcell Technologies). Only mTeSR1 sustained undifferentiated Shef3 proliferation on Matrigel. To assess why the Shef3 culture could be adapted in mTeSR1 but not in NutriStem is difficult because the commercially prepared media are complex combinations of growth factors and other additives and their exact formulation is proprietary and not published. It is reported though that both media contain key growth factors such as bFGF and TGF β or activin A, which stimulate the bFGF and TGF β signalling pathways known to maintain the pluripotent state in hESCs (Ludwig et al., 2006a; Villa-Diaz et al., 2013). However, mTeSR1 presumably also contains lithium chloride (LiCl), which mediates the activation of Wnt signalling, which is also implicated in the maintenance of pluripotency (Sato et al., 1996), and γ -aminobutyric acid (GABA), which is involved in stimulation of cell proliferation (Watanabe et al., 2002; Ludwig et al., 2006b). It is not published whether GABA and LiCl are also present in NutriStem.

However, in this study mTeSR1-cultures failed to produce any beating cardiomyocytes via the activin A/BMP4 protocol, which is discussed in the next section.

3.4.2. Cardiac differentiation

3.4.2.1. Cardiac differentiation of Shef3s via conventional and AggreWell EB formation

Shef3 hESCs, cultured on feeders in hESC medium or on Matrigel in mTeSR1 could be successfully differentiated into cardiomyocytes via conventional EB formation method, although with a low efficiency of 3-10 % of beating EBs per total number of plated EBs. This differentiation efficiency is in the same range as efficiencies reported by other studies, which used other hESC-lines and observed similar low yields of beating EBs differentiated in the presence of serum (Kehat et al., 2001; Xue et al., 2005). However, there are also reports that beating clusters could be identified in significantly higher numbers of EBs (10 to 70 %) (Xu et al., 2002; He et al., 2003). One possible explanation for such variability of cardiac differentiation efficiency is that EB formation by scraping or such as in our study by manual dissection of hESC colonies is not standardised and usually results in the formation of EBs widely heterogeneous in size and shape. Studies initially performed in mESCs and later in hESCs showed however that the cardiac differentiation potential of EBs might partially depend on the EB size (Bader et al., 2001; BurrIDGE et al., 2007) because EB size and total cell number within the EB may greatly influence the micro-environment of cells within EBs (Ungrin et al., 2008). With the aim to standardise the EB formation process and to control the EB size AggreWell plates were employed in this project to produce Shef3-EBs of uniform size and shape by forced aggregation (centrifugation). Shef3-EBs were formed in the serum-free, chemically defined, low-growth factor APEL medium. For cardiac differentiation, EBs were either cultured with serum according to the conventional EB-mediated differentiation method or cultured in APEL medium with sequential addition of growth factors, which are implicated in normal cardiac development, following a protocol by Stemcell Technologies (Stemcell Technologies, 2012b). None of these methods produced beating clusters. In contrast,

Stemcell Technologies reported that their growth-factor directed cardiac differentiation protocol using EBs formed in AggreWells and APEL medium results in 5 %-95 % of beating EBs with a cardiomyocyte yield of 3.2 % (± 2.3 %) (Antonchuk et al., 2011). However, the AggreWell protocols were designed by Stemcell Technology using the H7 and the H9 hESC lines (correspondence with Stemcell Technology) so the protocol might require optimisation to achieve cardiomyocyte induction in the Shef3 line. Critical parameters, which may influence the differentiation process, include for example input cell numbers to form EBs, the medium used for EB formation, the time point of EB plating in serum-induced protocols (Boheler et al. 2002). Apart from testing two different input cell numbers for EB formation (1000 and 2000 cells/EB) the protocol was not further optimised.

3.4.2.2. Non-EB based differentiation via activin A and BMP4

The non-EB-based activin A/BMP4 differentiation approach is reported to be considerably more efficient than conventional EB formation yielding consistently more than 30 % cardiomyocytes as shown with the H7 line (Laflamme et al., 2007). In this study this method generated only a few beating clusters with the Shef3 line. This is most likely due to the poor adaptation of Shef3s to Matrigel in MEF-conditioned medium (discussed in section 3.4.1), which prevented the establishment of a high-density hESC Matrigel culture essential for this method. In order to reach the high-density culture of Shef3s the cells were seeded on Matrigel in mTeSR1, which supported the growth of Shef3s in an undifferentiated state, and cardiac differentiation was induced according to the actA/BMP4 protocol. However, this approach was not successful as no spontaneously beating cells could be detected. mTeSR1 was described to contain large amounts of bFGF (100 ng/ml) and a relatively high concentration of insulin (0.023 g/l). Whilst bFGF and insulin can maintain hESCs in an undifferentiated state and promote cell proliferation (Xu et al., 2005; Levenstein et al., 2006; Beattie et al., 2005; Beattie et al., 2005; James et al., 2005; McDevitt et al., 2005) both factors (in high concentrations) might also influence the differentiation potential of the cells and interfere with the activin A/BMP4 protocol. Insulin was recently suggested to block cardiac differentiation by suppressing the formation of endoderm and mesoderm and to promote the expression of neuroectoderm in differentiating hESCs (Freund et al., 2008).

3.5. Conclusion

For downstream characterisation and maturation studies a reproducible and robust differentiation protocol was required to generate sufficient amounts of cardiomyocytes from the Shef3 hESC line. A long-term Shef3 hESC culture was successfully established on iMEFs in hESC medium and on Matrigel in mTeSR1. Spontaneously beating clusters could be generated from the Shef3 cells via directed differentiation with activin A/BMP4 (only after short-term culture in MEF-conditioned medium) and via conventional EB formation method in the presence of serum. However, both methods resulted in very low yields of beating clusters. These results urged us to look for alternative sources of hPSC-CMs.

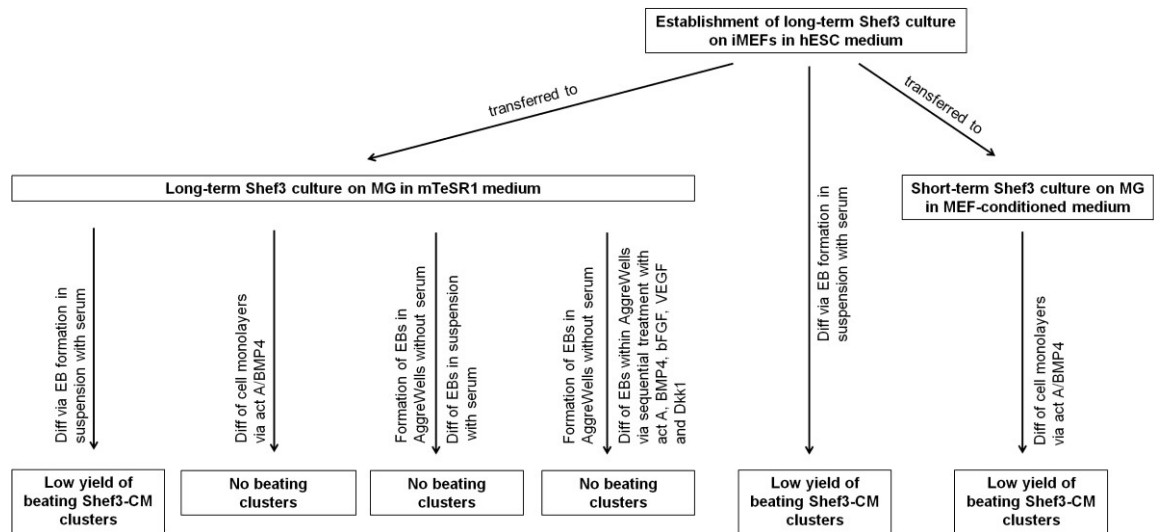


Fig. 3.5. Diagram summarising major results of chapter 3.

Cardiac differentiation of hESC monolayers via act A/BMP4 is based on the publication by Laflamme and co-workers (Laflamme et al., 2007). Cardiac differentiation of EBs within AggreWells via sequential treatment with act A, BMP4, bFGF, VEGF and Dkk1 is based on the publication by Yang et al. (Yang et al., 2008). act A: activin A, CM: cardiomyocyte, Diff: cardiac differentiation, EBs: embryoid bodies, hESC: human embryonic stem cell, MG: Matrigel, MEFs: mouse embryonic fibroblasts, iMEFs: mitotically-inactivated MEFs.

Chapter 4: Characterisation of Shef3-CMs and iCell-CMs

4.1. Introduction

In the previous chapter the challenges of Shef3 hESC culture and cardiomyocyte differentiation were discussed. Due to the difficulties in producing a sufficient quantity of pure Shef3-CMs as well as the limited access to alternative stem cell lines and the time restraints of this project it was finally decided to purchase pre-differentiated commercially produced hPSC-CMs for successive characterisation and maturation studies. In 2011 commercially produced hPSC-CMs were available from two major suppliers: GE Healthcare, which offered hESC-CMs at approximately 50 % purity, and Cellular Dynamics International (CDI), which offered hiPSC-CMs (iCell-CMs) at more than 98 % purity. Since the purity is a critical issue for studies such as qRT-PCR and TEM we decided to purchase iCell-CMs from CDI. CDI launched their iCell-CMs in December 2009. These cells are manufactured in large amounts in industrialised fashion via a CDI proprietary process (Anson et al., 2011), which includes: a) the generation of hiPSCs from human fibroblasts via a non viral-based protocol; b) standardised and scalable hiPSC culture; c) differentiation of hiPSCs into cardiomyocytes via a non EB-based directed differentiation method; d) purification of hiPSC-CMs via antibiotic (blasticidin) resistance gene, which is under the control of the cardiac α MHC promoter. The purified population of iCell-CMs represents a mixture of spontaneously beating atrial, nodal, and ventricular-like myocytes.

iCell-CMs are advocated as suitable cardiac model systems for drug discovery and cardiotoxicity assays (Anson et al., 2011). However, as described in section 0. hPSC-CMs are generally reported to be relatively immature compared to adult cardiomyocytes. For example, their gene expression pattern resembles that of developing cardiomyocytes (Gai et al., 2009; Synnergren et al., 2008), they are structurally immature as evident by the lack of TTs (Gherghiceanu et al., 2011; Snir et al., 2003) and they display immature electrophysiological features (Ma et al., 2011).

In this chapter the aim was to examine key molecular features, the overall ultrastructural cellular organisation and the basic electrophysiological properties of iCell-CMs. These characteristics may serve as a base line for the subsequent maturation studies. An additional aim was to analyse the cardiac protein expression in Shef3-CMs via immunofluorescence

microscopy. A more comprehensive characterisation of these cells was not possible due to the low numbers of the generated beating Shef3-clusters and the heterogeneity of the cell population within the beating Shef3-clusters.

During the analysis of morphology and (ultra)structure iCell-CMs and Shef3-CMs were compared to freshly isolated adult rat ventricular cardiomyocytes due to the lack of access to adult human ventricular cardiomyocytes. Adult rat ventricular cardiomyocytes demonstrate a similar morphology and (ultra)structure compared to their human counterparts, with all components of the excitation-contraction coupling system reproduced, as well as having a particularly well-formed TT system.

4.2. Methods

4.2.1. iCell-CMs culture

For detailed culture method see section 2.3. All iCell-CMs were cultured for no longer than approximately two weeks.

4.2.2. Culture of Shef3-EBs

See section 2.2.4.

4.2.3. qRT-PCR

For qRT-PCR experiments iCell-CMs were seeded into 6-well plates at densities of approximately 3×10^5 cells/well. For details see section 2.5.

4.2.4. Bright field and immunofluorescence analysis

For bright field and immunofluorescence microscopy beating outgrowths of Shef3-EBs, which were generated from Shef3 cultures on iMEFs via conventional EB formation, were

microdissected and enzymatically dispersed into smaller clusters or single cells. iCell-CMs were plated as single cells at low density. Freshly isolated adult rat cardiomyocytes, which were used as control cells, were seeded on laminin-coated plates and were not cultured for more than one day.

4.2.5. Plasma membrane staining

Cell plasma membranes of live cells were stained either via: a) WGA-conjugated to Alexa Fluor 488, which binds to specific sugar residues in the glycocalyx of cell membranes; b) the voltage-sensitive membrane dye Di-8-ANNEPS or c) the lipid dye CellMask Orange (CMO). Cells were not permeabilised prior to staining and incubated with the membrane dyes as recommended by the manufacturer's protocol to ensure that only the outer cell membranes were stained.

4.2.6. Transmission electron microscopy

For TEM iCell-CMs were embedded in resin either via a standard RT resin embedding method using cell suspensions or via RT resin embedding using cells attached to sapphire discs. Freshly isolated adult rat ventricular cardiomyocytes were used as controls.

4.2.7. Micro electrode array studies

For electrophysiological studies iCell-CMs were plated at low density (30×10^3 cells/well) on MEAs and their field potential was measured. To analyse the effect of caffeine and thapsigargin on iCell-CM contraction, 1 mM and 10 mM caffeine and 1 μ M and 10 μ M thapsigargin, respectively, were applied sequentially directly into the cell culture medium. The specified drug concentrations have been reported in various studies to be sufficient to affect hPSC-CMs (Sartiani et al., 2007; Liu et al., 2007a).

4.3. Results

4.3.1. Culture of iCell-CMs and Shef3-CMs and analysis via bright field microscopy

iCell-CMs were cultured as monolayers on gelatin-coated plates at densities of 3×10^4 - 6.5×10^4 cells/cm² and formed after 2-4 days in culture spontaneously-contracting syncytia with a rhythmic beating rate at 40-80 beats per minute (bpm). Shef3-EBs showing spontaneously contracting clusters (with beating rates of 27-74 bpm) were cultured on gelatin-coated plates with serum in standard differentiation medium.

Analysis via bright field microscopy showed that the morphology of dispersed Shef3-CMs and iCell-CMs differed significantly from adult rat cardiomyocytes, which were used as mature cardiomyocyte controls. Dispersed Shef3-CMs (Fig. 4.1. A) and iCell-CMs (Fig. 4.1. B) had a spindle, round, or multi-angular shape, usually displayed a “fried egg morphology” with a roughly spherical elevation (nucleus) centred on a flatter apron (Itzhaki et al., 2006) and were relatively flat (approximately 6 μ m at the nucleus and 3 μ m at the periphery). Adult rat cardiomyocytes on the other hand were approximately 10-20 μ m thick and displayed rod-shaped morphology and a characteristic regular striation pattern (Fig. 4.1. C). Shef3-derived cardiomyocyte were usually mononuclear whilst cultures of iCell-CMs showed mono- as well as binuclear cells.

4.3.2. qRT-PCR

qRT-PCR was used to examine the expression profiles of selected cardiac gene transcripts in order to elucidate the developmental stage of iCell-CMs. The analysed genes included: a) general cardiac markers and structural proteins; b) ion channels, which modulate the contractile activity of cardiomyocytes; c) genes coding for SR Ca²⁺ handling components, involved in mature ECC and d) genes coding for proteins implicated in the development or maintenance of TTs, a hallmark of mature ventricular cardiomyocytes. An overview of all analysed genes is given in Table 4.1. Gene expression levels measured in iCell-CMs were

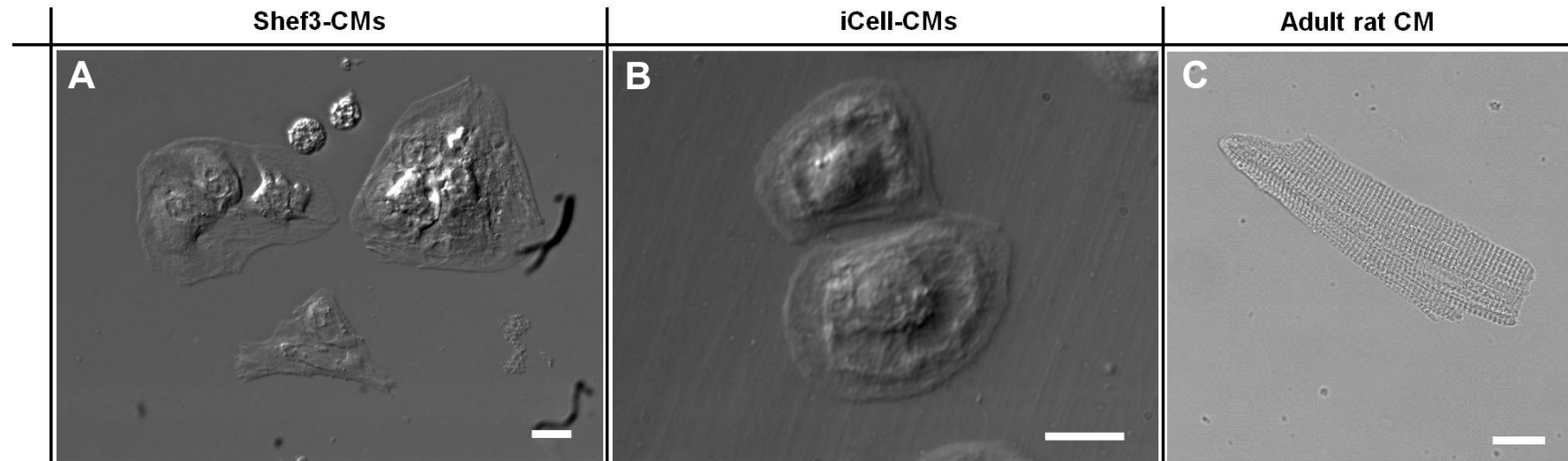


Fig. 4.1. Differences in cell morphology of adult cardiomyocytes and hPSC-CMs.

Dispersed single cells or small clusters of Shef3-CMs (A) and iCell-CMs (B), which were both approximately 30 days old, displayed similar roughly round or multi-angular morphology and no visible banding pattern. (C) An adult ventricular rat cardiomyocyte showed rod-shaped morphology and a highly aligned striation pattern throughout the cell typical for mature cardiac cells. Scale bars 20 μm . Bright field.

Contractile proteins and general cardiac markers	Gene	Encoded protein information
	α MHC	Alpha myosin heavy chain
	β MHC	Beta myosin heavy chain
	MLC2v	Cardiac ventricular myosin light chain 2
	cTnT	Cardiac troponin T type 2
	Nkx2.5	Cardiac tissue-restricted homeobox-containing transcription factor, early cardiac marker
	GATA4	Cardiac-specific zinc-finger transcription factor, early cardiac marker
Ion channels	ANP	Atrial natriuretic peptide
	Cav1.2	Alpha 1S pore-forming subunit of voltage-dependent L-type calcium channel
	Cav3.1	Alpha 1G subunit of voltage-dependent T-type calcium channel
	NCX1	Sodium/calcium exchanger
	Kir2.1	Inward rectifier potassium channel 2, responsible for IK1 current
	Kv4.3	Voltage-gated potassium channel subunit Kv4.3, mediates transient outward potassium currents Ito1
SR calcium handling components	RyR2	Cardiac ryanodine receptor 2
	IP3R2	Inositol 1,4,5-trisphosphate receptor type 2; transcript variant predominantly expressed in human ventricular cardiomyocytes (Kockskamper et al., 2008)
	SERCA2a	Cardiac sarcoplasmic reticulum calcium ATPase 2
	PLN	Phospholamban
	CSQ2	Cardiac calsequestrin 2
T-tubule associated genes	Bin1	Bridging integrator 1 (muscle specific)
	Cav3	Caveolin 3
	JP2	Junctophilin 2
	Tcap	Titin-cap, telethonin

Table 4.1. List of genes analysed via qRT-PCR.

SR: sarcoplasmic reticulum.

compared to gene expression levels measured in foetal (FH) and adult human heart (AH) controls.

The majority of the gene expression profiles measured in iCell-CMs resembled foetal heart controls. However, a number of genes were closer to adult controls or corresponded neither to the foetal nor to the adult phenotype. qRT-PCR analysis showed that in iCell-CMs the expression levels of early immature cardiac markers such as Nkx2.5 and GATA4 corresponded to the expression values detected in FH and were notably higher than in the AH controls (Fig. 4.2. A). Similarly, mRNA levels of the contractile proteins α MHC, β MHC and MLC2v correlated with the expression levels detected in FH: the expression of β MHC and MLC2v was approximately two-fold lower than in AH whereas the expression levels of the α MHC isoform were close to the values detected in AH. In contrast, the expression of cTNT, which regulates cardiac muscle contractility as part of the troponin complex, did not correlate with FH. Its expression corresponded to the AH levels being two-fold higher than in FH.

qRT-PCR analysis of a set of ion channels (Fig. 4.2. B) exhibited that both L-type Ca^{2+} channels (referred to as Cav1.2), which play a crucial role in ECC coupling in mature cardiomyocytes, and T-type Ca^{2+} channels (referred to as Cav3.1) were notably higher in iCell-CMs and FH controls relative to AH. The expression of Cav1.2 was approximately two-fold and that of Cav3.1 twenty-fold higher than in the AH controls. The expression of NCX1 in iCell-CMs and in FH expression levels correlated with the expression values detected in AH. In contrast the expression of Kir2.1 was markedly lower in iCell-CMs with mRNA levels five times lower compared to FH and more than ten times lower compared to the AH samples.

Gene expression analysis of major SR Ca^{2+} handling elements (Fig. 4.2. C) in iCell-CMs delivered diverse results. On the one hand, the expression levels of RyR2 and CSQ2 were considerably lower relative to the adult and foetal controls. On the other hand, the expression of SERCA2a was two-fold higher than in FH and close to the levels measured in AH. Moreover, the expression of PLN, which regulates SERCA2a, was two-fold higher in iCell-

CMs compared to AH and five-fold higher compared to FH. The expression of the SR Ca^{2+} channel IP3R2 corresponded to the expression levels of the FH controls.

Assessments of the expression profiles of TT-associated genes (Fig. 4.2. D) such as Bin1 and Cav3 demonstrated that their mRNA levels in iCell-CMs were significantly below the FH and AH controls. The expression of Bin1, which was shown to induce membrane invagination in muscles (Lee et al., 2002), was 10-fold lower than in FH and 30-fold lower than in AH. The expression level of the caveolae membrane protein Cav3 was almost 40-fold and 20-fold lower compared to FH and AH, respectively. In contrast, the expression of JP2 was almost five-fold and three-fold higher in iCell-CMs and FH, respectively, relative to the adult heart samples. The expression of Tcap correlated with the levels of FH and was approximately five-fold lower than in AH.

4.3.3. Immunofluorescence studies

4.3.3.1. Expression of sarcomeric and Ca^{2+} handling proteins

iCell-CMs and EB-derived Shef3-CMs were identified and characterised via immunostaining against cardiac markers including the myofibrillar protein α -actinin and SR components such as RyR2 and SERCA2a. Adult rat ventricular cardiomyocytes were used as controls.

The fluorescence images of spontaneously contracting Shef3 clusters dissected from EBs revealed that EB-derived cells represented α -actinin expressing myocytes (Fig. 4.3. A1-A4) but also cell types, which were α -actinin negative (arrows in Fig. 4.3. A2) confirming that EB aggregates did not exclusively contain cardiac cells. In contrast, the highly purified iCell-CMs showed consistent staining for α -actinin for virtually all cells (Fig. 4.3. B1-B2). Both cell types exhibited α -actinin stained fibres with characteristic sarcomeric striation, which were organised in separated bundles but also cell regions with rather disorganised α -actinin staining (arrows in Fig. 4.3. A3 and B1). In contrast, adult rat cardiomyocytes displayed a continuous highly aligned α -actinin banding pattern throughout the depth of the cell (Fig. 4.3. C1-C2). Negative controls, which did not receive a primary antibody (Fig. 4.3. D-F), showed

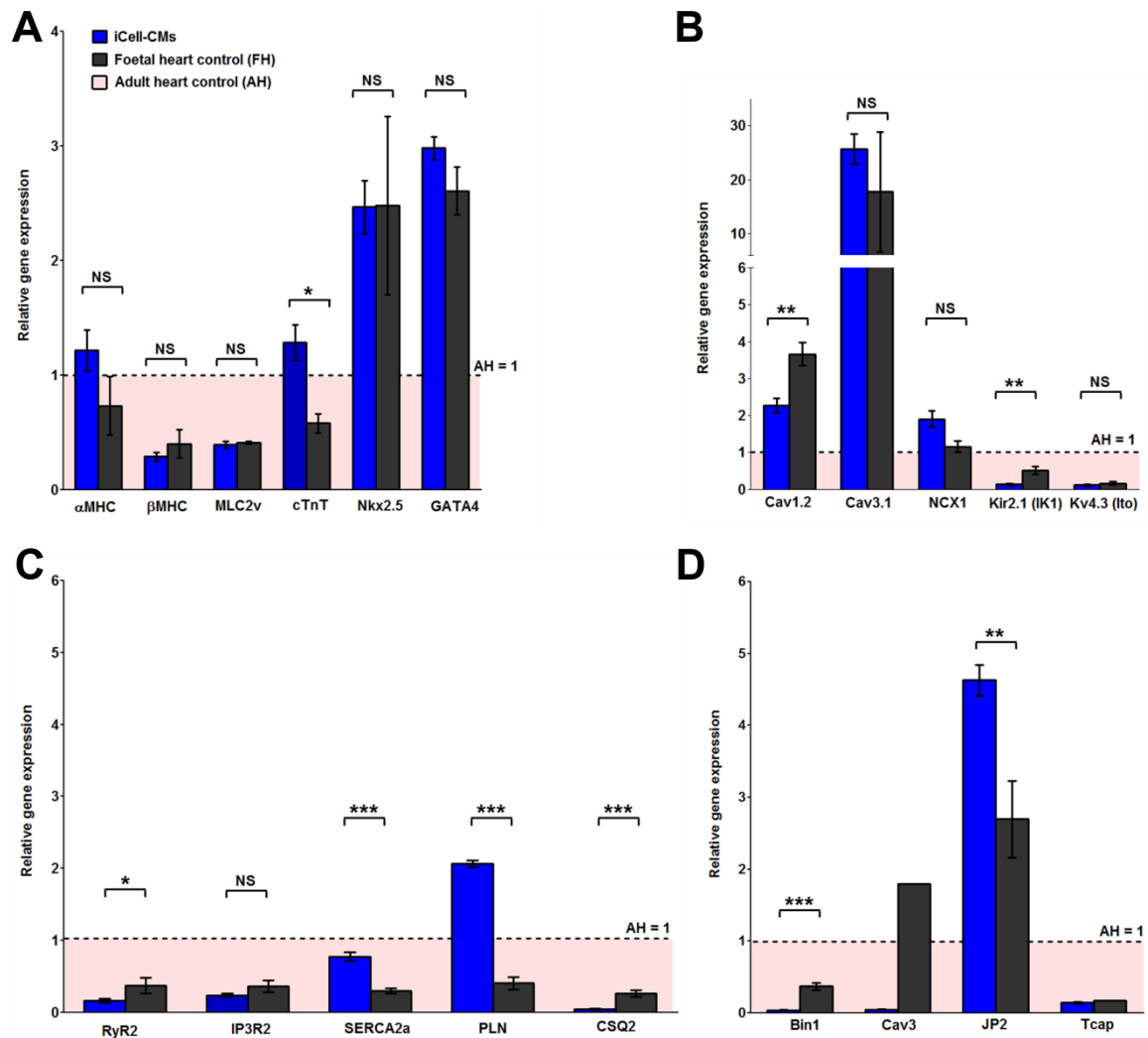


Fig. 4.2. mRNA expression of cardiac genes in iCell-CMs evaluated via qRT-PCR.

The graphs display mRNA expression levels of structural proteins and general cardiac markers (A), ion channels (B), genes coding for SR Ca^{2+} handling components (C) and genes coding for proteins implicated in TT development (D). qRT-PCR results from iCell-CM samples were obtained in triplicate (represent three independent culture preparations) and results from human foetal (FH) and adult heart (AH) controls in singlets, in $n=2$ qRT-PCR runs. All values were normalized with respect to GAPDH and shown relative to the corresponding values in AH (AH=1). Error bars represent $\pm$ SEM. Statistical significance between the two means was determined using unpaired Student's t-test (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

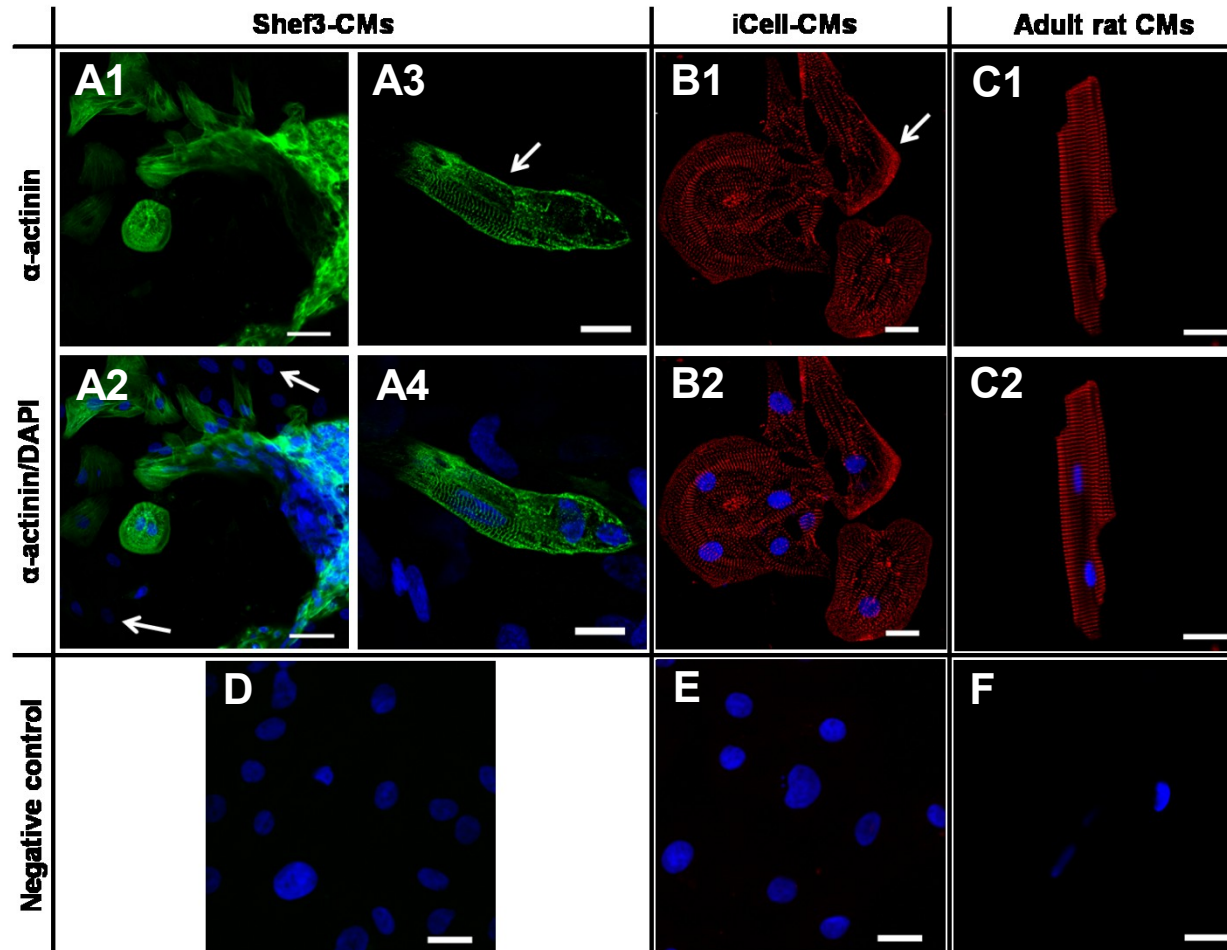


Fig. 4.3. Immunostaining against sarcomeric α -actinin.

The image shows Shef3-CMs (A1-A4), iCell-CMs (B1-B2) and an adult rat ventricular cardiomyocyte (adult rat CMs; C1-C2), which were stained with anti- α -actinin as primary antibody (A1-C2). The secondary antibody was Alexa Fluor 488 (green) and Alexa Fluor 647 (red), respectively. Negative controls received a secondary antibody but no primary antibody (D, E and F). Arrows in A2 show α -actinin negative cells within the Shef3-CM cluster. Arrows in A3 and B1 show cell areas with disorganised α -actinin staining pattern. Cell nuclei were stained with DAPI (blue). Scale bars 20 μ m except A1-A2 with 50 μ m.

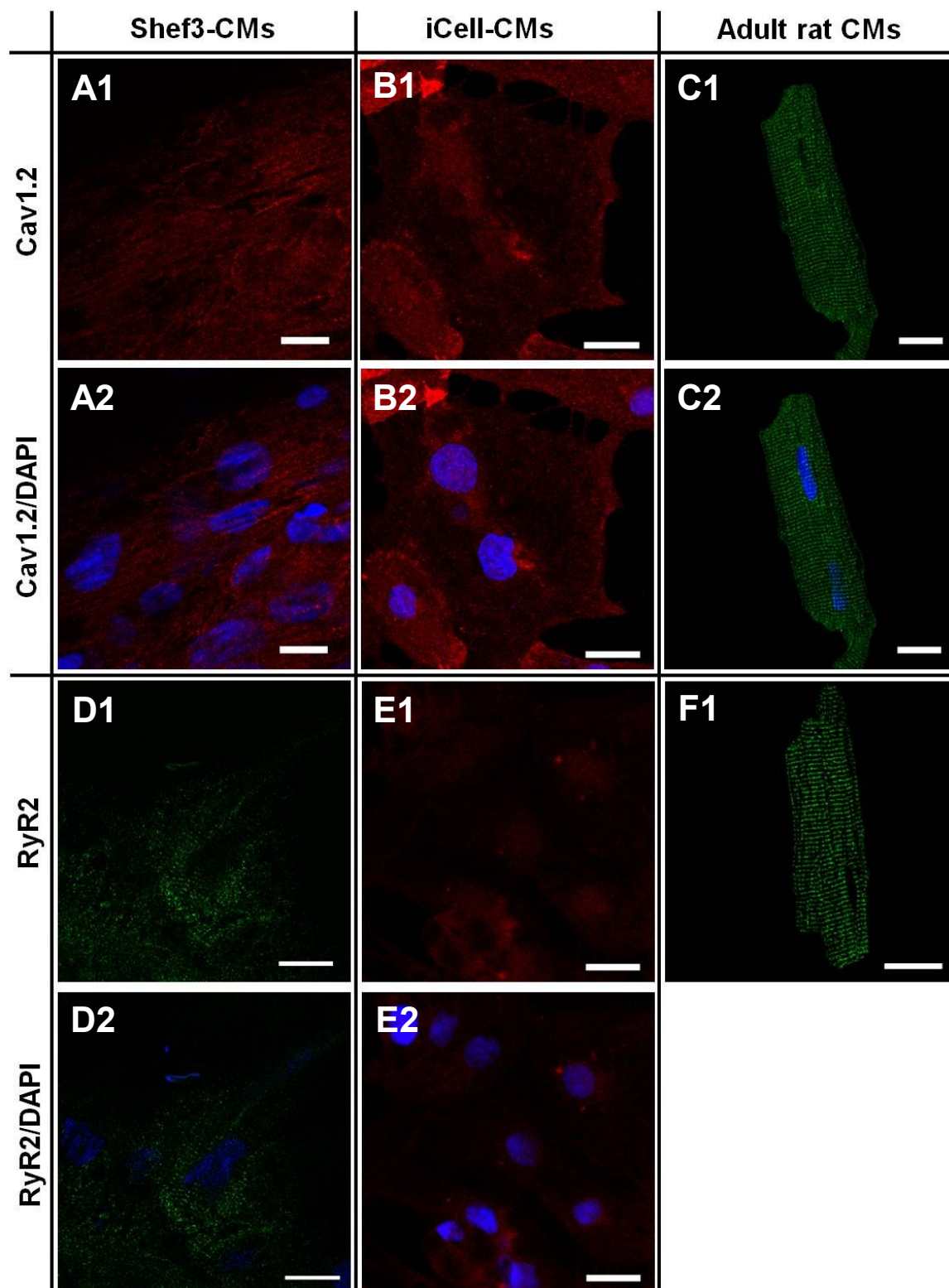
no fluorescence staining confirming that immunolabelling with the secondary antibody was specific.

Next the expression of Ca^{2+} handling molecules such as Cav1.2 and the SR components RyR2, SERCA2a and PLN was evaluated. As displayed in Fig. 4.4. all of these proteins could be detected and showed similar expression in Shef3-CMs and iCell-CMs. Immunolabelling of the sarcolemmal Cav1.2 showed punctate staining without a distinct pattern in both Shef3-CMs (Fig. 4.4. A1-A2) and iCell-CMs (Fig. 4.4. B1-B2). Expression of RyR2 (Fig. 4.4. D1-D2 and E1-E2), SERCA2a (Fig. 4.4. G1-G2 and H1) and PLN (Fig. 4.4. J1 and K1-K2) was exhibited throughout the cytosol of Shef3-CMs and iCell-CMs, respectively. However, the fluorescence signals for RyR2 were noticeably weaker compared to SERCA2a and PLN. PLN staining showed in some cell regions a defined banding pattern (arrows in Fig. 4.4. J1 and K1). In contrast, adult rat controls displayed a characteristic striated staining for Cav1.2 (Fig. 4.4. C1-C2), RyR2 (Fig. 4.4. F1), SERCA2a (Fig. 4.4. I1-I2) and PLN (Fig. 4.4. L1). The regular striation in the adult cells reflects the high degree of structural organisation of mature cardiomyocytes with highly aligned myofibrils, SR and TT structures.

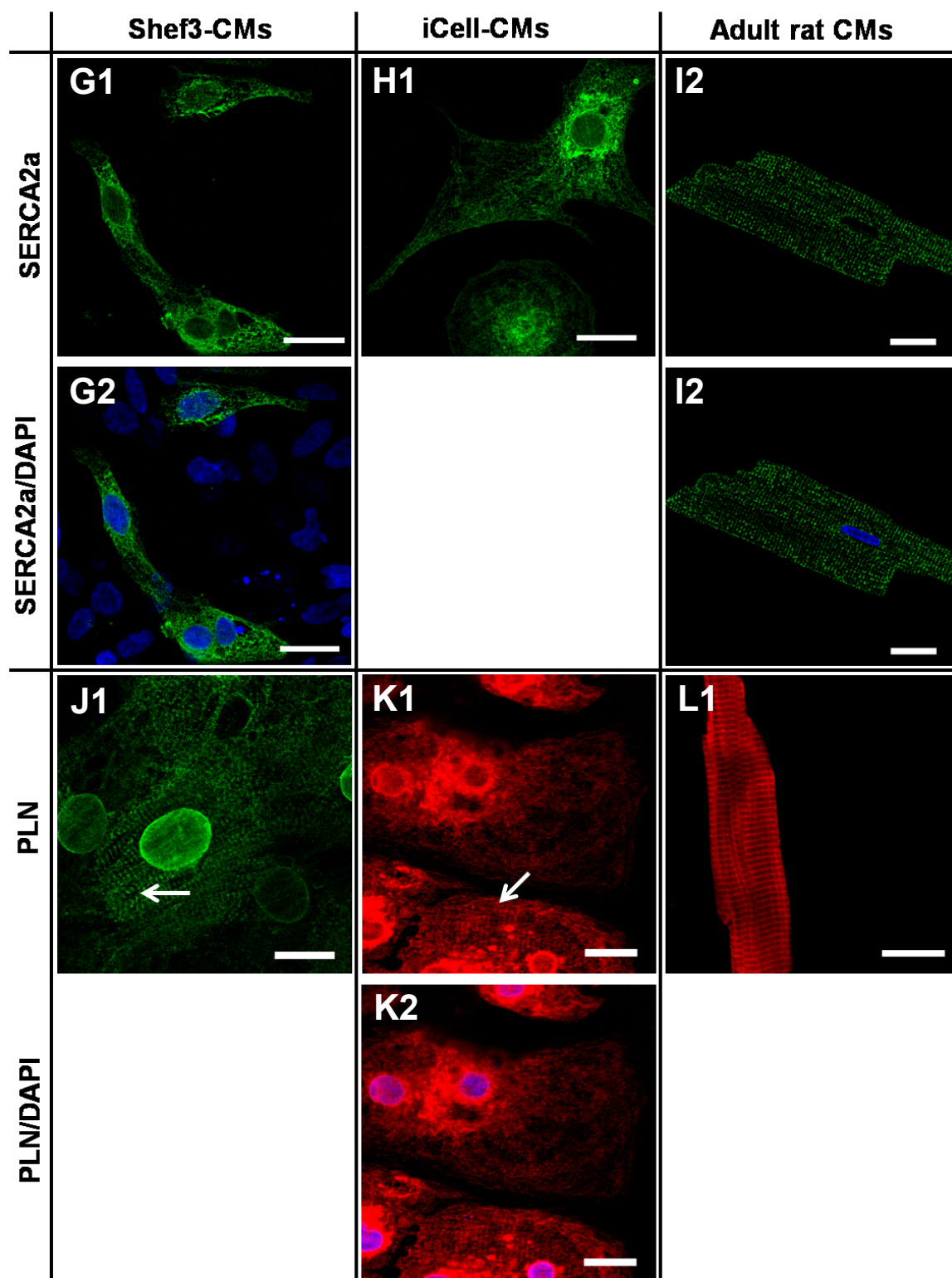
Double-immunostaining for Cav1.2 and RyR2 in iCell-CMs showed no visible spatial association of the two proteins (Fig. 4.5. A1-A3) suggesting that iCell-CMs did not possess any Cav1.2/RyR2 Ca^{2+} release units (couplons). On the contrary, adult rat cardiomyocytes showed overlapping of the Cav1.2 and RyR2 fluorescence signals throughout the cells (Fig. 4.5. B1-B3).

4.3.3.2. Staining of the cell plasma membrane

In order to reveal the presence of any TT invaginations non-permeabilised Shef3-CMs, iCell-CMs and adult rat cardiomyocytes were incubated with WGA-Alexa Fluor 488 (Fig. 4.6. A-C), which binds specific sugar residues in the sarcolemmal glycocalyx. However, since hPSC-CMs may not express the same repertoire of sugar components in their glycocalyx as adult cells iCell-CMs and adult rat cardiomyocytes were also stained with the membrane-dyes CMO (Fig. 4.6. D-E) and Di-8-ANNEPS (Fig. 4.6. F-G).



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Fig. 4.4. Immunostaining for Ca²⁺ handling proteins.

Clusters of Shef3-CMs (A1-A2, D1-D2, G1-G2 and J1), iCell-CMs (B1-B2, E1-E2, H1 and K1-K2) and adult rat ventricular cardiomyocytes (adult rat CMs; C1-C2, F1, I1-I2 and L1) were stained with anti-Cav1.2 (A1-C2), RyR2 (D1-F1), SERCA2a (G1-I2) and PLN (J1-L1) as primary antibodies. The secondary antibodies were either Alexa Fluor 488 (green) or Alexa Fluor 647 (red). Arrows in J1 and K1 depict cell regions showing a striated PLN staining pattern. Nuclei were stained with DAPI (blue). DAPI staining is missing in adult rat CMs stained for RyR2, in iCell-CMs stained for SERCA2a and in Shef3-CMs and adult rat CMs stained for PLN. Scale bars 20 μ m.

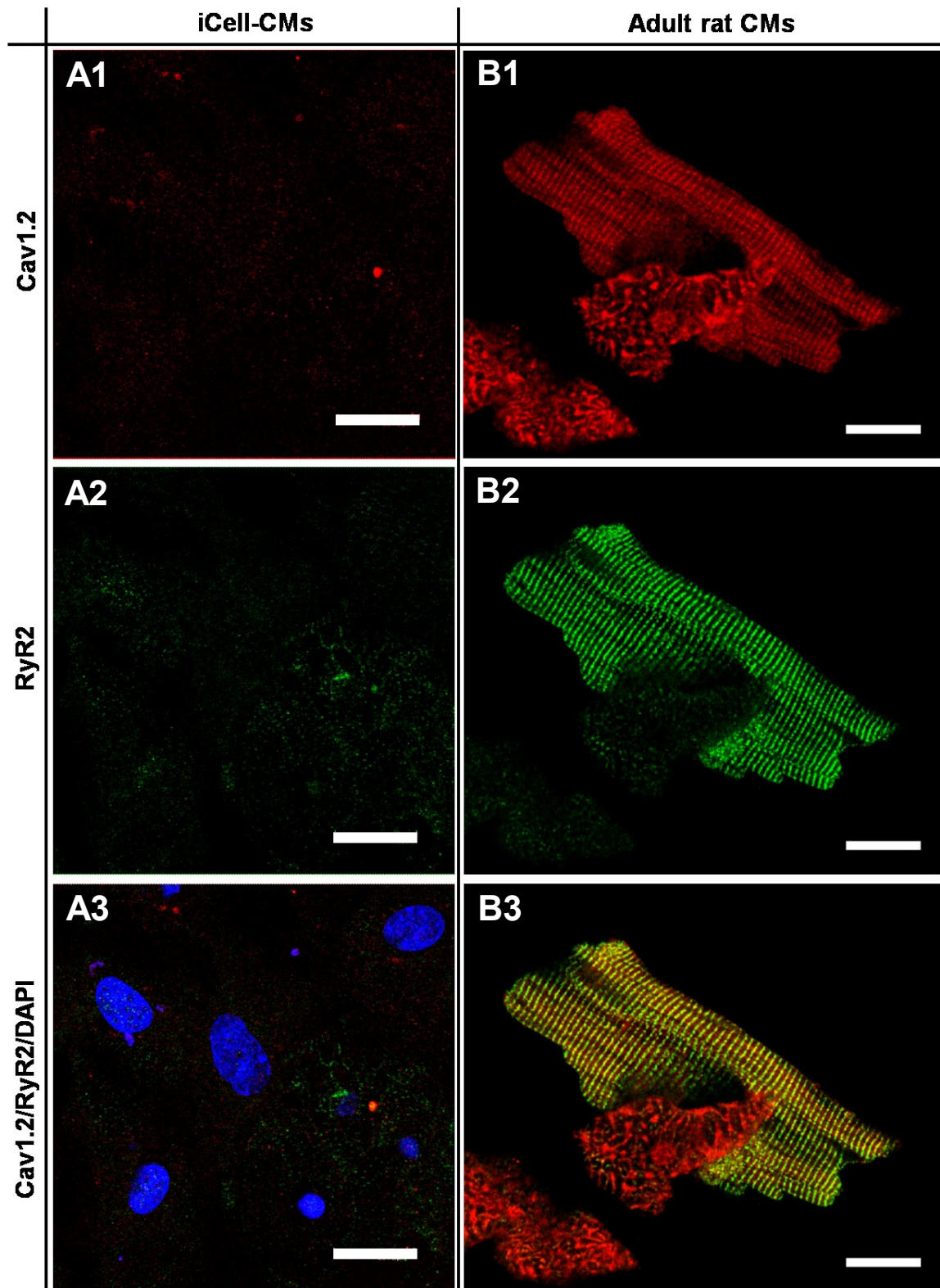


Fig. 4.5. Double immunostaining for Cav1.2 and RyR2.

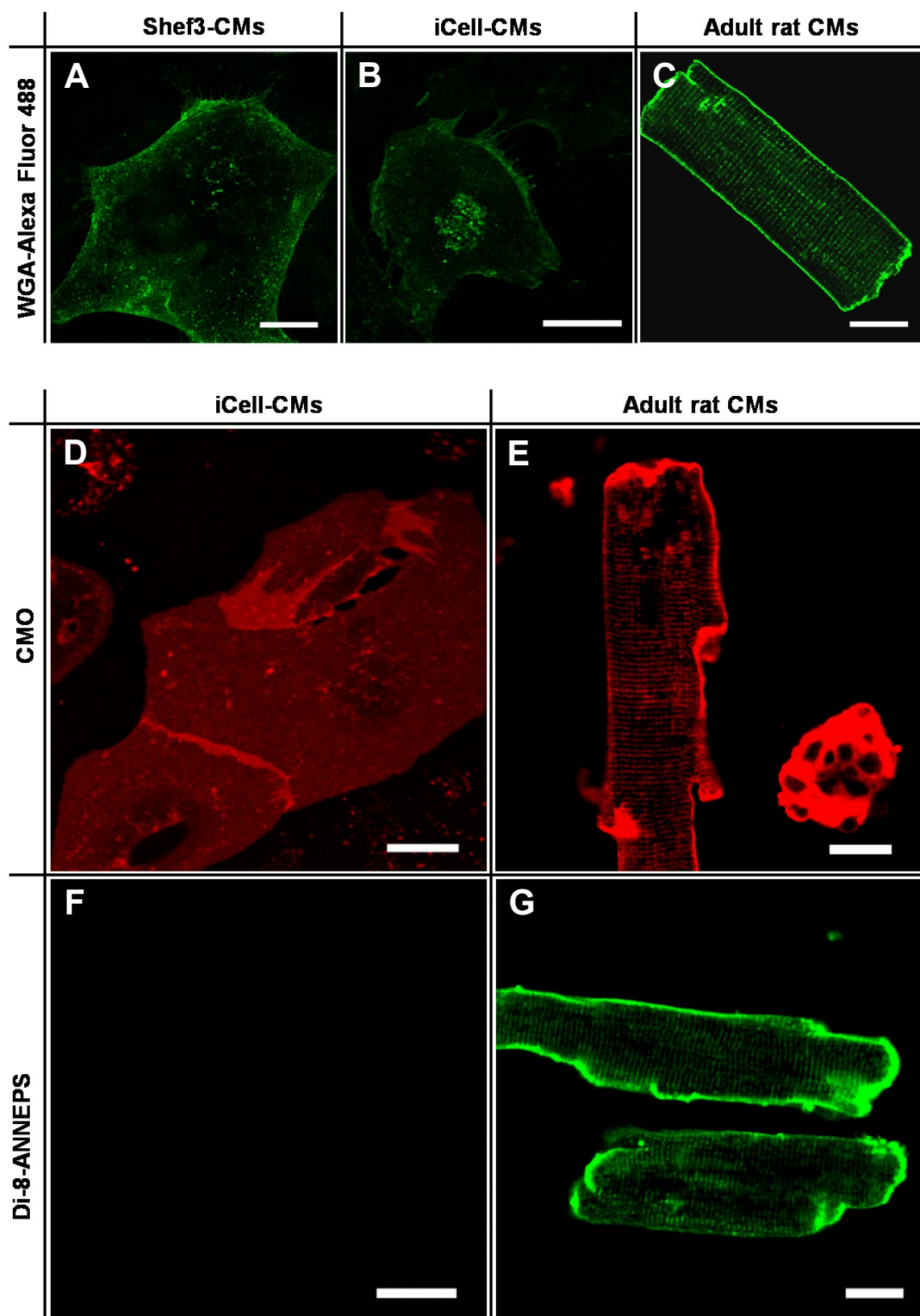
iCell-CMs (A1-A3) and adult rat cardiomyocytes (adult rat CMs; B1-B3) were double-stained with the primary antibodies anti-Cav1.2 (A1, B1, A3 and B3) and anti-RyR2 (A2, B2, A3 and B3). The secondary antibodies were Alexa Fluor 488 (green) and Alexa Fluor 647 (red). iCell-CMs were nuclear counterstained with DAPI (blue). Scale bars 20 μ m.

In Shef3-CMs (Fig. 4.6. A) and iCell-CMs (Fig. 4.6. B) staining with WGA-Alexa Fluor 488 resulted in occasional fluorescent dots but no fluorescent tubular structures penetrating the membrane were detected within the cells. Similarly, labelling of iCell-CMs with CMO (Fig. 4.6. D) also revealed infrequent punctual staining but no tubular invaginations. In further immunofluorescence studies with Di-8-ANNEPS no fluorescent signal could be detected in iCell-CMs (Fig. 4.6. F). The mature rat cardiomyocytes exhibited for all three dyes a typical striation pattern (Fig. 4.6. C, E and G) resulting from the diffusion of the dye into the TT invaginations, which penetrate the cell membrane in regular intervals.

4.3.3.3. Staining of iCell-CMs for TT-associated markers and other cardiac proteins

For further characterisation iCell-CMs were stained for proteins associated with TT development using antibodies against Bin1, Cav3, JP2 and Tcap. The cells were also labelled for the gap junction protein connexin 43. As shown in Fig. 4.7. A1-A2 immunolabelling against Bin1 in iCell-CMs gave a weak fluorescent signal, which appeared predominantly in the cell nuclei. Adult rat cells stained against Bin1 (Fig. 4.7. B1-B2) showed the characteristic banding pattern but also a nuclear expression. Cav3 and JP2 proteins could also be detected in iCell-CMs and displayed a punctuate expression pattern (Fig. 4.7. C1-C2 and E1-E2). The expression of the Z-disc protein Tcap could also be confirmed (Fig. 4.7. G1-G2) and some iCell-CMs showed a striated banding pattern. However, the staining intensity was low. In stark contrast, adult rat cardiomyocytes consistently showed intense positive staining for Cav3 (Fig. 4.7. D1-D2), JP2 (Fig. 4.7. F1-F2) and Tcap (Fig. 4.7. H1-H2), which was organised in regularly spaced bands.

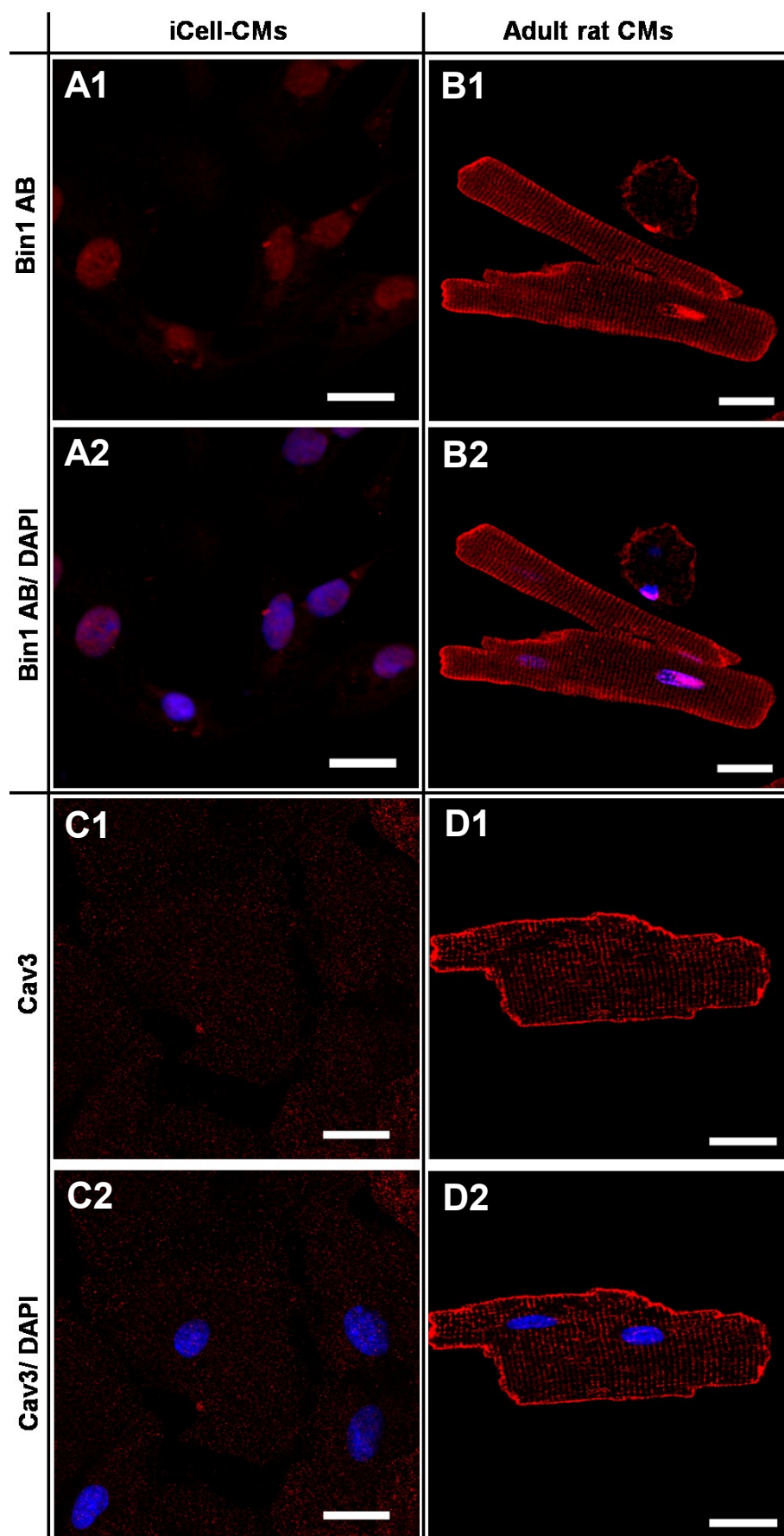
Connexin 43 in iCell-CMs was preferentially expressed at cell borders to neighbour cells (Fig. 4.8. A1-A2), though some staining could be visible randomly over the whole cell plasma membrane. Nevertheless, the concentrated expression at the cell-cell junctions suggests that iCell-CMs could couple electromechanically with neighbouring cells. In adult rat cardiomyocytes connexin 43 was predominantly expressed at the cell poles (Fig. 4.8. B1-B2).



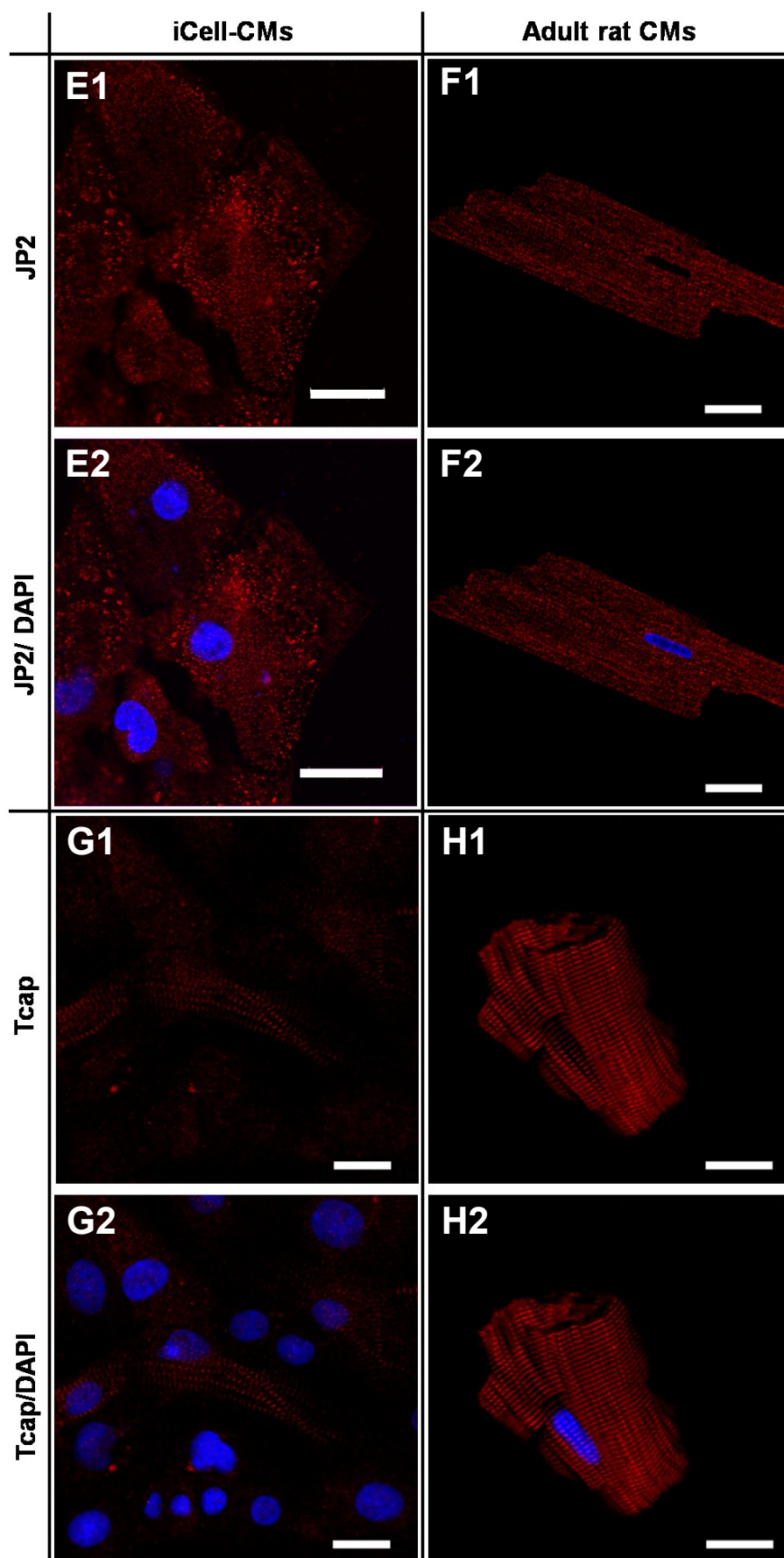
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Fig. 4.6. Staining of the cell plasma membrane.

Shef3-CMs (A) and iCell-CMs (B) stained with WGA-Alexa Fluor 488 showed fluorescent spots but no tubular like staining, suggesting that no TT invaginations were present. The absence of TTs in iCell-CMs was further confirmed by staining with CellMask Orange (CMO; D), which also did not reveal tubular shaped fluorescent structures. iCell-CMs labelled with Di-8-ANNEPS (F) did not reveal any detectable fluorescence. Adult rat cardiomyocytes (adult rat CMs) labelled with WGA-Alexa Fluor 488 (C), CMO (E) or Di-8-ANNEPS (G) showed for all dyes a regular striated staining pattern revealing its highly aligned TTs. Scale bars 20 μm .



For figure legend see page 135.



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Fig. 4.7. Expression of TT-associated proteins in iCell-CMs and adult rat cardiomyocytes.

iCell-CMs (A1-A2, C1-C2, E1-E2 and G1-G2) and adult rat cardiomyocytes (adult rat CMs; B1-B2, D1-D2, F1-F2 and H1 and H2) were stained with anti-Bin1 (Bin1 AB; A1-B2), caveolin 3 (Cav3; C1-D2), junctophilin 2 (JP2; E1-F2) and Tcap (G1-H2) as primary antibodies. As secondary antibodies either Alexa Fluor 488 (green) or Alexa Fluor 647 were used (red). The cell nuclei were stained with DAPI (blue). Scale bars 20 μ m.

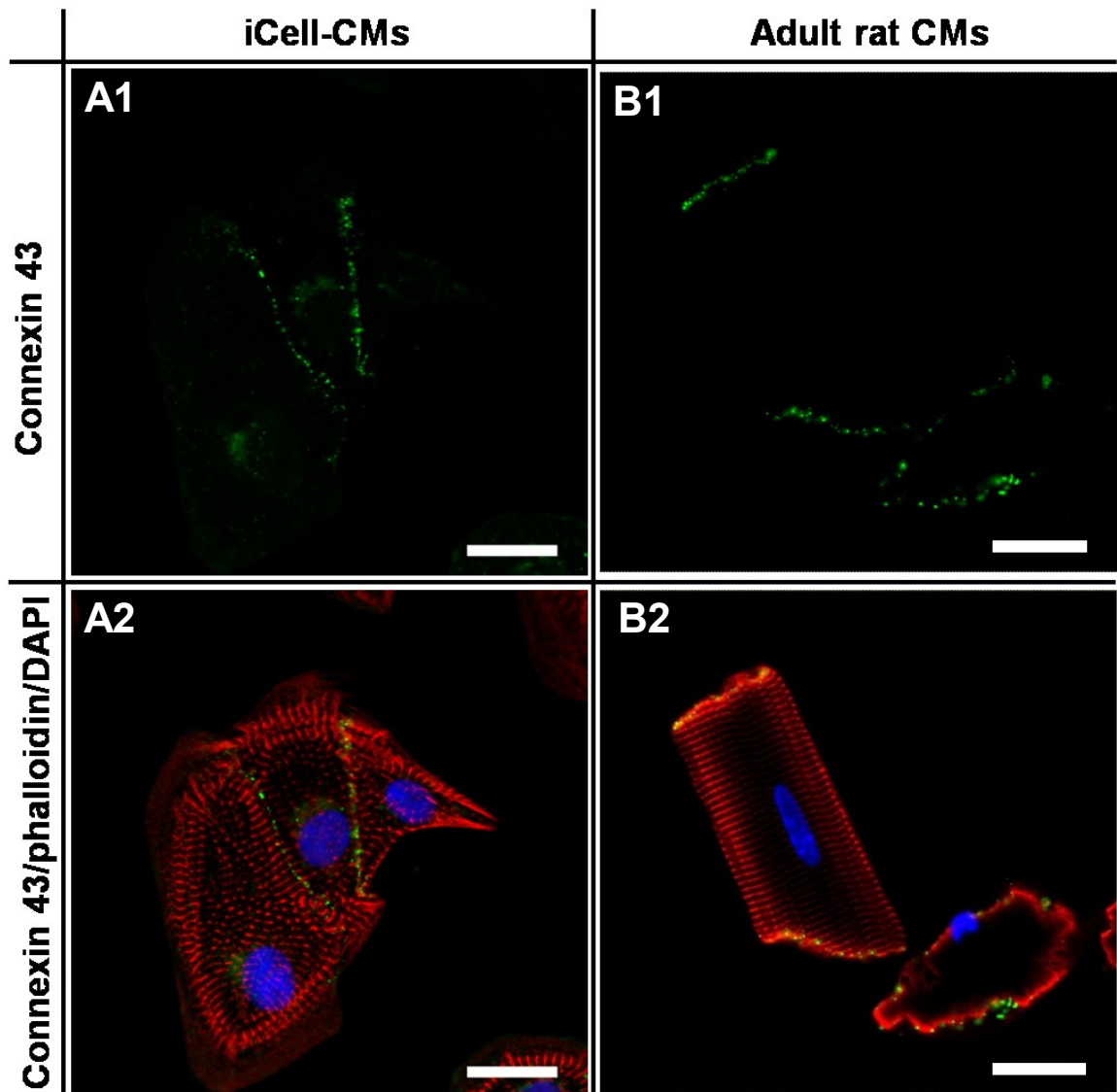


Fig. 4.8. Expression of connexin 43 in iCell-CMs and adult rat cardiomyocytes.

iCell-CMs (A1-A2) and adult rat cardiomyocytes (adult rat CMs; B1-B2) were labelled with the primary antibody anti-connexin 43 (A1-B2) and the secondary antibody Alexa Fluor 488 (green). Also, the actin cytoskeleton was stained with Alexa Fluor 594 phalloidin (red; A2 and B2). The cell nuclei were detected with DAPI (blue). Scale bars 20 μm .

4.3.4. Ultrastructural analysis via transmission electron microscopy

The ultrastructural characteristics of iCell-CMs were investigated via transmission electron microscopy and compared to adult rat cardiomyocyte controls. In initial experiments cell samples for TEM imaging were processed via a standard RT resin embedding protocol. For this method cell suspensions of adult rat cardiomyocytes and iCell-CMs, which were enzymatically removed from their culture substrate, were fixed and concentrated into a dense pellet in agarose before being further processed and infiltrated with resin. The TEM images of such resin embedded adult rat controls revealed a vast amount of damaged cells and cell debris (De) with recognisable free-floating rounded mitochondria (M) and dispersed myofilaments (Myfil; Fig. 4.9. A). However, a few intact rat cardiomyocytes could also be detected. These exhibited a highly organised ultrastructure with various subcellular structures recognisable (Fig. 4.10. A-C). The electron micrographs revealed the cell nucleus (N) and the cell cytoplasm packed with highly aligned myofibrils (Myf), which were in close association with mitochondria. The myofibrils contained parallel Z lines and I bands, which confine the typical sarcomeric pattern. The mitochondria appeared empty, due to contents leaching out during the sample preparation technique, probably due to osmotic effects of the media. Also, the nucleus showed some signs of dehydration artefacts (convoluted membrane appearance). Other membrane structures visible in close association with the sarcomeres might represent SR cisternae [25-100 nm; (Jorgensen et al., 1988)], which can appear in the interfibrillar spaces along the sarcomeres, and cross-sections of T-tubules [mean diameter in rat ventricular cardiomyocytes 200-300 nm; (Brette and Orchard, 2003)], which occur close to the Z lines of sarcomeres. In contrast, TEM images of the iCell-CM samples showed only poorly preserved cells and cell debris (Fig. 4.9. B). These results prompted us to test another sample preparation method. Adult cardiomyocytes and iCell-CMs, respectively, were plated as monolayers on small sapphire discs (3 mm diameter) and fixed, processed and embedded into resin in a similar way as with the standard RT resin embedding method but with the cells still attached to the sapphire discs. These discs were only removed after the cells were tightly anchored in cured resin blocks so that the morphology and orientation of the cells within the cell monolayer remained preserved. With this method a number of well-preserved rat adult cardiomyocytes and iCell-CMs could be detected via TEM.

The micrographs of adult rat cardiac cells from sapphire discs (Fig. 4.10. D) were of similar quality as the images of cells prepared via the standard RT resin embedding method. Several cellular structures, which are described above, were visible including parallel aligned myofibrils, mitochondria, cell nucleus, SR and TT structures.

In TEM images of iCell-CMs from sapphire discs (Fig. 4.11) various cell features could be observed. Generally these cells did not display the highly organised arrangement of subcellular components as seen in the adult rat controls. iCell-CMs displayed variable degrees of myofibrillar organisation. Some cell sections showed a scattered distribution of myofilaments bundles (Myofil) throughout the cytoplasm (Fig. 4.11. A), whilst other foci displayed parallel arrays of myofibrils organised into sarcomeres (Fig. 4.11. B-C), which were separated by Z bands. No I- or A- bands were recognisable in the myofibrils. Furthermore, various other cell components were visible in the cytoplasm: abundant mitochondria (Fig. 4.11. B and E), free ribosomes (Fig. 4.11. C), large glycogen deposits (Fig. 4.11. D), cell nucleus (Fig. 4.11. E) and various other membrane structures, which might represent the SR or the rough SR (Fig. 4.11. E-F) but seemed generally not to be associated with the myofibrils. No TTs-resembling structures, which have an average diameter of 400 nm in human adult ventricular cardiomyocytes (Jayasinghe et al., 2011), could be detected. Additionally the cells possessed clustered 50 nm structures morphologically consistent with caveolae (Fig. 4.12. A-B).

4.3.5. Electrophysiological studies: response of iCell-CMs to chronotropic drugs

In mature cardiomyocytes the SR Ca^{2+} has a major contribution to cell contraction. The immunofluorescence and qRT-PCR studies, presented above, demonstrated that iCell-CMs expressed major proteins implicated in Ca^{2+} cycling including L-type Ca^{2+} channels and the SR components SERCA2a and RyR2 (though in low amounts). In order to examine whether these Ca^{2+} handling proteins are functional and whether the SR Ca^{2+} release has a substantial contribution to ECC iCell-CMs were tested for their sensitivity to drugs, which affect SR function. The drugs tested included caffeine (Caf), a reversible activator of RyR2,

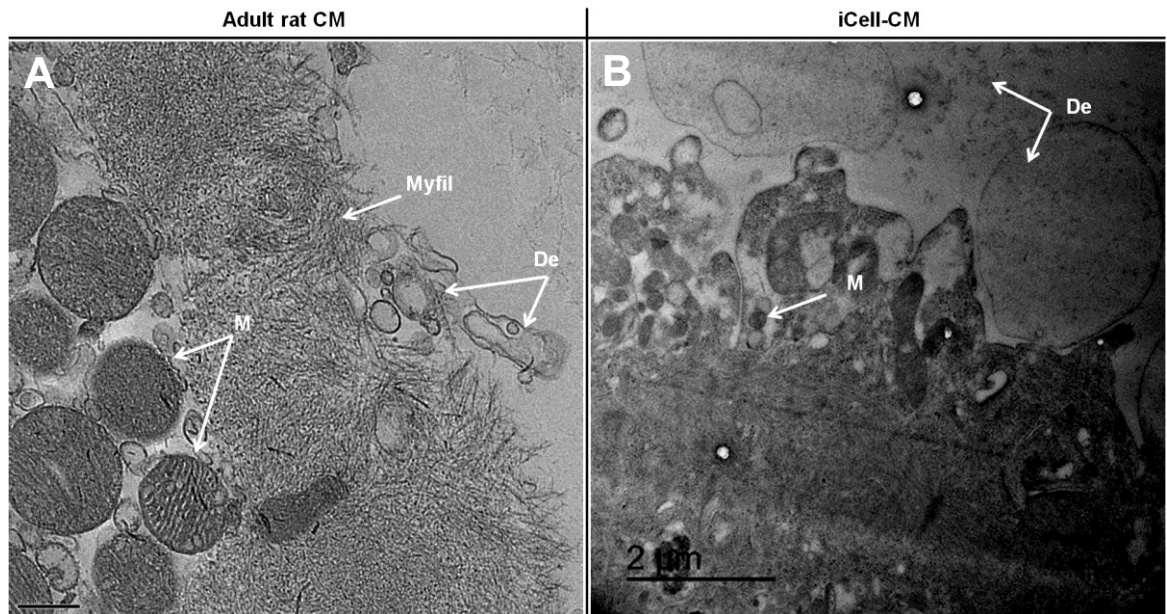


Fig. 4.9. TEM images of an adult rat cardiomyocyte and iCell-CM processed via standard RT resin embedding.

The images display a damaged adult rat cardiomyocyte (adult rat CM; A) and a poorly preserved iCell-CM (B) with free floating rounded mitochondria (M), scattered myofilaments (Myfil) and other cell debris (De). Scale bars 0.5 μm (A) and 2 μm (B).

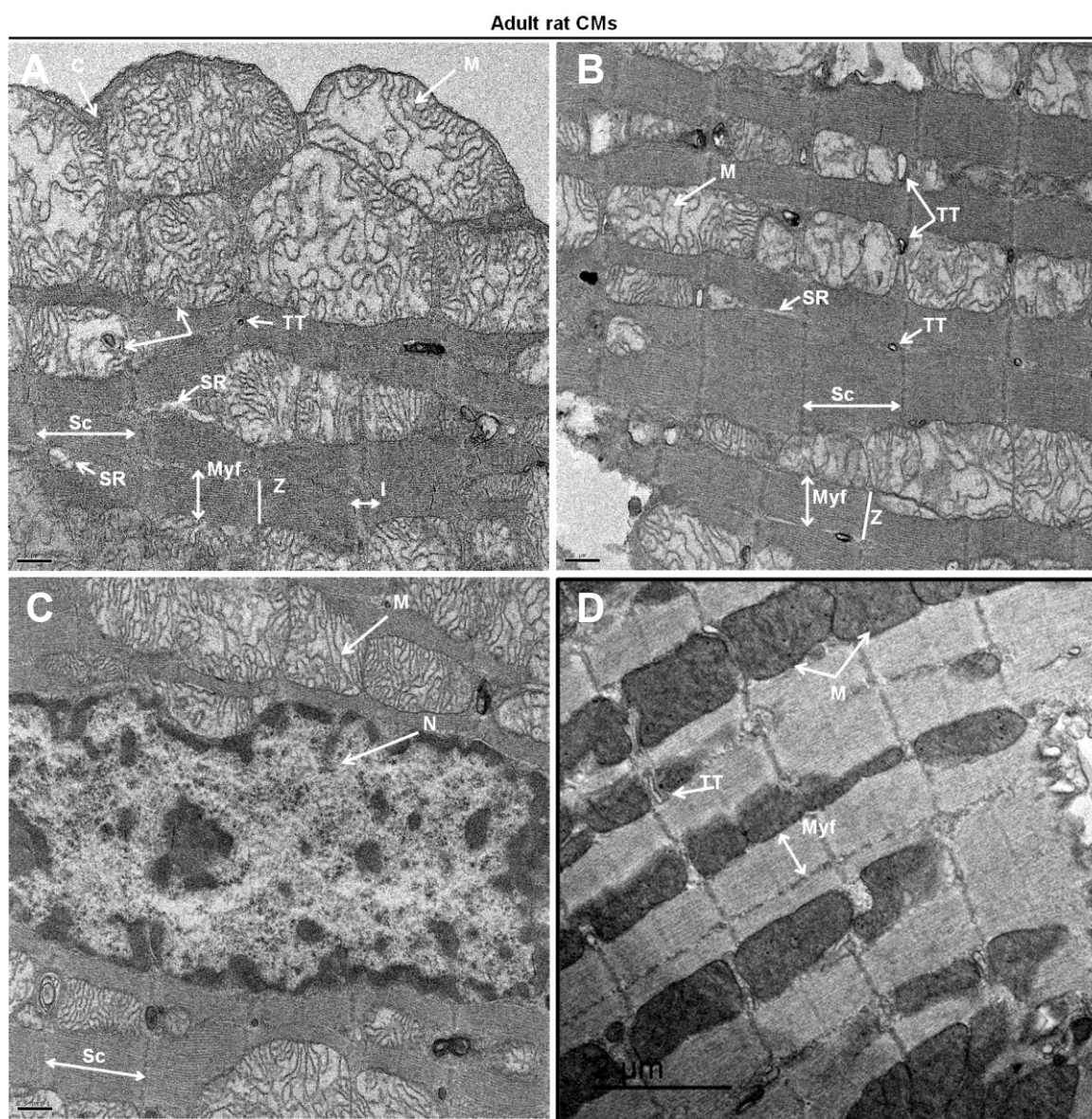
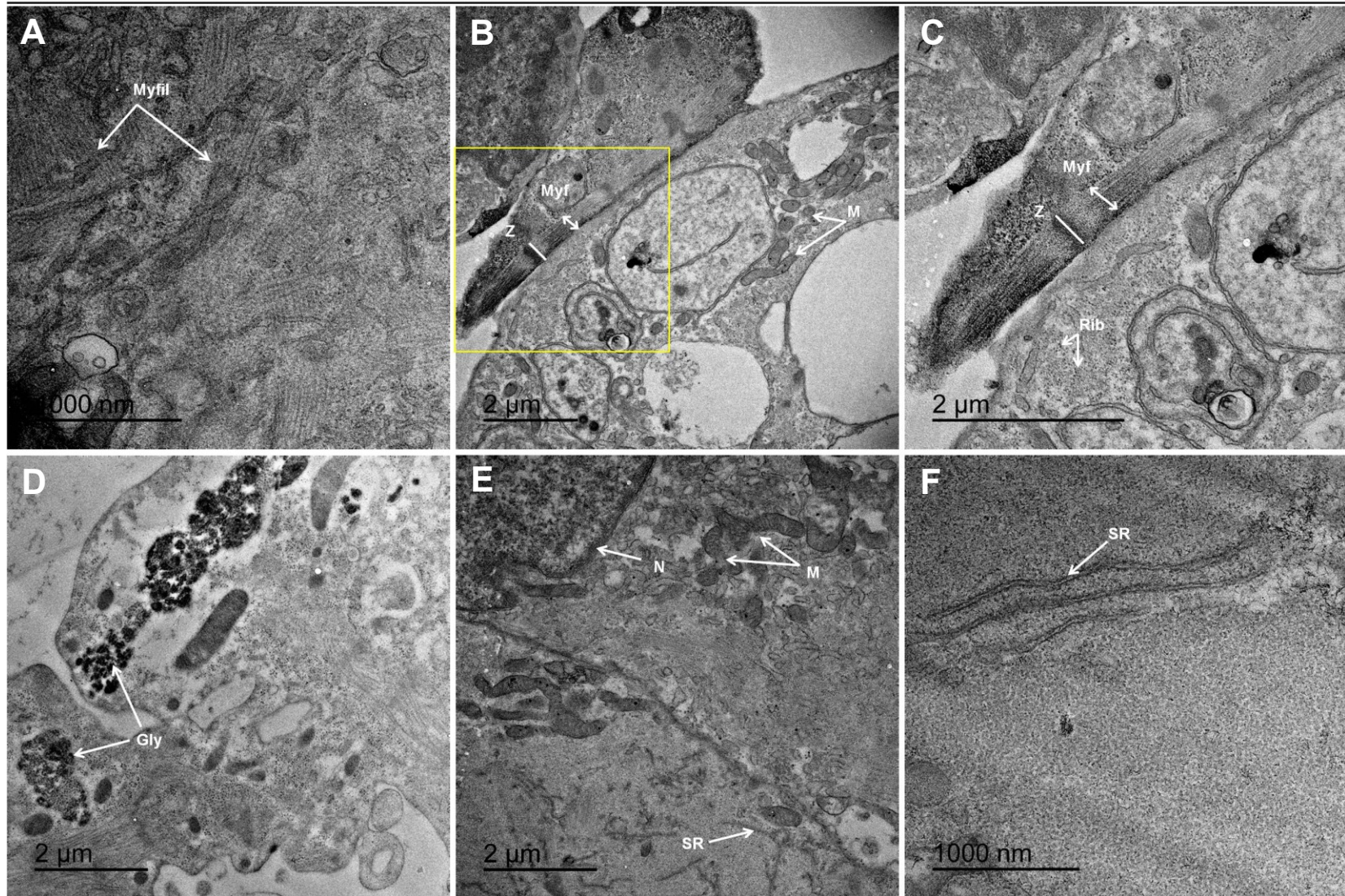


Fig. 4.10. TEM images of adult rat cardiomyocytes.

Image A, B and C show adult rat cardiomyocytes processed via standard RT resin embedding as cell suspensions. The cells are packed with mitochondria (M) in close association with parallel aligned myofibrils (Myf). The myofibrils contain sarcomeres (Sc), which are flanked by Z-lines (Z), and I-bands (I) are also visible. Sarcoplasmic reticulum (SR) structures are located adjacent to myofibrils and T-tubules (TT) occur at the Z-line junctions. Electron micrograph C displays the cell nucleus (N), which shows some signs of dehydration artefacts. D displays an adult rat cardiomyocyte processed and embedded in resin attached to a sapphire disc. Scale bars 0.5 μm (A-C) and 2 μm (D).

iCell-CMs



For figure legend see page 142.

Fig. 4.11. Electron micrographs of iCell-CMs from sapphire disc cultures.

The micrographs display iCell-CMs, which were grown and embedded in resin as monolayers on sapphire discs. iCell-CMs showed randomly dispersed bundles of myofilaments (Myfil) throughout the cytoplasm (A) as well as organised myofibrils (Myf) with recognisable Z-lines (Z; see B-C). The cells also showed the presence of mitochondria (M; see B and E), free ribosomes (Rib; in C), glycogen granules (Gly; see D) and sarcoplasmic reticulum (SR) cisternae (see E-F). N: cell nucleus. C shows a magnified view of the region shown in the yellow box in B. Scale bars 1000 nm (A and F) and 2 μ m (B-E).

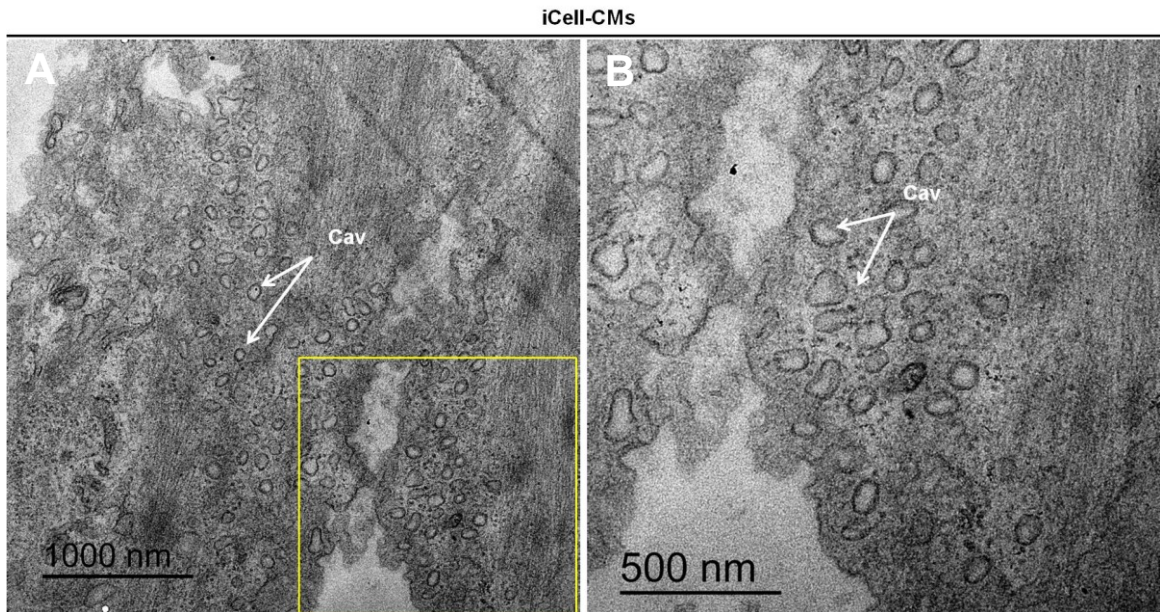


Fig. 4.12. TEM images of iCell-CMs displaying caveolae.

iCell-CMs (A-B) exhibited a large amount of caveolae (Cav), the membrane microdomains potentially implicated in TT development or organisation and cell signalling. B shows a magnified view of the region outlined by the yellow box in A. Scale bars 1000 nm (A) and 500 nm (B).

which empties SR Ca^{2+} stores and inhibits phosphodiesterase; and thapsigargin (Thg), a specific, high-affinity inhibitor of SERCA2a, which abolishes SERCA2a-mediated SR Ca^{2+} reuptake irreversibly. To measure the drug effect iCell-CMs were plated on micro electrode array probes (Fig. 4.13. A) and their beating rate was measured by recording the extracellular field potential of iCell-CM clusters.

Treatment with caffeine (10 mM) had a rapid effect on the cells (Fig. 4.13. B): It inhibited spontaneous contractility within one minute (n=3). This caffeine effect was reversible as spontaneous contraction reappeared after drug wash out. The addition of thapsigargin also interfered with the spontaneous contraction of the cells although only after longer incubation times (Fig. 4.13. C). Treatment with thapsigargin at 1 μM reduced the beating rate on average by 40 % after 15 min. The contraction was further reduced or completely ceased within 15 min in 10 μM Thg. These results imply that iCell-CMs were dependent on intracellular SR Ca^{2+} for contraction.

4.4. Discussion

4.4.1. Bright field analysis of Shef3-CMs and iCell-CMs

Bright field microscopy revealed that Shef3-CMs and iCell-CMs, which were dispersed into single cells or small clusters, exhibited a markedly different morphology compared to primary adult rat cardiomyocytes. These cells did not display the typical rod-shaped striated phenotype of the adult cardiomyocytes and were approximately three times thinner than the adult rat cells. These morphological differences may be partially due to the culture conditions. The *in vitro* culture on a two-dimensional matrix is not representative of the three dimensional *in vivo* environment of the heart muscle tissue, in which cardiomyocytes are joined end to end to other cardiomyocytes, are surrounded by extracellular matrix and tightly packed with other supporting cell types. Also, it is well known that with prolonged *in vitro* culture the primary adult cardiomyocytes undergo “dedifferentiation” with noticeable phenotypic changes: the cells gradually lose their *in vivo* rod-shaped morphology becoming more

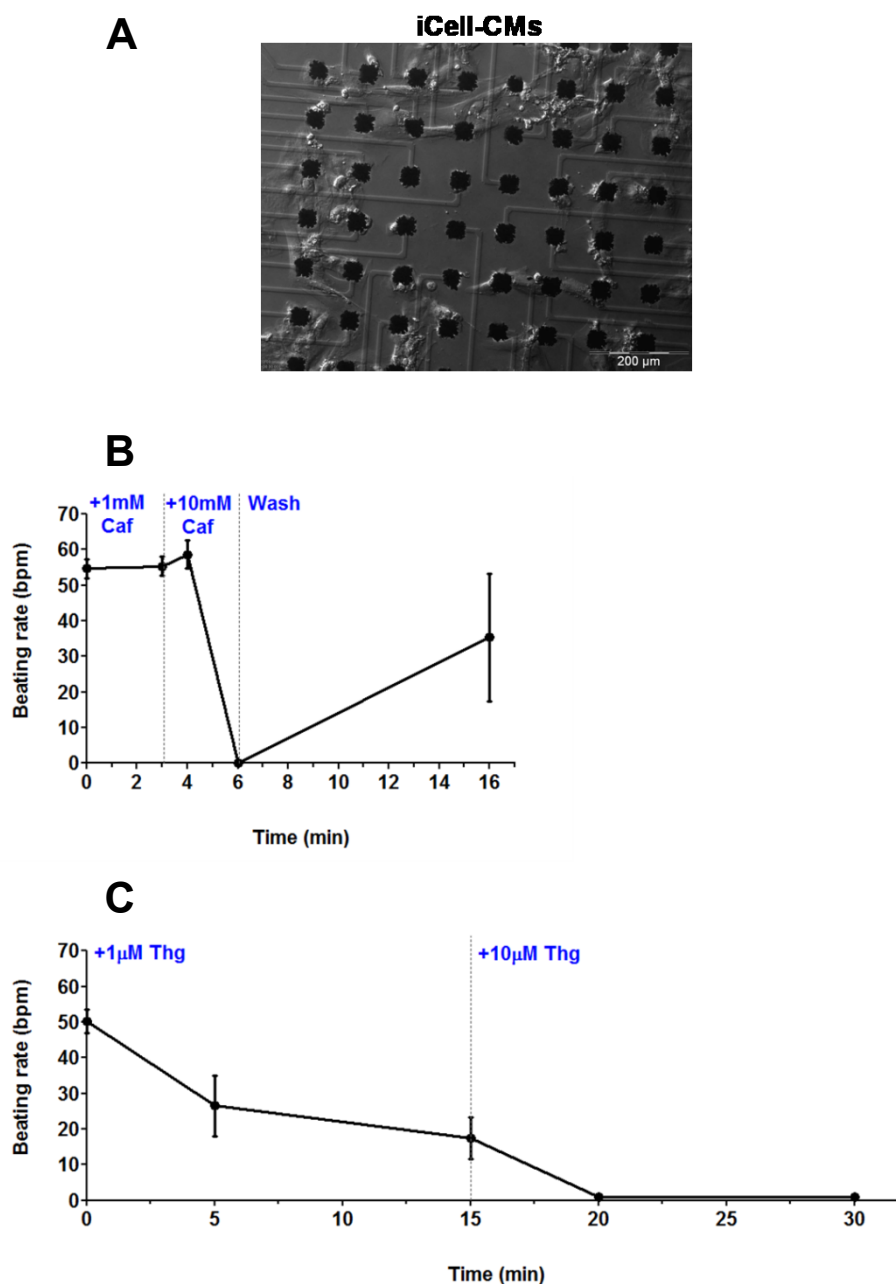


Fig. 4.13. Response of iCell-CMs to caffeine and thapsigargin.

(A) Monolayer clusters of contracting iCell-CMs on a micro electrode array probe. (B) Spontaneously beating clusters of iCell-CMs ($n=3$) were treated sequentially with 1 mM ($t=0$ min) and 10 mM caffeine (Caf; $t=3$ min) in culture medium and then washed once with medium ($t=6$ min). Spontaneous contractility was inhibited by Caf (10 mM) within 1 min but reappeared after drug wash out. (C) Clusters of spontaneously contracting iCell-CMs ($n=4$) were treated in a sequential manner with 1 μ M ($t=0$ min) and 10 μ M thapsigargin (Thg; $t=15$ min) in cell culture medium. Treatment with Thg (for 15 min at 1 μ M) reduced the beating rate on average by 40 %. With increased concentration of Thg (10 μ M) the contraction was further reduced or completely ceased within 15 min. The beating rate in beats per minute (bpm) was recorded via MEA.

rounded and flatter and show reduced density of the TT network (Piper et al., 1988; Lipp et al., 1996).

Shef3-CMs and iCell-CMs showed similar morphologies. However, although both cultures were not older than approximately 45 days and can be regarded as early-stage hPSC-CMs more binucleated cells could be observed in cultures of iCell-CMs than in cultures of Shef3-CMs. This suggests that the iCell-CMs may have been more mature than Shef3-CMs because the generation of a second nucleus usually occurs in a later stage of development (Jonker et al., 2007).

4.4.2. qRT-PCR

Gene expression analysis via qRT-PCR revealed that iCell-CMs expressed a number of cardiomyocyte-characteristic genes including those coding for transcription factors, ion channels, structural proteins, TT-proteins and Ca^{2+} handling components. Compared to adult and foetal human heart controls, the gene expression levels in iCell-CMs generally resembled those measured in the foetal heart tissue (19-21-week). These findings are consistent with other studies, which analysed the gene expression profiles in hESC-CMs and showed that cardiac gene expression in hESC-CMs was similar to foetal human heart (Asp et al., 2010; Cao et al., 2008).

Relative to AH and similar to FH iCell-CMs showed high expression of immature transcription factors such as GATA4 and Nkx2.5, which are highly expressed in early developmental stages playing a significant role in early cardiogenesis (Hiroi et al., 2001; Watt et al., 2004). Furthermore, the cells demonstrated decreased expression of contractile proteins including β MHC, which is the predominant isoform in mature human ventricles [whereas in adult ventricular rat cardiomyocytes the α MHC isoform predominates (Weiss and Leinwand, 1996; Lompre et al., 1984)], and MLC2v suggesting an underdeveloped contractile machinery. Also, iCell-CMs showed high expression of T-type (Cav3.1) and L-type (Cav1.2) Ca^{2+} channels but several fold lower expression of Kv4.3 and Kir2.1. High T-type Ca^{2+} channel expression levels were also shown in hESC-CMs (Otsuji et al., 2010). Indeed, the high expression is typical for embryonic hearts and diminishes during development in ventricular

cardiomyocytes (Ono and Iijima, 2010; Qu and Boutjdir, 2001). In contrast, the detection of high expression levels of L-type Ca^{2+} channels was surprising since Cav1.2 expression is generally reported to increase during development. Interestingly, high expression levels of Cav1.2 were also observed in hESC-CMs by Asp and co-workers (Asp et al., 2010). An explanation for the high expression of Cav1.2 seen in this qRT-PCR study might lie in the primer choice. The Cav1.2 subunit undergoes extensive alternative splicing, which results in the generation of at least 40 different transcript variants (Tang et al., 2004). The primer used for qRT-PCR recognised only twenty transcript variants. It is possible that these might be a predominant transcript variant combination in the foetal but not in the adult heart.

Kir2.1 contributes to the generation of the inwardly rectifying potassium current IK_1 , which plays a role in the stabilisation of the resting membrane potential and the late repolarisation phase of the action potential (Chen et al., 1991). The relatively low expression of Kir2.1 observed in this study in iCell-CMs is in line with the results of other reports, which demonstrated low expression or even complete absence of Kir2.1 and the IK_1 current in early hESC-CMs (Satin et al., 2004; Otsuji et al., 2010). The absence of the inwardly rectifying potassium current is suggested to be responsible for the spontaneous activity of hPSC-CMs (Satin et al., 2004).

Apart from PLN, the expression levels of most of the Ca^{2+} handling proteins were below AH. Moreover, the expression levels of RyR2, the major SR Ca^{2+} release channel, and CSQ2, which is important for Ca^{2+} storage in the SR lumen (Scott et al., 1988) and can modulate the activity of RyR2 (Knollmann, 2009), were even significantly lower than in FH. This relatively low expression of these key SR Ca^{2+} regulatory components implies low Ca^{2+} content in the SR and altered Ca^{2+} handling properties in iCell-CMs. Interestingly, Lee et. al. (Lee et al., 2011) reported recently that cardiomyocytes derived from two different hiPSC lines exhibited significantly lower expression of RyR2 and SERCA2a than hESC-CMs. The authors argue further that the Ca^{2+} handling properties of hiPSC-CMs are immature even compared to hESC-CMs.

The expression of IP3R2 in iCell-CMs was comparable to FH. In adult cardiomyocytes ECC relies principally on CICR from the SR stores via RyR2. In immature cardiomyocytes IP3R2-

mediated SR Ca^{2+} release may stimulate CICR and contribute to the activation of contraction (Janowski et al., 2010).

In contrast to RyR2 and CSQ2 SERCA2a and NCX1, which allow relaxation of cardiac cells by removing Ca^{2+} from the cytosol after contraction, were expressed at levels comparable to AH. The high expression of the SERCA2a regulator PLN in iCell-CMs (approximately five fold higher than in FH) corresponds to the relatively high expression level of PLN seen in hESC-CMs (Asp et al., 2010) but is nevertheless unexpected, given that the expression of SERCA2a does not reflect the same level of relative increase. Such high expression of PLN may have a strong inhibiting effect on the function of SERCA2a. The expression of PLN is limited to the SR and as a SERCA2a regulator (Koss and Kranias, 1996) PLN can bind SERCA2a and lower its affinity for Ca^{2+} and as a result decrease the uptake of Ca^{2+} into the SR and reduce contractility. However, the qRT-PCR experiments do not provide any information about the phosphorylation status of the expressed proteins thus the PLN protein may be phosphorylated in iCell-CMs and have no effect on SERCA2a.

Finally, qRT-PCR analysis showed that iCell-CMs expressed markedly low levels (even compared to FH) of Bin1 and Cav3, which may be implicated in TT development or organisation (Lee et al., 2002; Parton et al., 1997; Carozzi et al., 2000; Galbiati et al., 2001). The low expression of Bin1 and Cav3 indicated that iCell-CMs are unlikely to possess any TTs at this stage of development. Thus the high expression of JP2 (approximately five-fold higher in iCell-CMs than in AH), which is reported to link the TT with the SR membrane, was surprising. This is further discussed in section 4.4.3.2. Also surprising was the large difference between the relatively low Cav3 expression level in iCell-CMs and the high expression level detected in FH (almost twice higher than in AH).

4.4.2.1. General qRT-PCR limitations

Expression levels of cardiac genes may vary dramatically depending on the heart region and healthiness of the heart. In this study the aim was to compare gene expression levels of iCell-CMs with healthy human ventricular cardiomyocytes. iCell-CMs represent a mixture of nodal, atrial and ventricular cardiomyocytes but with 50 % of the cells showing the ventricular-like

action potential (correspondence with CDI), qRT-PCR results most likely have relevance to gene expression pattern in the ventricles.

With regard to the FH and AH controls, which were used in this qRT-PCR study, the manufacturer (Stratagene) reassured us that the samples were derived from the ventricles but could not exclude that some samples were derived from diseased myocardium. However, generally there was a good correlation between the qRT-PCR results obtained in this study and previously published data on relevant gene expression pattern in healthy foetal and adult human ventricular heart tissue.

In this study GAPDH was used as a housekeeping gene and was used for normalisation of all qRT-PCR values. GAPDH was chosen because it was used as a reference gene previously in a number of publication measuring gene expression pattern in human embryonic and induced stem cell-derived cardiomyocytes (Sartiani et al., 2007; Földes et al., 2011; Germanguz et al., 2011). It is reported that cell proliferation and density can alter the expression of housekeeping genes, including GAPDH (Goldsworthy et al., 1993; Greer et al., 2010). However, iCell-CMs are non-proliferative (as specified by the manufacturer CDI) and were plated onto 6-well plates with equal densities per well. Thus cell density was assumed relatively stable during the course of the experiments and was unlikely to affect the expression of GAPDH.

qRT-PCR data does not give any evidence on mRNA stability, posttranslational modifications or subunit assembly thus protein levels may differ from mRNA transcript levels in the cells. Protein assays and functional analysis would provide further information to put data into physiological context.

4.4.3. Immunofluorescence studies

4.4.3.1. Expression of sarcomeric and Ca^{2+} handling proteins

Immunofluorescence studies of beating Shef3-CMs and iCell-CMs confirmed that these cells expressed appropriate cardiac proteins such as the sarcomeric protein α -actinin and the SR

Ca²⁺ handling proteins RyR2, SERCA2a, PLN and Cav1.2. However, the staining pattern of these proteins in the hPSC-CMs did not represent the highly organised staining pattern, which was detected in the adult cardiomyocytes.

The striated staining pattern of α -actinin and PLN (less defined for RyR2 and SERCA2A) in some cell areas revealed that Shef3-CMs and iCell-CMs possessed myofibrils with sarcomeric organisation and that the SR was at least partially associated with the myofibrils. However, the irregularity of the α -actinin and PLN banding pattern indicates a relatively disorganised myofibrillar and SR organisation, which is typical for immature early stage cardiomyocytes. Also, RyR2 staining showed a very low fluorescence signal, which indicates a low expression of RyR2 at this developmental stage of the cells (30-45 days old).

Staining for Cav1.2 revealed a punctate expression pattern. Double-labelling for RYR2 and Cav1.2 in iCell-CMs exhibited no co-localisation of the two proteins in any cell area. This implies that no Cav1.2/RyR2 couplons, which are essential for CICR (Bers, 2002), were present in iCell-CMs. This result is surprising because it was reported that in developing mammalian cardiomyocytes, which lack TTs, L-type Ca²⁺ channel/RyR couplons can form at the cell periphery at the sarcolemma (Sedarat et al., 2000). However, in the present study the fluorescent signals of RyR2 and Cav1.2 were weak and the punctate fluorescent signals of Cav1.2 were small so that the detection of co-localisation signals was visually difficult.

4.4.3.2. Plasma membrane staining and expression of TT-associated proteins

Staining of Shef3-CMs and iCell-CMs with WGA-Alexa Fluor 488 and CMO (iCell-CMs only) revealed no evidence that TT-like invaginations were present in these cells. The lack of TTs is a feature iCell-CMs share with ventricular cardiomyocytes from embryonic and newborn animals and highlights the immature phenotype of these cells. TTs are considered to form very late during cardiac development. It was shown in smaller mammals such as rats and rabbits that TTs are absent or underdeveloped at birth but develop rapidly after birth (Sedarat et al., 2000; Ziman et al., 2010). Also, this study showed that iCell-CMs were relatively thin even compared to adult rat cardiomyocytes (3-6 μ m versus 10-20 μ m; see section 4.3.1.). The formation of TT invaginations may not be beneficial in such flat cells but may occur with

hypertrophic growth of the cells. Indeed, adult ventricular cardiomyocytes of birds, fish and small lizards, which are also only approximately 5 μm thick, also lack TTs (Myklebust et al., 1980; Myklebust and Kryvi, 1979; Perni et al., 2012). However, despite the lack of TT invaginations iCell-CMs showed positive staining for the TT-associated proteins Bin1, Cav3, JP2 and Tcap although their staining pattern in iCell-CMs did not correspond to the regular striated staining pattern seen in the adult rat cardiomyocytes. In iCell-CMs the Bin1 staining was confined almost exclusively to the nucleus, Cav3 and JP2 showed a punctate staining throughout the cytosol of the cells and Tcap displayed striated staining only in some cell regions. These results suggest that the detected proteins might play TT-independent roles or might be not functional in iCell-CMs at this developmental stage.

The positive staining for Bin1 in iCell-CMs may also be due to a ubiquitously expressed Bin1 isoform because the Bin1 antibody used for immunolabelling in this study was not exclusive for the muscle-specific Bin1 protein. Bin1 is described to have several isoforms, which show a nuclear localisation and were for example implicated in cell growth regulation through both Myc-dependent and Myc-independent mechanisms (Elliott et al., 1999). However, so far only the muscle-specific isoform was shown to be targeted to the outer cell membrane and to induce membrane invaginations.

Cav3, the scaffolding protein of caveolae, is generally considered as a TT protein marker, since it localises on the TT membrane but it is also considered to associate with various regulatory proteins and to be important for cell signal transduction (Smart et al., 1999).

JP2 is thought to complex between the plasma membrane and the membrane of the SR (Pozzan et al., 1994; Takeshima et al., 2000). However, since no co-localisation of RyR2 and Cav1.2 could be detected in iCell-CMs the strong expression of JP2 mRNA (almost two-fold and five-fold higher than in FH and AH, respectively) and the robust staining for the JP2 protein seen in the immunofluorescence images suggest that JP2 may play additional, yet unknown, TTs-independent roles in developing cardiomyocytes.

4.4.4. Ultrastructural analysis via transmission electron microscopy

For ultrastructural analysis of iCell-CMs and adult rat cardiomyocytes via TEM two sample preparation methods were evaluated including a RT resin embedding method using cell suspensions and a RT resin modified embedding technique using cell samples attached to sapphire discs. The standard RT resin embedding technique was successfully used in various previous studies for TEM preparation of EBs containing PSC-CMs (Kehat et al., 2001; Snir et al., 2003). However, in this study it was shown that this preparation technique was less suitable for single cell suspensions of iCell-CMs possibly due to the sensitivity of the single cells to mechanical stress. Whilst a number of intact adult rat cardiomyocytes could be identified no well preserved intact iCell-CMs could be detected in the respective TEM images.

Growing cells on sapphire discs and processing and embedding them into resin directly with the discs showed to be a more suitable strategy. With this technique both iCell-CMs and adult rat cardiomyocytes showed improved preservation. Processing the cells on sapphire discs has also the benefit of conserving the cell orientation and structure of the cell culture and allows light/fluorescence microscope examination of the cell samples prior to TEM preparation.

TEM images of adult rat cardiomyocytes revealed that the cells possessed a highly organised ultrastructure with parallel aligned myofibrils and mitochondria, TTs and SR compartments (including junctional SR) associated with the myofibrils. In contrast iCell-CMs showed a relatively immature structural phenotype. They demonstrated sparse stacks of myofibrils with varying degrees of sarcomeric organisation and random orientation within a cell, a high content of glycogen, which is characteristic for foetal heart cells (Gutierrez-Correa et al., 1991), abundant amount of free ribosomes throughout the cytosol, which implies a highly active protein syntheses within the cells, mitochondria scattered randomly in the cytosol, some SR cisternae and no TTs.

Furthermore, the ultrastructural analysis revealed the presence of caveolae. These specialized lipid rafts in the plasma membrane are not only described to be involved in TT development (Galbiati et al., 2001) but are also thought to play an important role in cell

signalling in ventricular cardiomyocytes by hosting a wide range of regulatory proteins and ion channels including beta 2-adrenergic receptors and Na⁺ channels (Xiang et al., 2002; Yarbrough et al., 2002).

The findings of this study compare to other studies analysing the ultrastructure of hiPSC-CMs (Gherghiceanu et al., 2011) and hESC-CMs (EB clusters up to 27 days post plating) (Snir et al., 2003; Kehat et al., 2001). These studies found similar cell features and describe hPSC-CMs as being phenotypically immature. Moreover, Gherghiceanu et. al. (Gherghiceanu et al., 2011), who analysed the ultrastructure of hiPSC-CMs, reported to have identified structures resembling “Ca²⁺ release units” (couplons; also discussed above), which connected the sarcolemma with the peripheral SR cistern. In this study no couplons could be detected via TEM in iCell-CMs though since the authors also describe them to be rare it cannot be excluded that they appear in iCell-CMs but were not present in the examined TEM cell sections.

4.4.5. Calcium handling properties in iCell-CMs

To determine whether iCell-CMs exhibited loaded SR Ca²⁺ stores, which release and uptake Ca²⁺ via functional RyR2 and SERCA2a, respectively, it was tested whether iCell-CMs responded to caffeine and thapsigargin. Caffeine rapidly releases SR Ca²⁺ by opening RyR2 whereas thapsigargin inhibits SERCA2a thus blocking SR Ca²⁺ reuptake. The MEA recordings revealed that iCell-CMs were sensitive to both drugs. Caffeine (10 mM) ceased contraction. Thapsigargin (10 µM) decreased and finally ceased spontaneous beating in a concentration dependent manner and after prolonged incubation times. These findings suggest that functional RyR2- and SERCA2a-mediated SR Ca²⁺ stores were present in iCell-CMs and contributed to contraction.

These results are in agreement with a previous study by Itzhaki et al. (Itzhaki et al., 2011a), who analysed the Ca²⁺ handling properties in hiPSC-CMs and also found that hiPSC-CMs (30-50 days old) were sensitive to caffeine and thapsigargin treatment. The authors tested the effects of the drugs on whole-cell Ca²⁺ transients and showed that caffeine application triggered a rapid increase in cytosolic Ca²⁺ whilst thapsigargin decreased the amplitude of

whole cell Ca^{2+} slowly and progressively. Furthermore, various studies showed that hiPSC-CMs were also sensitive to the RyR-inhibitor ryanodine (Itzhaki et al., 2011a; Germanguz et al., 2011; Lee et al., 2011). Collectively, these findings indicate that hiPSC-CMs express functional SR Ca^{2+} stores and that the SR Ca^{2+} release contributes at least partially to ECC. However, contraction of iCell-CMs may also depend on transsarcolemmal Ca^{2+} influx similar to foetal cardiomyocytes. Ca^{2+} entry into the cytosol can be mediated not only by L-type but also by T-type Ca^{2+} channels (Cav3.1) and NCX1. Ca^{2+} removal from the cytosol can depend more on NCX1 (in forward mode) and the plasmalemmal Ca^{2+} ATPase (Tohse et al., 2004). As shown via qRT-PCR iCell-CMs expressed relatively high levels of both Cav3.1 and NCX1 (see section 4.3.2.).

4.5. Conclusions

In summary the characterisation study of Shef3-CMs and a more comprehensive study of iCell-CMs revealed that these cell types show generally an immature phenotype resembling embryonic cardiomyocytes. However, some adult-like features are present.

Immunofluorescence analysis showed that Shef3-CMs and iCell-CMs expressed characteristic cardiac contractile proteins such as α -actinin and α MHC and SR Ca^{2+} handling components including RyR2 and SERCA2a. However, the highly organised mature sarcomeric pattern for myofibrils and SR has not been established at this developmental stage yet. Also, no TTs could be detected. qRT-PCR results for iCell-CMs demonstrated with few exceptions generally a foetal-like gene expression profile. TEM analysis revealed overall disorganised cell architecture in iCell-CMs unlike the well ordered and aligned ultrastructure in adult cardiac myocytes. MEA studies revealed that iCell-CMs showed dependence on intracellular Ca^{2+} stores for contraction, which implies mature calcium handling properties.

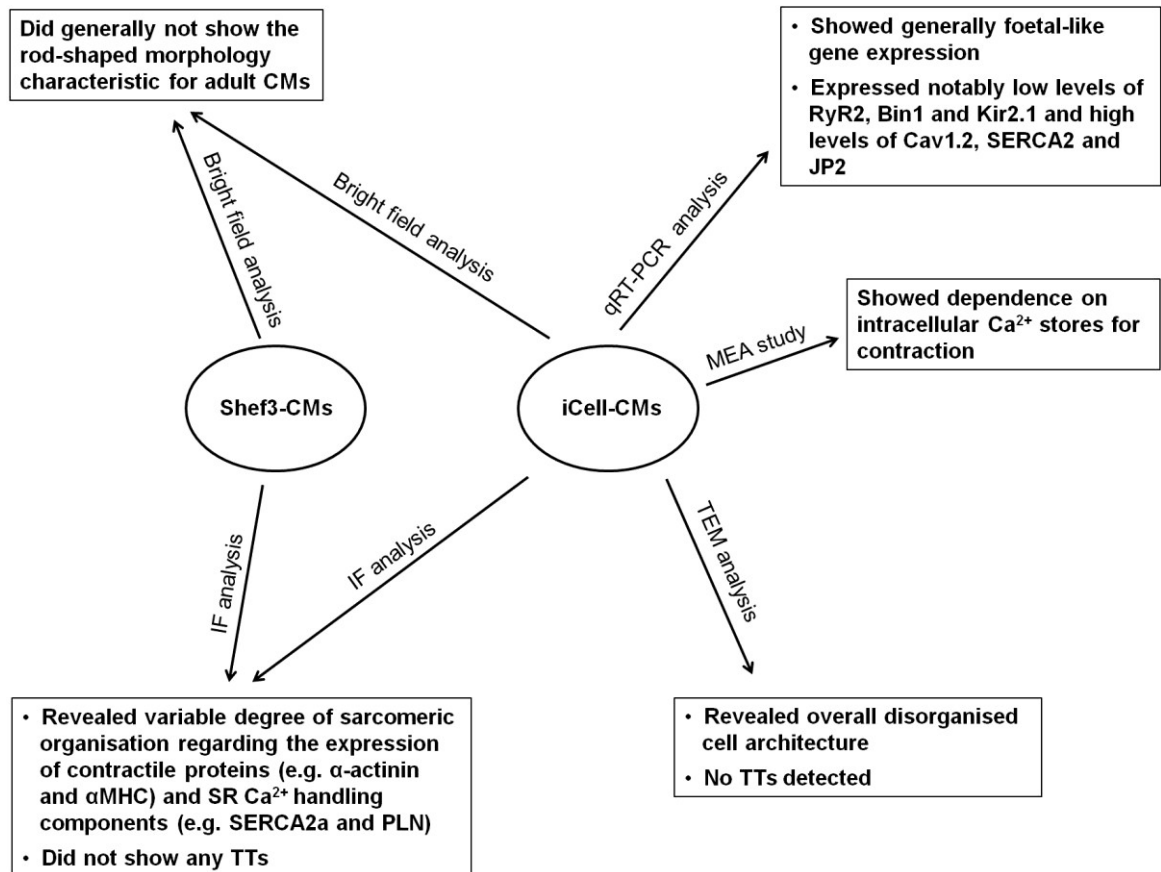


Fig. 4.14. Diagram summarising major results of chapter 4.

CMs: cardiomyocytes, IF: immunofluorescence microscopy, TEM: transmission electron microscopy, TTs: transverse tubules.

Chapter 5: Maturation of iCell-CMs

5.1. Introduction

In the previous chapter it was demonstrated that iCell-CMs revealed overall an immature foetal-like phenotype with regards to their gene expression and (ultra)structural and electrophysiological properties. In this chapter the aim was to evaluate strategies to drive iCell-CMs towards a more mature phenotype.

First the effect of T3 administration was investigated. T3 is known to be a key mediator in cardiac differentiation and maturation [(Klein, 1990); see section 1.4.4. for details]. T3 signalling is described to drive cardiomyocyte maturation among other by regulating the expression of a variety of cardiac genes on a transcriptional or postranscriptional level. T3-responsive genes include ion channels such as the voltage-gated-potassium channels Kv1.5, Kv4.2 and Kv4.3 (Ojamaa et al., 1999; Kahaly and Dillmann, 2005), Ca²⁺ handling proteins including SERCA2a (Cernohorsky et al., 1998) and structural proteins such as α - β MHC (Morkin, 1993) [effect seen predominantly in animals (Kahaly and Dillmann, 2005)]. The transcriptional effects of T3 are mediated by the T3 nuclear receptors THR α and THR β .

Second the effect of the overexpression of Bin1(-EGFP) was evaluated. Bin1 has been implicated in the induction of tubules in biological membranes (Takei et al., 1999; Razzaq et al., 2001) and also localisation of L-type Ca²⁺ channels (Cav1.2) to Bin1-induced membrane tubules (Hong et al., 2010). iCell-CMs were shown to express only low levels of Bin1 and to lack TTs (see section 4.2.3. and 4.2.5.). In order to investigate whether Bin1 can induce the formation of TTs and promote the maturation of Ca²⁺ handling properties in iCell-CMs Bin1 tagged to EGFP was overexpressed in iCell-CMs.

5.2. Methods

5.2.1. T3-treatment

iCell-CMs were seeded in 6-well plates at 1.25×10^5 - 1.5×10^5 cells/cm², cultured for 7-10 days in iCell-CMs Maintenance Medium and incubated with T3 at 3 nM for 48 h.

5.2.2. qRT-PCR

See section 2.5.3.

5.2.3. Immunostaining

See section 2.6.

5.2.4. Transfection of iCell-CMs with Bin1-EGFP

iCell-CMs were transfected using Lipofectamine 2000. For transfection iCell-CMs were typically seeded at densities of 1×10^5 - 1.5×10^5 cells/cm² and cultured for 7-10 days before being transfected with the Bin1-EGFP plasmid.

5.2.5. Transmission electron microscopy

The immunogold labelling was performed on cell sections embedded in epoxy resin. Anti-Bin1 (1:5) or anti- α -actinin (1:10) was used as primary antibodies and antibodies conjugated with 10 nm gold particles were used as secondary antibodies. For amplification of the gold signal the resin sections were incubated with a silver enhancement kit.

5.2.6. Whole-cell patch-clamping

For all patch-clamping experiments iCell-CMs were seeded on glass-bottom MatTek dishes at low cell densities at 4.5×10^4 - 6.2×10^4 cells/cm² so that individual transfected and non-transfected iCell-CMs could be studied. The experimental set up also allowed the use of fluorescence microscopy when it was necessary to identify Bin1-EGFP transfected iCell-CMs, which displayed green fluorescence.

5.3. Results

5.3.1. The effect of triiodothyronine hormone on iCell-CM maturation

5.3.1.1. Molecular analysis of T3-treated iCell-CMs via qRT-PCR

qRT-PCR analysis was used to compare mRNA expression levels of non-treated (control) with T3-treated iCell-CMs. At 3 nM (2 ng/ml, for 48 h) the administrated T3 concentration corresponded to the normal T3 concentration found in human serum at birth (1.9 ± 0.96 ng/ml) and was similar to the concentrations used by other investigators, who studied the maturational T3 effect on sheep foetal cardiomyocytes (Chattergoon et al., 2012). The group of genes analysed in this chapter comprised a) the nuclear T3 receptors $\text{THR}\alpha$ and $\text{THR}\beta$; b) deiodinase type 3 (D3), which is the main enzyme involved in T3-inactivation (Pol et al., 2010) and might thereby inhibit the T3 action; c) the panel of genes listed in Table 4.1. including contractile proteins, ion channels, TTs- and SR Ca^{2+} -handling associated genes and d) atrial natriuretic peptide (ANP), a cardiomyocytes marker of hypertrophy (Schaub et al., 1997).

qRT-PCR analysis of the mRNA expression pattern of $\text{THR}\alpha$, $\text{THR}\beta$ and D3 showed the following. The expression levels of $\text{THR}\alpha$, $\text{THR}\beta$ and D3 in T3-treated iCell-CMs did not differ from expression levels in control iCell-CMs (Fig. 5.1. A). The expression levels of $\text{THR}\alpha$ in iCell-CMs corresponded to the adult heart controls and were approximately 1.6-fold lower than in the foetal heart controls. In contrary, the expression levels of $\text{THR}\beta$ corresponded to the foetal control levels and were more than 15-fold lower than in adult control samples. The expression of D3 in iCell-CMs was significantly below adult and foetal controls with expression levels approximately 10-fold and 35-fold lower than in the adult and foetal heart tissue samples.

Furthermore, the qRT-PCR results revealed that T3-treatment increased the expression of the hypertrophic marker ANP (Fig. 5.1. B), the potassium channel Kv4.3 (Ito) (Fig. 5.1. C), and the SR Ca^{2+} -ATPase SERCA2a (Fig. 5.1. D) by approximately 1.7-fold, 2-fold and 1.6-

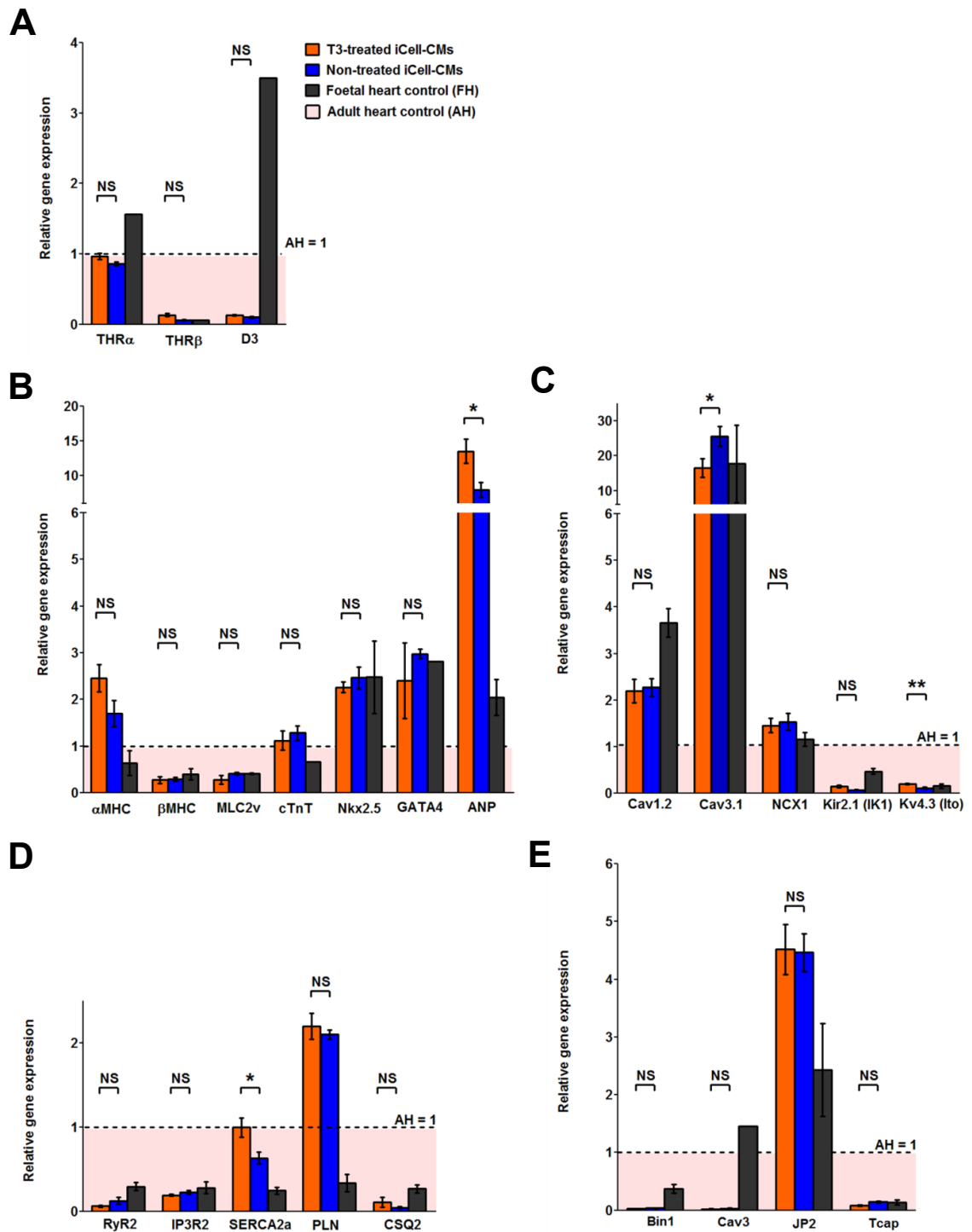
fold, respectively, and down-regulated the expression of the T-type Ca^{2+} channel Cav3.1 (Fig. 5.1. C) by more than 1.5-fold. T3 did not increase the expression of any other Ca^{2+} -handling (e.g. RyR2; Fig. 5.1. D) or TTs-related genes (e.g. Bin1 and JP2 Fig. 5.1. E).

5.3.1.2. Immunofluorescence analysis of SERCA2a expression in T3-treated iCell-CMs

In order to investigate whether the increased SERCA2a expression in the T3-treated iCell-CMs was accompanied by a remodelling of the SR towards a more mature structural organisation control and T3-treated samples were immunolabelled against SERCA2a and visualised via confocal microscopy. The resulting fluorescence images revealed that there was no detectable difference in the strength of the fluorescent signal of SERCA2a between the T3-treated (Fig. 5.2. A-B) and control iCell-CMs (Fig. 5.2. C-D). Also, the T3-treated cells did not show a visible higher degree of SR organisation. Both samples revealed cells with variable degrees of structural organisation and only a minority of cells with a recognisable banding pattern (arrows in Fig. 5.2. A and D).

5.3.1.3. Plasma membrane staining of T3-treated iCell-CMs

Finally, it was examined whether T3-treated iCell-CMs showed any evidence of TTs formation. For this T3-treated and control iCell-CMs were stained with the membrane dye CMO. The resulting immunofluorescence images showed no evidence of TTs formations. T3-treated (Fig. 5.3. A) as well as control iCell-CMs (Fig. 5.3. B) demonstrated only few fluorescent dots and streaks, which represent presumably non-specific staining, but no repetitive tubule-like structures could be detected.



For figure legend see page 162.

Fig. 5.1. Effect of T3 on gene expression in iCell-CMs.

iCell-CMs were incubated with 3 nM triiodothyronine (T3). After 48 h total RNA was isolated and assayed by qRT-PCR to measure the expression levels of (A) T3 receptor alpha and beta (THR α and THR β) and deiodinase type 3 (D3); (B) structural proteins and general cardiac markers; (C) ion channels; (D) genes coding for SR Ca²⁺ handling components and (E) genes coding for proteins implicated in TT development or maintenance. T3-treated iCell-CMs were compared with non-treated cells. Adult human heart (AH) and foetal human heart tissue (FH) were used as controls. qRT-PCR results from iCell-CM samples were obtained in triplicate and results from AH and FH in singlets, in n=2 preparations. All values were normalised with respect to GAPDH and shown relative to the corresponding values in AH (AH=1). Error bars represent $\pm$ SEM. Statistical significance between the two means was determined using unpaired Student's t-test (*p<0.05, **p<0.01, ***p<0.001).

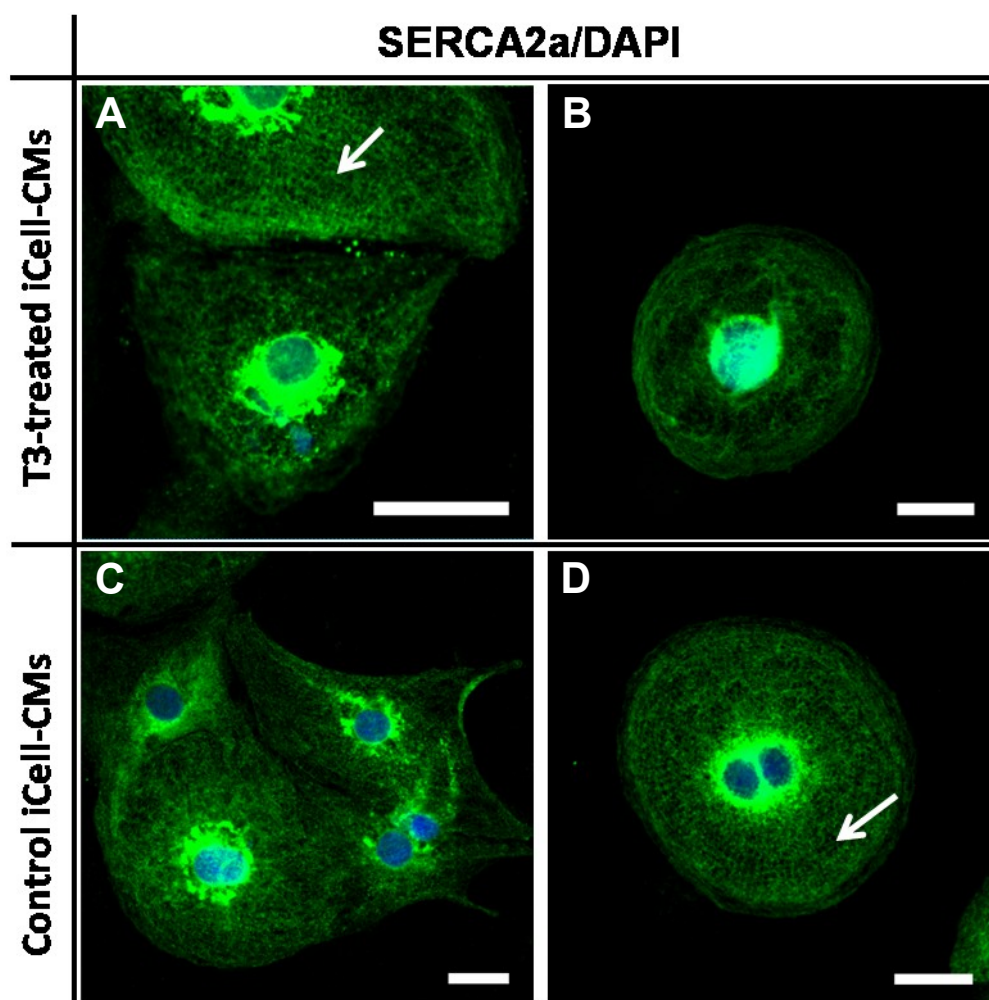


Fig. 5.2. Immunostaining of SERCA2a in T3-treated and control iCell-CMs.

T3-treated (A-B) and control iCell-CMs (C-D) were labelled with a primary antibody against SERCA2a. Alexa Fluor 488 (green) was used as secondary antibody. Arrows in A and D show cell regions with a striated SERCA2a staining pattern. Nuclei were stained with DAPI (blue). Scale bars 30 μ m in A and 20 μ m in B-D.

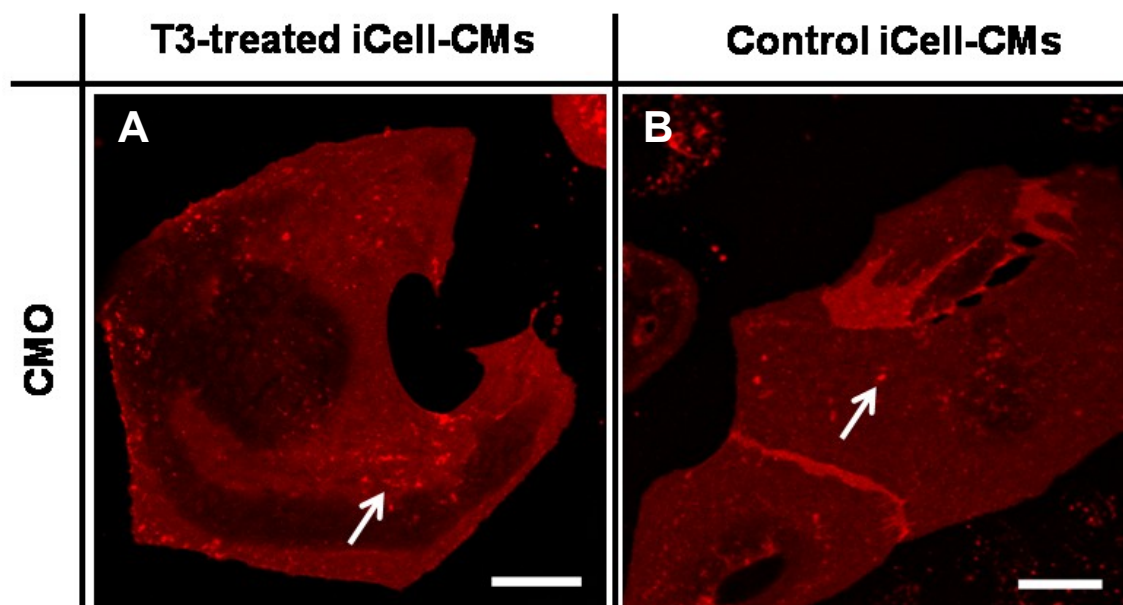


Fig. 5.3. Staining of the cell plasma membrane of T3-treated iCell-CMs with CellMask Orange.

Non-permeabilised T3-treated (A) and control iCell-CMs (B) were incubated with the plasma membrane dye CellMask Orange (CMO) and analysed via immunofluorescence microscopy. Arrows show fluorescent dots and streaks within T3-treated and non-treated cells representing presumably non-specific staining. Scale bars 20 μm .

5.3.2. Overexpression of Bin1-EGFP in iCell-CMs

5.3.2.1. Transfection of iCell-CMs: Formation of tubular structures

In order to investigate the effect of Bin1 on cell structure iCell-CMs (Fig. 5.4. A) were transfected with a expression plasmid encoding Bin1-EGFP. 48 h after transfection distinctive green fluorescent cells could be detected via immunofluorescence microscopy in 30-50 % of the plated iCell-CMs (Fig. 5.4. B-C). Higher magnifications of the green fluorescent iCell-CMs revealed that the cells contained numerous green fluorescent tubular structures throughout the cell body (Fig. 5.5. A1 and A4).

Immunolabelling of transfected and non-transfected (control) iCell-CMs with an antibody against the Bin1 protein (Fig. 5.5. A2, A5 and B2) revealed that in transfected cells the Bin1-antibody staining overlapped with the tubular Bin1-EGFP expression pattern throughout the cells (Fig. 5.5. A3 and A6) whereas in control cells it was predominantly confined to the cell nucleus (Fig. 5.5. B3). These results imply that the tubular structures detected in transfected iCell-CMs were induced by Bin1-EGFP.

5.3.2.2. Staining of the plasma membrane of transfected iCell-CMs

In order to determine whether the tubules detected in Bin1-EGFP expressing iCell-CMs were continuous with the cell surface membrane the cells were labelled with the lipid dye CMO or the glycocalyx binding WGA-TRITC. Immunofluorescence microscopy revealed that the tubular structures showed positive staining for CMO (Fig. 5.6. A1-A6) demonstrating that they were accessible for the membrane impermeable dye and thus connected to the plasma membrane. In contrast, surprisingly, no tubular structures could be detected using WGA-TRITC which is inconsistent with the CMO data (Fig. 5.6. B1-B6). These observations are discussed in section 5.4.3.

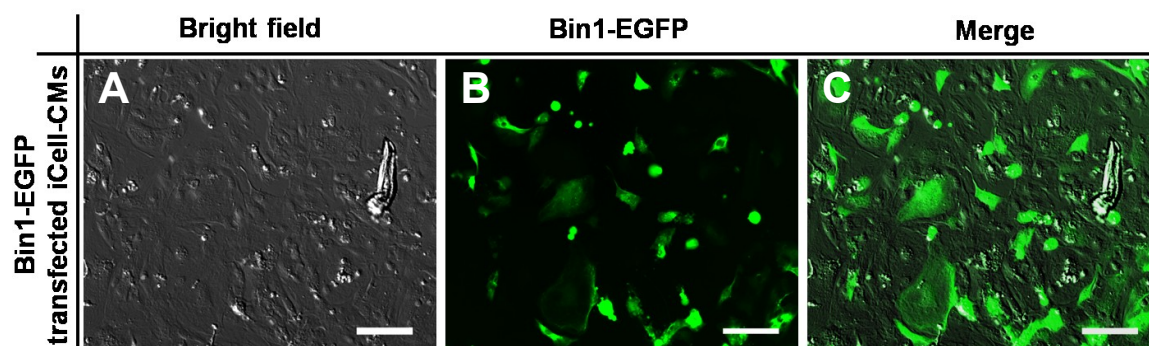


Fig. 5.4. Overexpression of Bin1-EGFP in iCell-CMs.

iCell-CMs (A, bright field) were transfected with a plasmid expressing Bin1-EGFP. Analysis of the cells via immunofluorescence microscopy revealed that 48 h after transfection bright green fluorescent cells emerged (B). The transfection efficiency represented 30-50 % as evaluated by the number of green fluorescent cells (C). Scale bars 100 μ m.

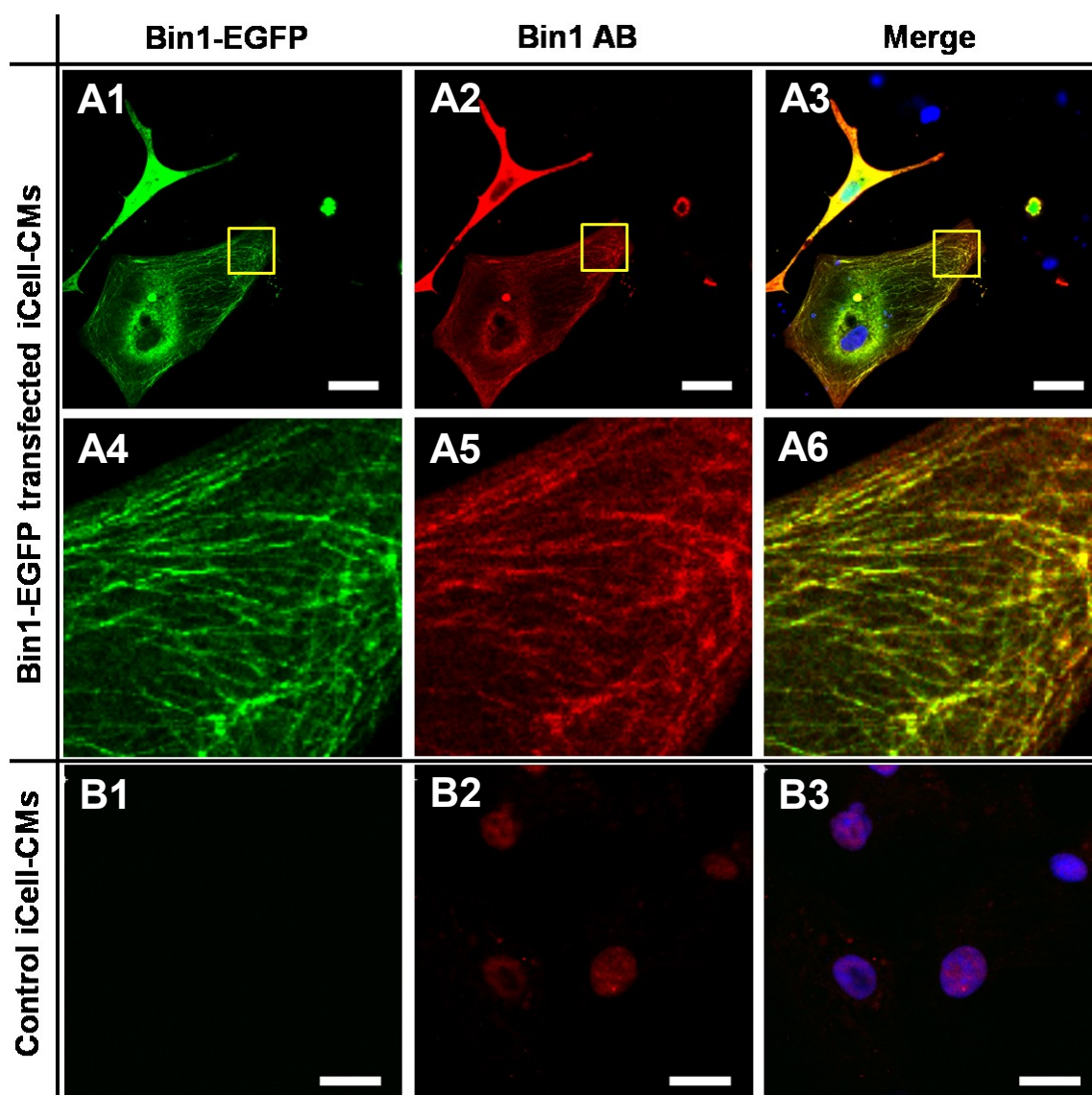


Fig. 5.5. Immunostaining of transfected and control iCell-CMs against Bin1.

iCell-CMs transfected with Bin1-EGFP (A1-A6; green) and non-transfected control iCell-CMs (B1-B3) were stained with a primary antibody against Bin1 (Bin1 AB). Alexa Fluor 555 was used as secondary antibody (red). Nuclei were visualised via DAPI (blue). A4-A6 represent a magnified view of the region outlined by the yellow box in A1-A3. Scale bars 20 μ m.

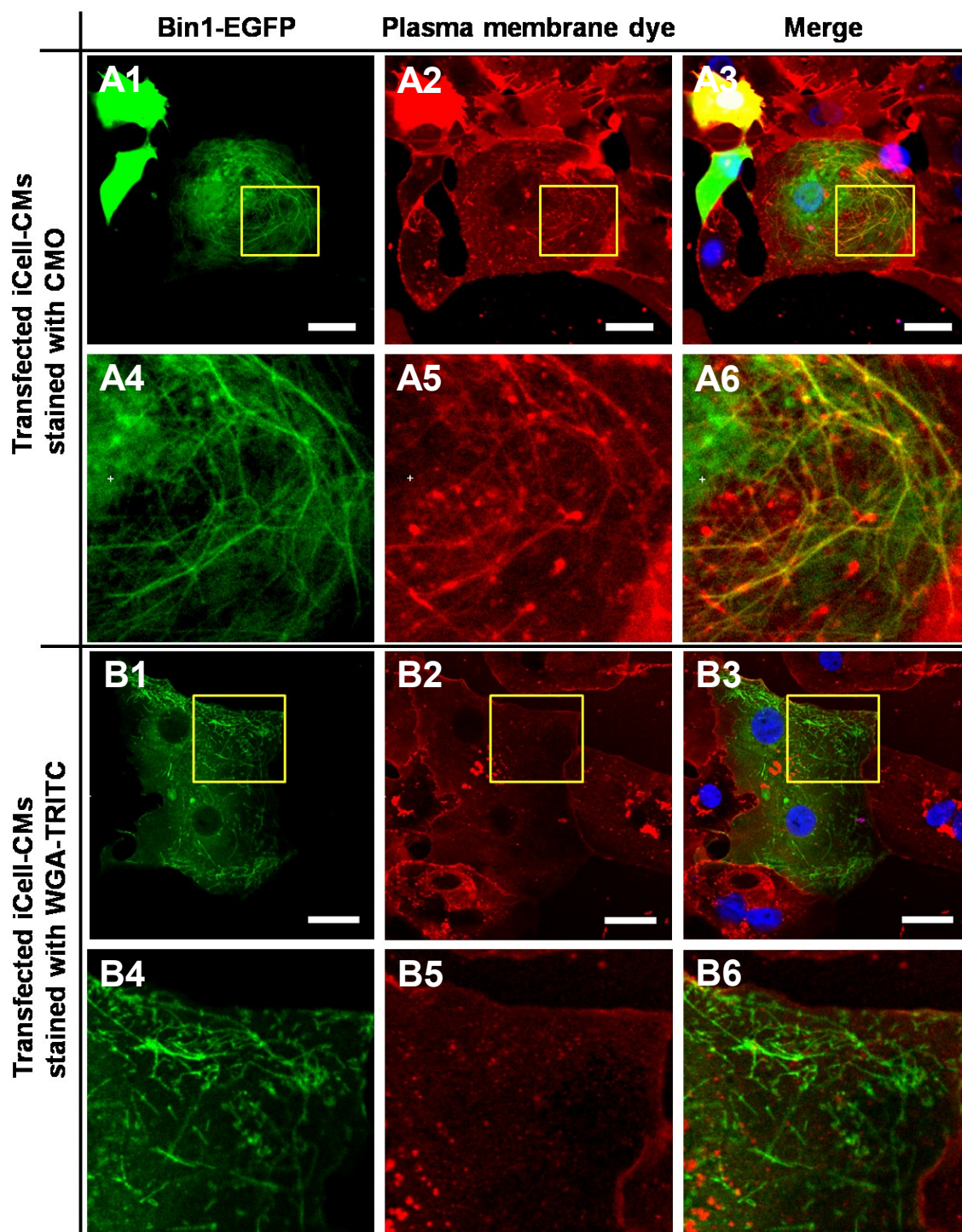


Fig. 5.6. Labelling of the plasma membrane of Bin1-EGFP expressing iCell-CMs. iCell-CMs expressing Bin1-EGFP (green; A1, A4, B1, B4) were stained with the plasma membrane dyes CellMask Orange (CMO; A2 and A5) or WGA-TRITC (red; B2 and B5) and analysed via immunofluorescence microscopy. Labelling with CMO produced a staining pattern, which overlapped with the green fluorescent tubule structures (A3 and A6). In contrast, staining with WGA-TRITC did not reveal any tubule like staining, which overlapped with the green fluorescent structures (B3 and B6). A4-A6 and B4-B6 display a magnified view of the region shown by the yellow box in A1-A3 and B1-B3, respectively. Scale bars 20 μm .

5.3.2.3. Characterisation of the Bin1-EGFP induced tubules

To elucidate the structural properties of the tubules induced in Bin1-EGFP expressing cells, it was first investigated whether the tubules were associated with the myofibrils within iCell-CMs. Immunofluorescence analysis of transfected cells stained for sarcomeric α -actinin (Fig. 5.7. A1-A6) revealed that the green fluorescent tubules showed no continuous overlapping with the α -actinin banding pattern (Fig. 5.7. A3 and A6) but a number of foci with overlapping fluorescent signals (arrows in Fig. 5.7. A6) could be observed.

Next it was examined whether the tubules showed any co-localisation with Cav1.2 channels, which are found abundantly in TTs of adult cardiomyocytes. Immunofluorescence staining for Cav1.2 (Fig. 5.8. A1-A6) in transfected cells showed a punctate Cav1.2 staining pattern (A2 and A5) similar to that seen in non-transfected iCell-CMs (see section 4.3.3.1.). However, only few of the Cav1.2-immunoreactive puncta showed co-localisation with the green fluorescent structures (arrows in Fig. 5.8. A6).

Furthermore, transfected iCell-CMs were labelled against the SR proteins RyR2 (Fig. 5.8. B1-B6), SERCA2a (Fig. 5.8. C1-C6) and PLN (Fig. 5.8. D1-D6) to investigate whether the tubular structures in the transfected cells were connected with the cellular SR system. Similar to the results with Cav1.2 the staining pattern of RyR2, SERCA2a and PLN in transfected iCell-CMs resembled the pattern seen in non-transfected cells described in section 4.3.3.1: RyR2 gave only a weak fluorescent signal (Fig. 5.8. B2 and B5) indicating a low RyR2 expression; SERCA2a (Fig. 5.8. C2 and C5) and PLN (Fig. 5.8. D2 and D5) gave a stronger signal throughout the cells and PLN staining showed a banding pattern in some cell areas. None of the SR protein staining in the transfected cells showed continuous alignment with the green fluorescent tubules (see Fig. 5.8. RyR2 in B3 and B6, SERCA2a in C3 and C6 and PLN in D3 and D6). However, some overlapping fluorescence signals for the robustly expressed PLN and the green fluorescent tubules could be detected (arrows in Fig. 5.8. D6) suggesting that a few contact points between the SR and the Bin1-EGFP induced tubules might have been present.

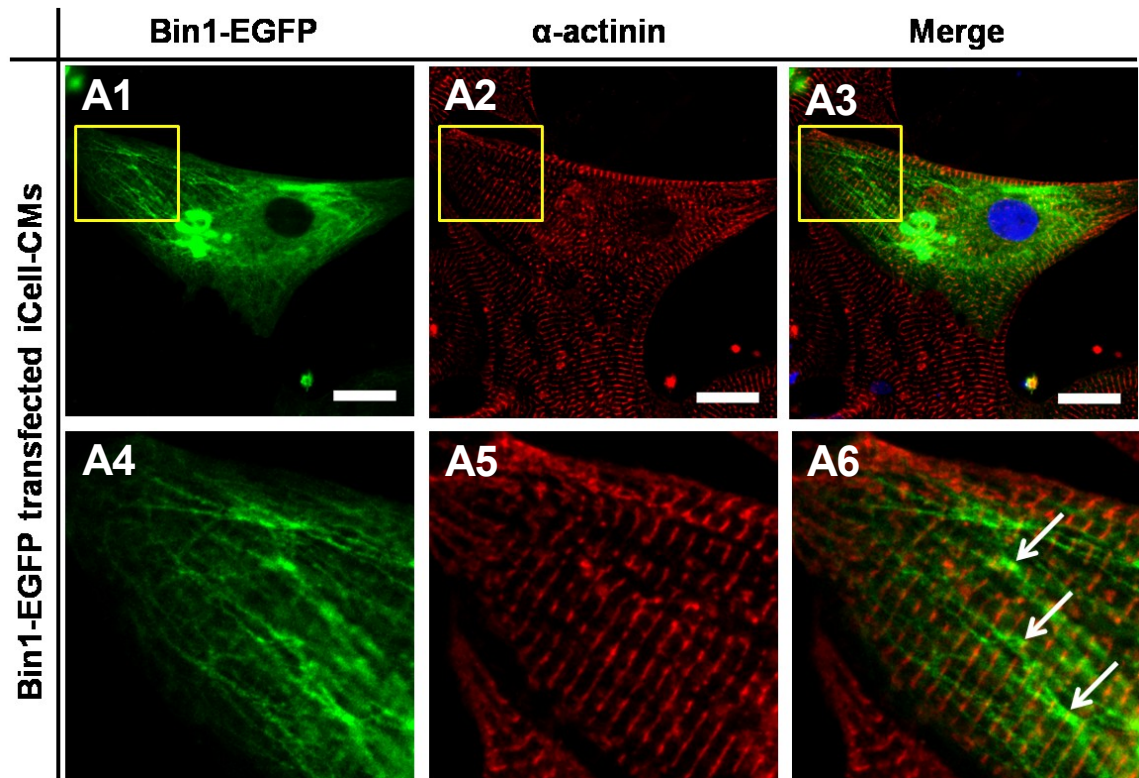
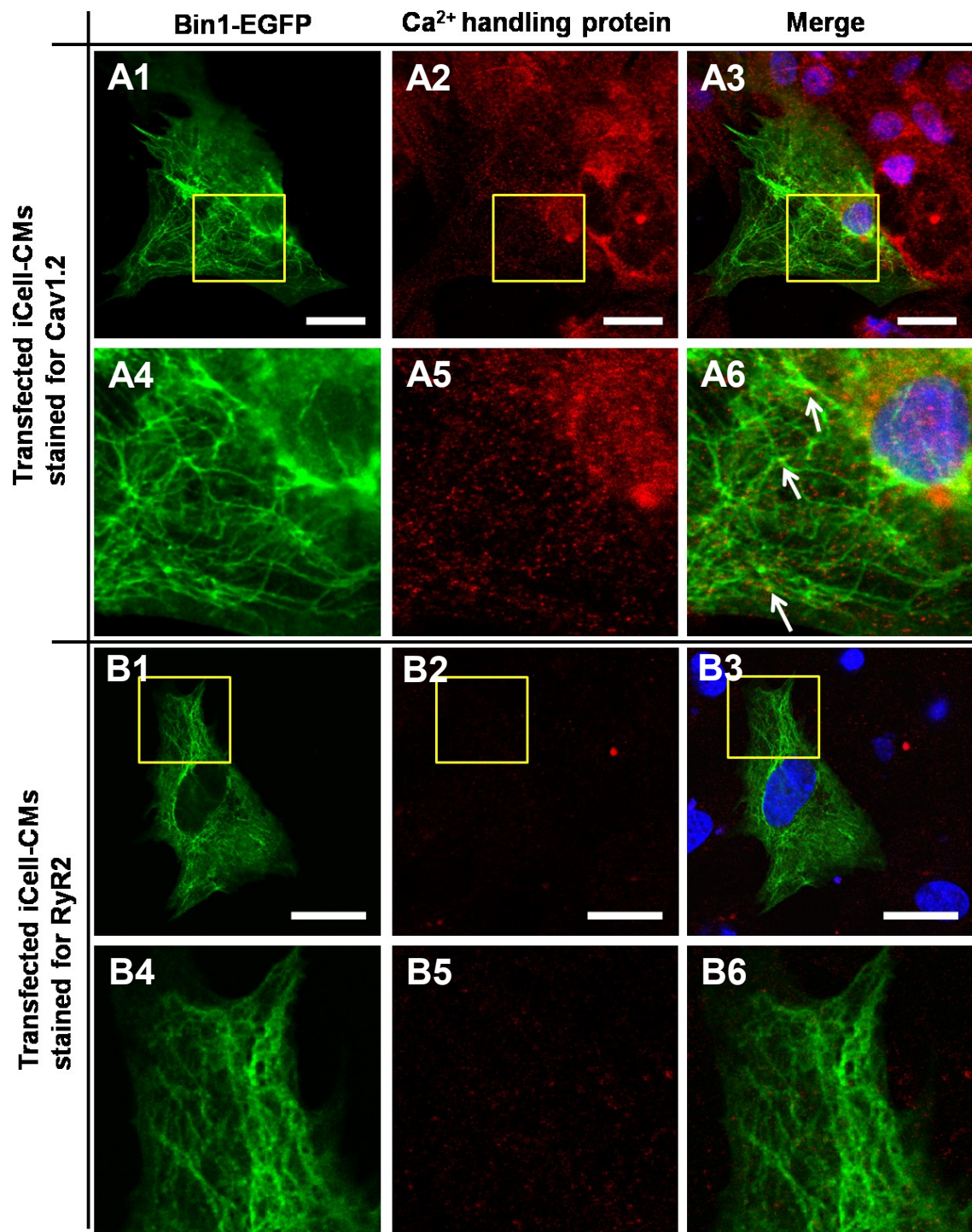


Fig. 5.7. Immunostaining for α -actinin in Bin1-EGFP expressing iCell-CMs.

iCell-CMs overexpressing Bin1-EGFP (green; A1 and A4) were labelled with a primary antibody against α -actinin (A2 and A5) and the secondary antibody AlexaFluor 555 (red). Nuclei were stained with DAPI (blue). A4-A6 show a magnified view of the region outlined by the yellow box in A1-A3. Arrows in A6 show overlapping regions of α -actinin and Bin1-EGFP. Scale bars 20 μ m.



For figure legend see page 172.

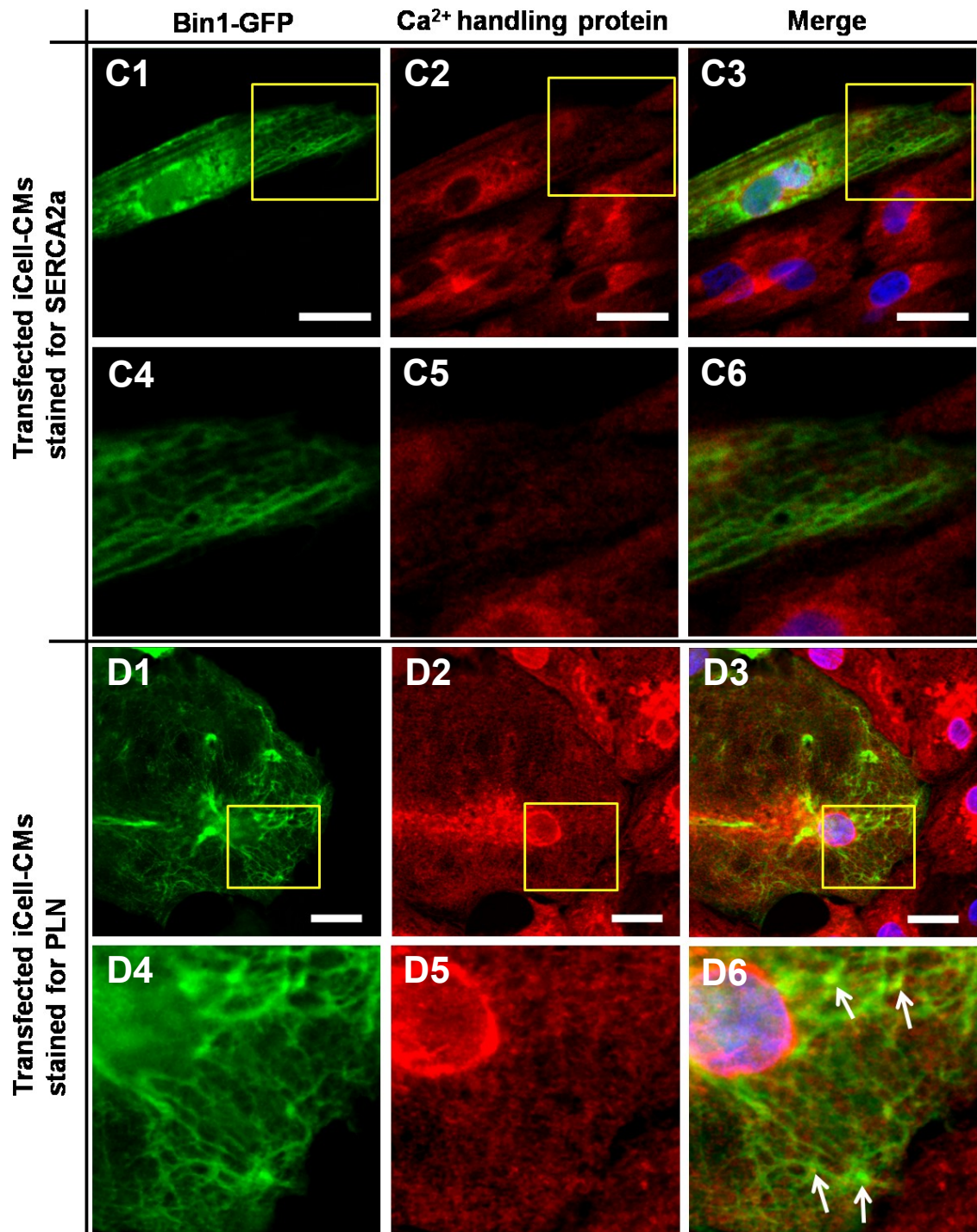


Fig. 5.8. Expression of Ca^{2+} handling proteins in Bin1-EGFP transfected iCell-CMs. iCell-CMs overexpressing Bin1-EGFP (green; A1, A4, B1, B4, C1, C4, D1 and D4) were labelled with primary antibodies against Cav1.2 (A2 and A5), RyR2 (B2 and B5), SERCA2a (C2 and C5) and PLN (D2 and D5). AlexaFluor 555 (red) was used as secondary antibody. Nuclei were stained with DAPI (blue). A4-A6, B4-B6, C4-C6 and D4-D6 display a magnified view of the region outlined by the yellow box in A1-A3, B1-B3, C1-C3 and D1-D3, respectively. Arrows in A6 and D6 show overlapping regions of Cav1.2 and Bin1-EGFP and PLN and Bin1-EGFP, respectively. Scale bars 20 μm .

5.3.2.4. Molecular analysis of Bin-EGFP expressing iCell-CMs via qRT-PCR

Overexpression of Bin1-EGFP in iCell-CMs induced a strong upregulation of the Bin1 expression level relative to non-transfected iCell-CMs (more than a 2×10^6 -fold increase) and the adult heart control (more than a 8×10^4 -fold increase; Fig. 5.9. A). However, the expression of the TT-associated genes Cav3, JP2 and Tcap in transfected cells did not change compared to non-transfected samples. The overexpression of Bin1-EGFP had also no effect on the expression of the key SR Ca^{2+} release and uptake proteins RyR2 and SERC2a, respectively, and the regulatory protein PLN but induced a minor increase in the expression of the SR proteins IP3R2 (increased by 1.7-fold) and CSQ2 (increased by 1.6-fold; Fig. 5.9. B).

Overexpression of Bin1-EGFP also increased the expression of the potassium channels Kir2.1 (2.8-fold increase) and Kv4.3 (3-fold increase; Fig. 5.9. C) and the cardiac transcription factor GATA4 (1.3-fold increase; Fig. 5.9. D). However, Bin1-EGFP did not alter the expression of Ca^{2+} channels such as Cav1.2 (Fig. 5.9. C) and structural proteins including α - and β MHC (Fig. 5.9. D).

5.3.3. TEM analysis of transfected iCell-CMs

For further structural analysis of the tubules induced in Bin1-EGFP transfected iCell-CMs the transfected cells were grown on sapphire discs, embedded in epoxy resin via a RT resin embedding method and sectioned to be analysed via transmission electron microscopy. The electron micrographs of transfected samples revealed that these cells had similar features to non-transfected cells (described in section 4.3.4.). Various cell organelles could be identified including myofibrils with a variable degree of organisation, mitochondria and the cell nucleus (Fig. 5.10. A). However, in contrast to non-transfected cells the transfected samples also revealed cells, which showed an abundance of tubular membrane structures (thick arrows in Fig. 5.10. A-B) with random orientation within the cell and a diameter of approximately 50 nm, which is similar to caveolae buds. Moreover, in a few cell sections the tubular structures appeared to be connected to the plasma membrane (thick arrows in Fig. 5.10. B).

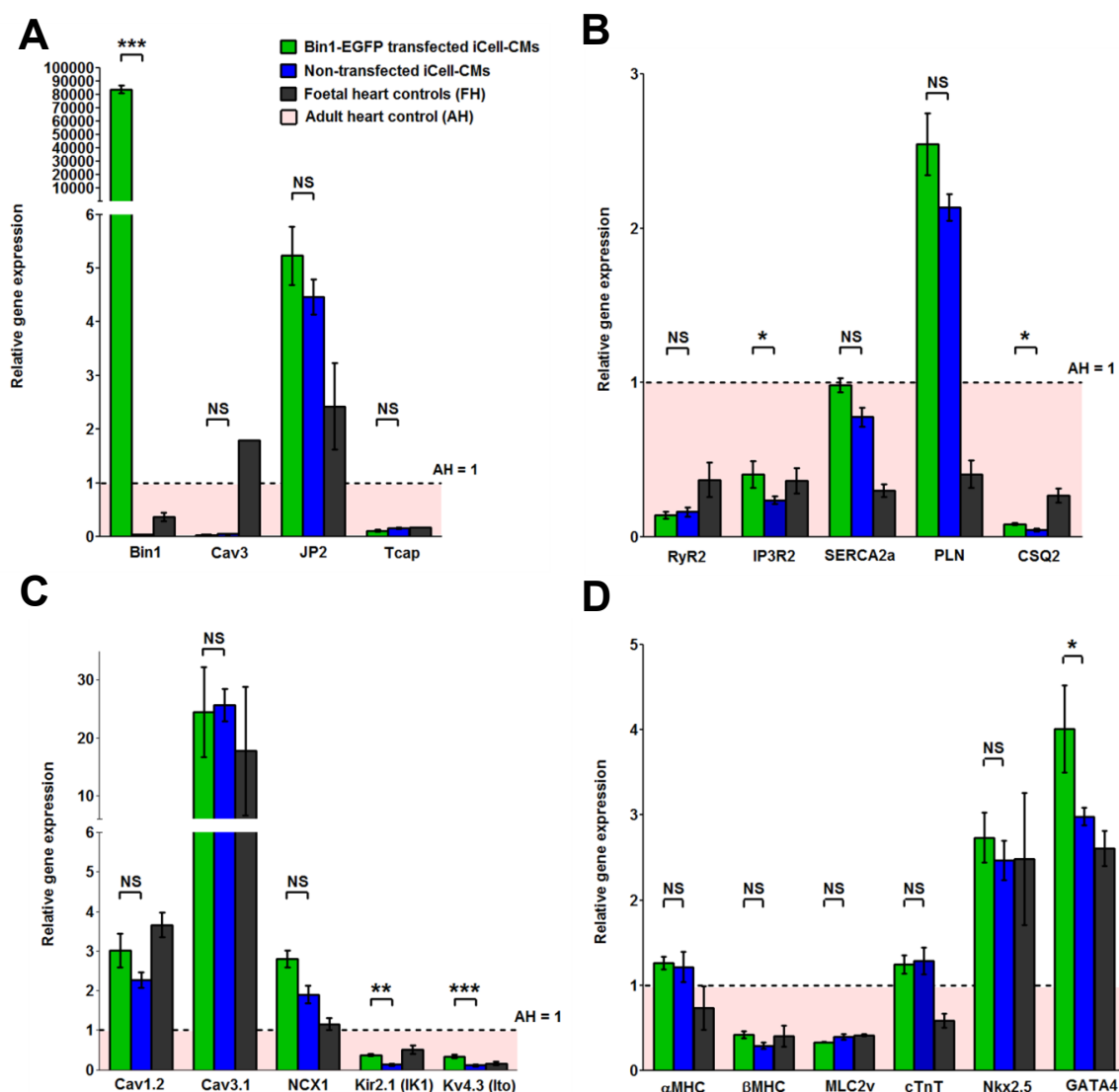


Fig. 5.9. qRT-PCR analysis of iCell-CMs transfected with Bin1-EGFP.

Displayed are mRNA expression levels of (A) TTs-associated proteins, (B) SR Ca^{2+} handling components, (C) ion channels and (D) structural proteins and general cardiac markers in iCell-CMs transfected with Bin1-EGFP. The gene expression levels from transfected iCell-CMs were compared with the expression levels in non-transfected samples. Human foetal (FH) and adult heart (AH) tissue were used as controls. qRT-PCR data from iCell-CMs were obtained in triplicates (represent three independent culture preparations) and results from FH and AH in singlets, in $n=2$ different qRT-PCR runs. All values were normalised with respect to GAPDH and shown relative to the corresponding values in AH (AH=1). Error bars represent $\pm$ SEM. Statistical significance between the two means was determined using unpaired Student's t-test (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

Next, in order to confirm that the tubular membrane structures were associated with Bin1-EGFP immunogold labelling was performed to tag Bin1 in transfected iCell-CMs. Adult rat cardiomyocytes immunogold labelled for α -actinin, which is expressed abundantly at the Z-lines of sarcomeres, were used as control. The labelling was carried out on the epoxy resin sections of transfected iCell-CMs and adult rat cardiomyocytes, respectively, using primary antibodies against Bin1 and α -actinin, respectively, and secondary antibodies conjugated to 10 nm gold particles. However, when analysing the samples via TEM no gold particles or only few gold particles showing non-specific binding (arrow in Fig. 5.11. A) could be detected in the cell sections of both transfected iCell-CMs (Fig. 5.11. A) and adult rat controls (Fig. 5.11. B). This suggests that the immunogold-labelling protocol using resin sections was not successful.

5.3.4. Measurement of cell capacitance and Ca^{2+} current

In order to determine whether the Bin1-EGFP induced tubules altered the electrophysiological properties of iCell-CMs the whole-cell patch-clamping technique was used to measure the transmembrane Ca^{2+} current (ICa ; in pA), which plays a key role in initiating ECC, and cell capacitance (in pF), which can serve as an index for the cell surface membrane area, in transfected and non-transfected (control) cells. All patch-clamp recordings were kindly conducted by James Cartledge, a PhD student within the cell electrophysiology group at the National Heart and Lung Institute, Imperial College London.

The patch-clamp results revealed that compared to control iCell-CMs the membrane capacitance in transfected iCell-CMs was increased by almost 30 % (Fig. 5.12. A) indicating an enlarged surface membrane area in transfected cells. Furthermore, patch-clamp recordings showed that ICa density (obtained by normalisation of the peak ICa to the cell capacitance) was approximately 35 % larger in transfected iCell-CMs than in control cells (Fig. 5.12. B), suggesting an increased transmembrane Ca^{2+} flux in the transfected cells. In order to assess whether the measured Ca^{2+} current derived from L-type Ca^{2+} channels ICa was also measured in cells, which were perfused with nitrendipine (0.1 mM, 5 min), a blocker of L-type Ca^{2+} channels. The current recordings revealed that nitrendipine blocked ICa

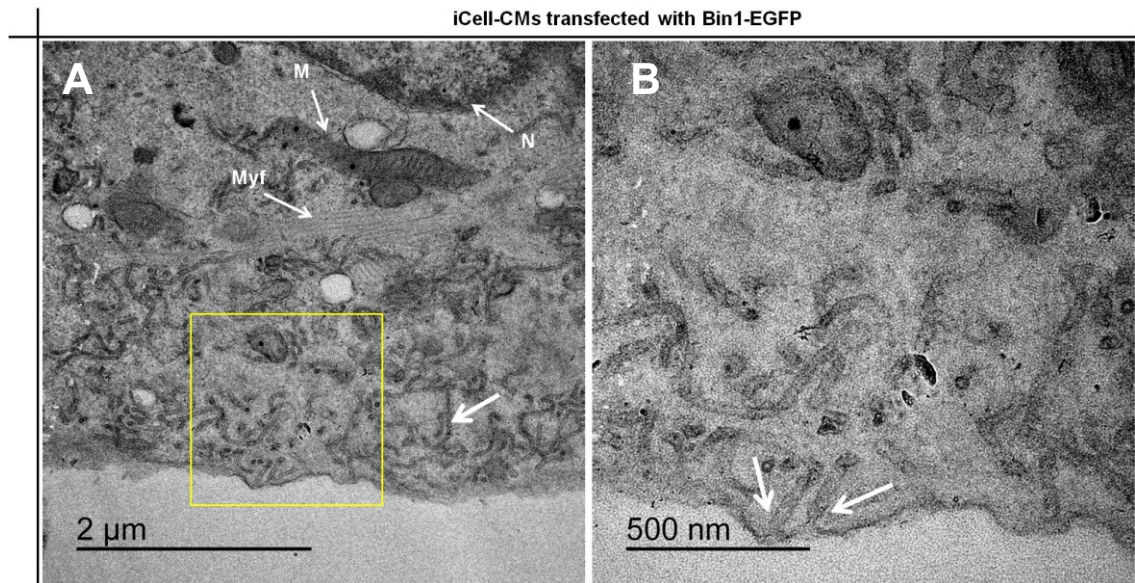


Fig. 5.10. Electron micrographs of iCell-CMs transfected with Bin1-EGFP.

iCell-CMs, which were transfected with Bin1-EGFP (A and B), displayed various cell organelles including mitochondria (M), randomly dispersed myofibrils (Myf) and a cell nucleus (N). The transfected samples also revealed an increased abundance of tubular membrane structures (A and B, thick arrows). B displays a magnified view of the region outlined by the yellow box in A. Arrows in B display tubular structures showing continuity with the cell plasma membrane. Scale bars 2 μ m in A and 500 nm in B.

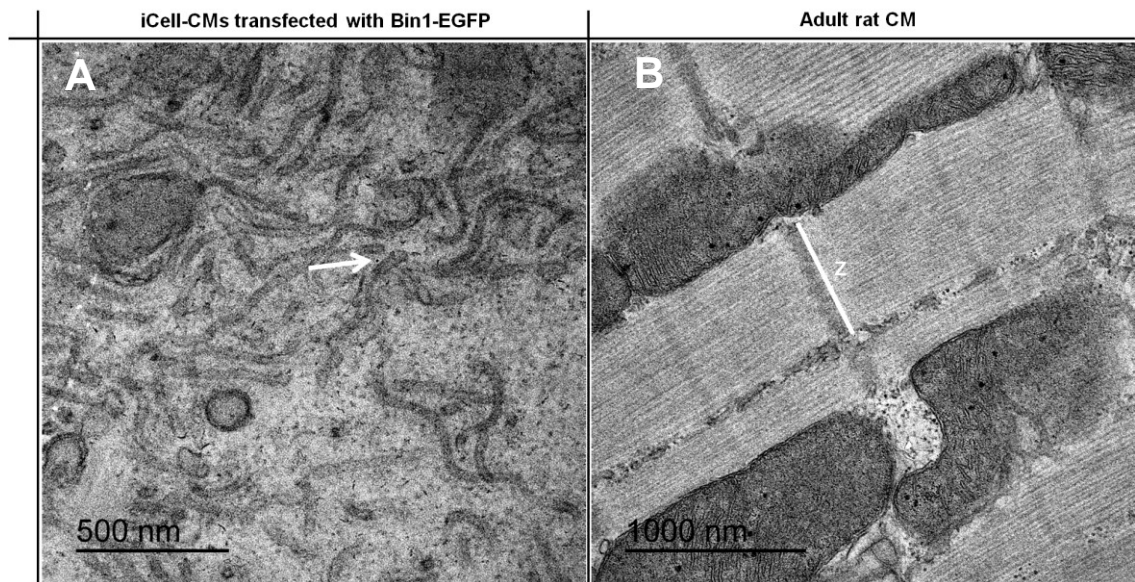


Fig. 5.11. Immunogold labelling of transfected iCell-CMs and adult rat cardiomyocytes for Bin1 and α -actinin.

Resin section of Bin1-EGFP transfected iCell-CMs (A) and adult rat cardiomyocytes (B) were labelled with anti-Bin1 (A) and anti- α -actinin (B), respectively, as primary antibodies, incubated with secondary antibodies conjugated to 10 nm gold particles and silver-enhanced. However, no specific immunogold labelling could be detected in A or B. Arrow in A shows a non-specifically bound gold particle. Z: Z-line. Scale bars 500 nm in A and 1000 nm in B.

almost completely (Fig. 5.12. C, grey curve). I-V curves depicting the I_{Ca} density-voltage relationship in control and transfected iCell-CMs (Fig. 5.12. C, red and blue curve) exhibited a bell-shaped pattern, which showed that the I_{Ca} density in these cells was voltage-dependent with peaks at 5 mV and 10 mV, respectively. This reflects the voltage-dependent properties of L-type Ca²⁺ channels. In contrary, the I_{Ca} density obtained from nitrendipine-treated cells remained close to 0 pA/pF across all depolarisation voltages.

5.4. Discussion

5.4.1. Effect of T3 on gene transcript expression in iCell-CMs

In the present study qRT-PCR analysis was used to investigate the effect of T3 on cardiac gene expression in iCell-CMs. First, it was shown that both control and T3-treated iCell-CMs expressed high levels of THR α and low levels of THR β and D3. This indicates that the receptors mediating the transcriptional effects of T3 were present in the cells and that the biological activity of T3 was not inhibited by degradation through D3. The high expression of THR α is in agreement with the literature as THR α is described to be the predominant receptor isoform in the heart, which in the absence of T3 represses (and bound to T3 activates) the transcription of a number of genes including SERCA2a (Gloss et al., 2001).

Second, the qRT-PCR results suggest that T3 treatment enhanced maturation of iCell-CMs in some measure as indicated by the shift of the expression of Kv4.3, SERCA2a and Cav3.1 closer to the gene expression levels of adult heart controls. However, T3 did not alter the expression of other gene transcripts, which are implicated in TTs-development (e.g. Bin1 and Cav3) or mature Ca²⁺-handling (e.g. RyR2 and CSQ2). Most of these gene expression levels remained below adult levels. The increased expression of the hypertrophic marker ANP in T3-treated cells suggests that T3 promoted the hypertrophic growth of iCell-CMs, which is part of the normal cardiac maturation process.

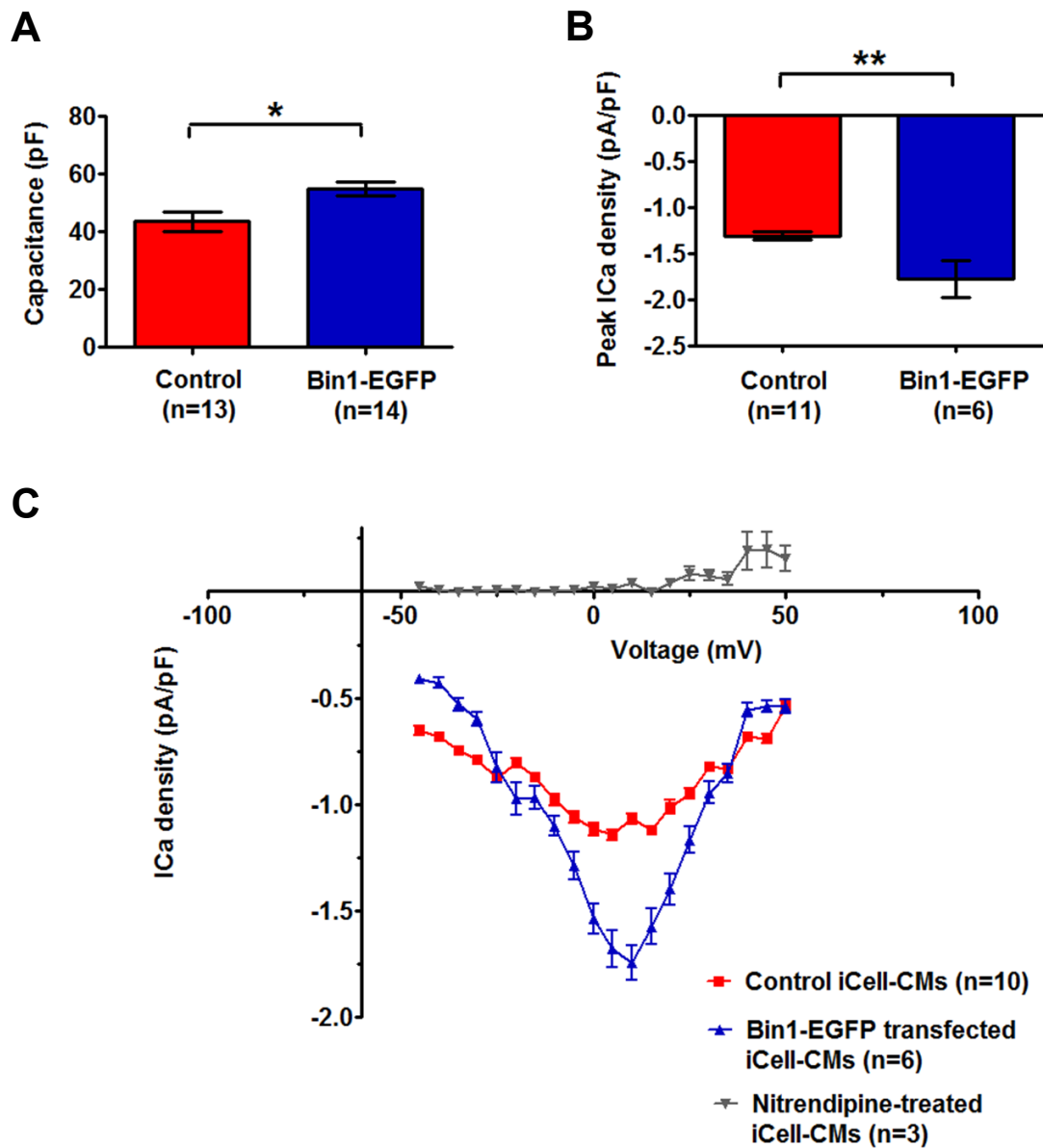


Fig. 5.12. Cell capacitance and Ca^{2+} currents in iCell-CMs measured via the whole-cell patch-clamping method.

The whole-cell patch-clamping technique was used to measure cell capacitance (A) and peak Ca^{2+} current (ICa) in control and Bin1-EGFP transfected iCell-CMs. ICa is displayed as ICa density (B), determined by normalisation of the peak Ca^{2+} current to the cell capacitance. ICa density-voltage relationship in control, transfected and nitrendipine-treated iCell-CMs is displayed in I-V curves (C). Error bars represent $\pm\text{SEM}$. Statistical significance between the two means was determined using unpaired Student's t-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

5.4.2. Staining for SERCA2a and labelling of the plasma membrane

Immunofluorescence analysis of T3-treated iCell-CMs revealed that, despite the higher mRNA levels of SERCA2a, no difference in the strength of the fluorescence signal could be detected between T3-treated and untreated cells. Also, the increased expression of SERCA2a was not paralleled by a reorganisation of the SR towards a more mature sarcomeric structure. Furthermore, staining of the plasma membrane of T3-treated cells with CMO did not exhibit the presence of any TT invaginations. The fluorescent dots and streaks detected in both T3-treated and non-treated iCell-CMs may be due to membrane folds or contaminations. These results imply that T3 did not promote the structural maturation of iCell-CMs.

5.4.3. Bin1 transfection and immunofluorescence studies

Bin1 can bind and invaginate cell membranes into narrow tubules and was linked to TT biogenesis (in skeletal muscle) (Takei et al., 1999; Farsad et al., 2001; Razzaq et al., 2001). The present study demonstrated that overexpression of Bin1-EGFP in iCell-CMs, which lack a TT system, resulted in the formation of green fluorescent tubular structures. Immunostaining of the transfected cells for Bin1 confirmed that Bin1 was localised continuously along these structures and positive staining of transfected cells with CMO implied that these tubules were connected to the cell plasma membrane. Collectively these results suggest that the overexpression of Bin1-EGFP induced the formation of plasma membrane invagination in iCell-CMs.

The difficulties to detect the Bin1-induced structures via WGA-TRITC may be explained by a change in the glycosylation of the membrane in the nascent membrane tubules, which precluded the labelling with WGA-TRITC. Other investigators reported similar problems with WGA when labelling thin TTs in human cardiomyocytes (Crossman et al., 2011).

A limitation of this study is the lack of additional robust controls. In addition to non-transfected iCell-CMs cells incubated with Lipofectamine 2000 only, an empty vector or a vector containing EGFP only should have been used as transfection controls to confirm that the

green fluorescent structures resulted exclusively from the expression of Bin1 and were not due to the expression of EGFP or the treatment with the transfection reagent Lipofectamine 2000.

5.4.4. Staining for Cav1.2 and SR Ca²⁺ handling proteins

Hong and colleagues (Hong et al., 2010) reported recently that in addition to its potential to initiate membrane curvature Bin1 also recruits L-type Ca²⁺ channels to the Bin1-induced invaginations via interaction with the microtubule trafficking network. In the present study immunostaining for Cav1.2 in iCell-CMs, which overexpressed Bin1-EGFP, showed only poor co-localisation with the Bin1-induced tubular structures. However, regions of co-localisation of the Cav1.2-immunoreactive puncta with the green fluorescent tubules were visually difficult to identify because although Cav1.2 puncta were present abundantly in the cells they appeared very small (as already discussed in section 4.4.3.1.).

In adult cardiomyocytes mature TTs are aligned approximately at the Z-lines of all myofibrils and are closely associated with the SR network. Immunostaining of transfected iCell-CMs for α -actinin and the SR proteins RyR2, SERCA2a and PLN aiming to reveal whether the Bin1-EGFP-induced tubules were connected to myofibrils and the SR showed no continuous alignment with the green fluorescent tubules. However, in cells stained for α -actinin and the abundantly expressed protein PLN a few co-localisation foci could be detected, which suggest that the Bin1-EGFP induced membrane invaginations might have been associated at some regions with the contractile apparatus and the SR. In order to confirm the spatial coincidence of both Cav1.2 with Bin1 and PLN with BIN1 additional studies (e.g. TEM with double immune-gold labelling) are required. Furthermore, iCell-CMs transfected with Bin1-EGFP were usually analysed 48 h after transfection. However, longer incubation times may deliver more conclusive results with regards to the localisation of Cav1.2 at the Bin1-EGFP tubules.

5.4.5. qRT-PCR result in transfected iCell-CMs

qRT-PCR analysis of the mRNA expression profile in transfected iCell-CMs showed that the expression of Bin1 was substantially higher in transfected iCell-CMs compared to control iCell-CMs and foetal and adult heart controls. This confirms that the transcription of the Bin1-EGFP plasmid was highly active within the transfected iCell-CMs.

Furthermore the qRT-PCR results revealed that overexpression of Bin1-EGFP was associated with a relatively strong increase in the expression levels of the potassium channels Kir2.1 and Kv4.3 and a minor increase in the expression levels of the cardiac transcription factor GATA4, the Ca^{2+} channel IP3R2 and the SR Ca^{2+} binding protein CSQ2. The overexpression of Bin1-EGFP did not alter the expression of any TT-associated genes (except Bin1), other major SR Ca^{2+} handling proteins including RyR2 and SERCA2a or any structural genes such as α - and β MHC.

Also, as discussed previously all samples (non-transfected and transfected with Bin1-EGFP) were normalised to GAPDH. However, to exclude that transfection using Lipofectamine or the overexpression of Bin1-EGFP may have affected the expression of GAPDH in transfected iCell-CMs future qRT-PCR experiments should include additional controls: (a) additional housekeeping genes (e.g. β -actin and ribosomal RNA 18S); (b) iCell-CMs treated with Lipofectamine only or cell samples transfected with a vector expressing for example EGFP only.

5.4.6. Analysis via transmission electron microscopy

Although the culture of iCell-CMs on transparent sapphire discs allowed the detection of transfected cells, which expressed Bin1-EGFP, via immunofluorescence microscopy it was not possible to distinguish between transfected and non-transfected iCell-CMs on cell resin sections via electron microscopy. However, the high abundance of membrane tubules was found only in cell sections of transfected samples, which indicates that the membrane structures were associated with the expression of Bin1-EGFP.

The attempt to label Bin1 on the cell resin sections via immunogold labelling in order to confirm that Bin1 was localised at the detected tubules was not successful. This was presumably due to the labelling protocol since the immunogold labelling of control adult rat cardiomyocytes for the sarcomeric α -actinin was not successful either. The immunogold labelling was performed directly on epoxy resin sections of transfected iCell-CMs and adult rat cardiomyocytes. However, it is reported that immunogold labelling of epoxy resin sections might be inefficient due to co-polymerisation of epoxy resins with the antigens (Brorson et al., 1999). Due to time restrictions an alternative labelling protocol, in which the samples are for example immunogold labelled before resin embedding, could not be evaluated.

The diameter of the detected membrane tubules in the captured TEM images of transfected iCell-CMs was approximately 50 nm. This diameter size is in line with the data of another study (Lee et al., 2002), which demonstrated tubules formation in Bin1-GFP transfected Chinese hamster ovary (CHO) cells, but is considerably smaller than the average diameter of TTs in adult cardiomyocytes [400 nm (Jayasinghe et al., 2011)]. However, iCell-CMs are immature and thin compared to adult cardiomyocytes (as shown in the previous chapter) thus the Bin1-EGFP induced tubules might widen with further maturation and hypertrophic growth of the cells.

5.4.7. Measurement of cell capacitance and I_{Ca} density

The whole-cell patch-clamp studies demonstrated that the membrane capacitance and the I_{Ca} density were significantly increased in iCell-CMs, which had been transfected with Bin1-EGFP and showed tubule structure formation. The increased membrane capacitance, which may be proportional to the cell membrane surface area, supports the evidence that the tubules formed in transfected iCell-CMs were connected to the cell plasma membrane thus resembling at least structurally TTs in adult cardiomyocytes.

The increase in I_{Ca} density, which is inversely correlated with the membrane capacitance, indicated that the formation of the tubules in Bin1-EGFP transfected cells did not only increase the plasma membrane surface area but was also accompanied by an increase in the number of functional L-type Ca^{2+} channels within the plasma membrane (e.g. via

increased gene expression) or an upregulation in channel activity in transfected cells. Given that Hong and colleagues (Hong et al., 2010) described that Bin1 can target L-type Ca^{2+} channels to Bin1-induced membrane invaginations the increased I_{Ca} density in transfected iCell-CMs implies that L-type Ca^{2+} may have been recruited to the Bin1-EGFP induced tubules and contributed to the I_{Ca} current in transfected cells.

5.5. Conclusion

5.5.1. Effect of T3 on iCell-CMs

T3 treatment enhanced maturation of iCell-CMs, as measured by a shift in the gene expression of various ion channels and SERCA2a, closer to the expression levels of adult heart controls. However, T3 treatment did not alter the expression of other genes, which are implicated in mature Ca^{2+} handling (i.e. RyR2, CSQ2) or TTs-development (e.g. Bin1 and Tcap). Also, T3 did not induce any TT formation in iCell-CMs.

5.5.2. Overexpression of Bin1-EGFP in iCell-CMs

Overexpression of Bin1-EGFP in iCell-CMs induced the formation of membrane invaginations, which were reminiscent of TT investigations in adult cardiomyocytes. Although immunostaining for Cav1.2 did not indicate clearly that L-type Ca^{2+} channels co-localised with the Bin1-EGFP tubules the increased I_{Ca} densities measured in transfected cells implied that L-type Ca^{2+} channels may have been targeted to the nascent tubules and contributed to the cell calcium current. qRT-PCR showed that overexpression of Bin1-EGFP did not alter the expression of other TT-associated genes or major SR Ca^{2+} handling components.

Collectively these results show that T3-treatment and overexpression of Bin1-EGFP, respectively, enhanced the maturation of iCell-CMs in some measure but seemed not to be sufficient to generate a fully mature phenotype.

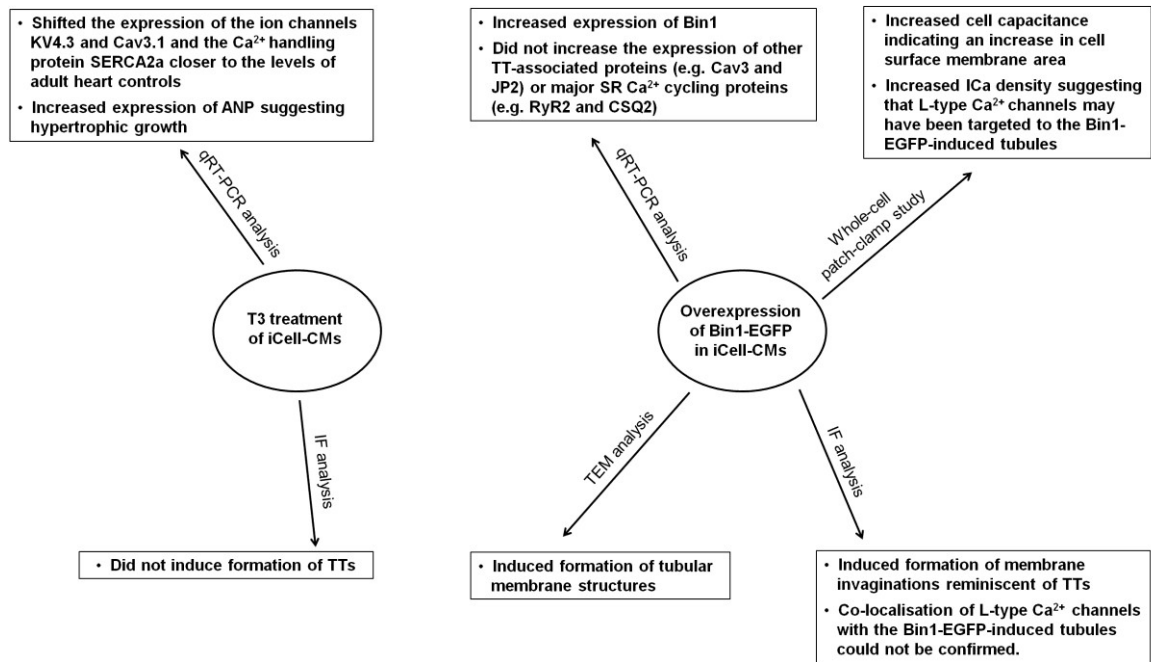


Fig. 5.13. Diagram summarising major results of chapter 5.

CMs: cardiomyocytes, ICa: Ca²⁺ current, IF: immunofluorescence microscopy, T3: triiodothyronine, TEM: transmission electron microscopy, TTs: transverse tubules.

Chapter 6: General discussion

6.1. Factors limiting the use of hPSC-CMs

hPSCs, which include hESCs and hiPSCs, have the capacity for self-renewal and can differentiate into almost any cell type of the human body (Thomson, 1998; Takahashi et al., 2007). Thus they can potentially represent a renewable source of human cardiomyocytes. Cardiomyocytes derived from hPSCs could have a variety of applications. They could serve as normal cardiac cell models or models of cardiac congenital diseases to study normal cardiac development or the pathobiology of cardiac conditions and may eventually replace animal models currently used to investigate drug efficacy and cardiac side-effects (Harding et al., 2007). hPSC-CMs have also the potential to be used in future in cell-based therapies to regenerate damaged heart tissue (Robinton and Daley, 2012). However, for the extensive use of hPSC-CMs in pharmacology and in clinical applications there are several limitations to overcome.

First, the culture conditions of hPSCs needs to be further optimised. Most of the hESC and hiPSC lines are currently routinely maintained as monolayers on mouse feeders (Thomson, 1998; Reubinoff et al., 2000) or on Matrigel (Xu et al., 2001; Ludwig et al., 2006a). However, the maintenance and expansion of MEFs are laborious, time consuming and hamper the scale up of hPSC cultures and animal components in MEFs and Matrigel might limit the use of hPSCs in regenerative medicine. However, although much progress has been made to develop fully defined, xeno-free culture components [e.g. chemically defined, xeno-free culture media and synthetic growth matrices (Villa-Diaz et al., 2013)] a standard widely excepted culture method for hPSC lines has not been determined yet, which hampers the development of standard differentiation protocols. Also, culture protocols published for some lines are often challenging to adapt for other lines (Denning et al., 2006; Allegrucci and Young, 2007).

Second, the cardiac differentiation protocols need to be improved. The introduction of directed cardiac differentiation methods, which employ signalling pathways (Activin/Nodal/TGF β , Wnt and BMP) known to drive cardiogenesis have greatly improved the efficiency of cardiac differentiation (approximately up to 50 %) (Laflamme et al., 2007; Paige et al., 2010). However, it is still not possible to generate pure and lineage-specific cardiac

populations. The differentiated cultures typically contain heterogeneous populations of different cardiomyocytes subtypes (ventricular, nodal and atrial) and other non-cardiac cell types. To obtain relatively pure cardiomyocyte populations, which are for example desirable for accurate and consistent drug testing, an additional enrichment step is required (Xu et al., 2002; Huber et al., 2007). Furthermore, there can be significant line-to-line-variability with regards to the efficiency of a cardiomyocyte differentiation protocol (Osafune et al., 2008; Pekkanen-Mattila et al., 2009). This may be an issue particularly for the generation of cardiomyocytes from different patient-specific hiPSC lines.

Third, the maturity of hPSC-CMs needs to be enhanced. hPSC-CMs show initially immature structural and functional characteristics, which resemble more foetal than adult cardiomyocytes (Snir et al., 2003; Liu et al., 2007a; Gherghiceanu et al., 2011). For a safe application in potential cell-based therapies, reliable prediction of drug efficacy and toxicity hPSC-CMs need to be matured towards a more adult-like phenotype. Although, to some extent, maturation of hPSC-CMs occurs during prolonged *in vitro* culture (Snir et al., 2003; Sartiani et al., 2007; Lundy et al., 2013) and a number of pro-maturation approaches have been already evaluated by various research groups (described in section 1.6.1), the establishment of a fully adult-like phenotype has not been observed yet.

This study focused on the assessment of two alternative pro-maturation approaches: administration of T3, which is described to play an important role in cardiac development and maturation (Klein and Ojamaa, 2001; Pantos et al., 2008), and overexpression of Bin1(-EGFP), which has been reported to initiate membrane curvature in skeletal muscle and suggested to be involved in TT biogenesis (Lee et al., 2002).

For studying the effect of the pro-maturation stimuli initially cardiomyocytes were generated by differentiating the Shef3 hESC line. However, due to the inefficiency of cardiac differentiation of the Shef3 line all maturation studies were performed on the commercially produced iCell-CMs (hiPSC-CMs). In the following paragraphs the most important findings and limitation of this study will be discussed and future directions highlighted.

6.2. Adaptation of hESC lines to feeder-free culture conditions

The Shef3 hESC line was originally derived and routinely maintained on mouse feeders (Draper et al., 2004). In this study considerable effort was applied to adapting Shef3 hESCs to feeder-free culture with the target to overcome the limitations associated with the handling of MEFs (tedious maintenance, difficulties of culture scale-up) and the aim to evaluate directed cardiac differentiation methods, which require feeder-free conditions.

The Shef3 line was transferred on Matrigel in MEF-conditioned medium, which had been prepared beforehand in our laboratory, and the commercially available culture medium mTeSR1 and NutriStem, respectively. However, long-term feeder-independent culture of Shef3 could only be established in mTeSR1. Neither MEF-conditioned medium nor NutriStem supported the undifferentiated growth of Shef3 hESCs long-term.

An explanation as to why stable long-term culture of Shef3 hESCs could not be established in MEF-conditioned medium may lie in the quality of the medium. First, the latter highly depends on the density of MEFs used for conditioning. In this study the density of MEFs (80000 cells/cm²) corresponded to values, which were used for feeder-free culture of the H7 line in our laboratory at Imperial College. It is possible, however, that this density was not high enough for the feeder-free maintenance of the Shef3 line. Future culture set-ups with medium conditioned by higher MEF-densities may elucidate this point. Second, MEF-conditioned medium is not defined and prone to significant batch-to-batch variability, which also may have interfered with the undifferentiated growth of Shef3 hESCs. Second, the quality of the MEF-conditioned medium may have been affected by the freeze-thaw cycle of the medium (it was frozen/thawed once and stored at -80 °C), which could have destroyed several cytokines, and the use of mitomycin C for the inactivation of MEFs prior to conditioning. Recent studies indicate that different inactivation methods such as treatment with mitomycin C and γ -irradiation have a different impact on the metabolic activity of MEFs and their expression of cytokines and ligands, which are important for the undifferentiated growth of ESCs (Roy et al., 2001; Fleischmann et al., 2009). Although mitomycin C treatment

of MEFs and freezing of MEF-conditioned medium have been used routinely within our laboratory when preparing conditioned medium for the feeder-free propagation of the H7 line the initial adaptation of the Shef3 line to feeder-free conditions may require the use of γ -irradiated MEFs and freshly prepared MEF-conditioned medium. Finally, MEF-conditioned medium is not defined and prone to significant batch-to-batch variability, which also may have interfered with the undifferentiated growth of Shef3 hESCs.

In contrast to MEF-conditioned medium, mTeSR1 and NutriStem are both chemically fully defined and principally do not show the quality variations associated with MEF-conditioned medium. However, although it is known that both media contain growth factors, which trigger bFGF and TGF β signalling pathways to sustain undifferentiated growth of hPSCs, the specific combination and concentration of all components is proprietary. The difficulties to adapt the Shef3 line to a feeder-free culture in MEF-conditioned medium and NutriStem and the successful adaptation to mTeSR1 may be also explained by both the specific composition of the different media and intrinsic properties of the Shef3 line. It is reported that each hESC line has a unique gene expression signature showing distinct expression levels for a variety of genes (Abeyta et al., 2004; Skottman et al., 2005). In particular potential differences in the expression levels of bFGF and TGF β receptor subtypes and key cell adhesion factors [e.g. integrins (Meng et al., 2010)] may lead to distinct factor requirements of different hESC lines for undifferentiated growth in culture. Thus some media formulations may be more suitable than others for the maintenance of a particular hESC line.

Furthermore, the transfer of hPSCs to different culture conditions is often challenging because it is generally a stressful event for the cells. Recently, it was shown that the transfer to a feeder-free culture is associated with considerable changes in gene transcription and DNA methylation in hESCs as the cells adapt to the new culture conditions (Tompkins et al., 2012).

Data on unsuccessful attempts to adapt an hPSC line to feeder-independent culture are generally scarce in the literature. As far as our laboratory is concerned, apart from the Shef3 line we also encountered difficulties to adapt the H7 line (WA07), which was purchased from WiCell Research Institute (Madison, USA), to a long-term Matrigel-culture in MEF-

conditioned medium (data not published) although the H7 hESCs, which were provided by Geron Corporation (Menlo Park, CA), were maintained on Matrigel in MEF-conditioned medium routinely in our laboratory. This finding indicates that even the same cell line but cultured in different laboratories can behave differently in altered culture conditions. Moreover, a recent study by the International Stem Cell Initiative Consortium (Akopian et al., 2010) compared the potential of eight different published, defined culture media (including mTeSR1 and StemPro) in combination with Matrigel or other substrates to support the feeder-free culture of several hESC-lines, which were previously maintained on mouse feeders. The results of the study revealed that only the commercially prepared media mTeSR1 and StemPro supported the undifferentiated proliferation of most tested hESC lines long-term. These results highlight that adaption of different hESC lines to feeder-free conditions is not straight-forward and also that commercially prepared media, which undergo extensive testing and quality controls, are usually superior in the maintenance of hPSCs in a feeder-free culture system.

6.3. Challenges to reproduce cardiac differentiation protocols

In the present study various published cardiac differentiation approaches were evaluated for the Shef3 hESC line: the conventional EB differentiation method, which relies on spontaneous differentiation in serum (Itskovitz-Eldor et al., 2000); directed cardiac differentiation of cell monolayers with activin A/BMP4 based on the protocol by Laflamme and colleagues (Laflamme et al., 2007); and directed cardiac differentiation of AggreWell-formed EBs with APEL medium and a combination of growth factors (Stemcell Technologies, 2012b). Overall, for all applied methods the Shef3 line exhibited low cardiac differentiation propensity.

Beating clusters of Shef3-CMs could be successfully produced via the conventional EB formation method but at 8-10 % the proportion of EBs containing contracting clusters was low compared to studies, which reported to produce beating clusters in 10-70 % of EBs (Xu et al., 2002; He et al., 2003). Similarly, there were difficulties with the Shef3 line to reproduce the

high yield of cardiomyocytes and beating clusters reported for the directed cardiac differentiation protocols by Laflamme et al. and Stemcell Technologies (Laflamme et al., 2007; Stemcell Technologies, 2012b). The success of the directed cardiac differentiation method by Laflamme et al. seemed at least partially to depend on culture conditions. Directed differentiation of Shef3 hESCs, which were routinely propagated on feeders but transferred on Matrigel and exposed to MEF-conditioned medium shortly before induction of differentiation with activin A, produced spontaneously contracting clusters, albeit with low efficiency. In contrast, directed differentiation of Shef3 cells, which were adapted to long-term Matrigel culture in mTeSR1 medium, did not generate any contracting clusters when using the protocol by Laflamme et al. The influence of culture conditions on the differentiation potential of hPSC lines has been also observed by others and was evaluated in the study by Ojala and co-workers (Ojala et al., 2012). Although, Ojala et al. used other hPSC lines (H7 and four hiPSC lines) and another cardiac differentiation method (co-culture with END-2) it is interesting to note that the authors observed that cells cultured on feeder layers showed the highest and cells cultured on Matrigel in mTeSR1 showed the lowest cardiac differentiation potential.

The second directed cardiac differentiation protocol, which was evaluated for the Shef3 line in this study, was developed by Stemcell Technologies and described to be highly efficient for mTeSR1-cultures of H7 and H9 (Stemcell Technologies, 2012b). This method was a novel protocol at the time introduced by Stemcell Technologies, and has not been extensively evaluated by other independent research groups yet. However, this method did not generate any contracting clusters with the Shef3 line, although the line was well adapted to mTeSR1 medium.

A possible explanation for failing to reproduce a specific differentiation protocol in an individual hESC line may also lie in line-intrinsic properties. As discussed in the introduction, individual hPSC lines show transcriptional and epigenetic variations, which may lead to different propensities to differentiate into cardiomyocytes and other cell types (Allegrucci and Young, 2007; Osafune et al., 2008). A limitation of this study is that no other hESC line, which was grown on Matrigel in mTeSR1 medium, was available in our laboratory at the time

of the differentiation studies to repeat the differentiation protocol. This may have shed light whether the difficulties to adapt the differentiation protocol provided by Stem Cell Technologies was associated with intrinsic characteristics of the Shef3 line.

Furthermore, a key issue for the efficient cardiac differentiation is cardiac mesoderm formation. The induction of cardiac differentiation in PSCs via treatment with growth factors, which mediate mesoderm formation such as activin A, BMP4, bFGF and DKK1 (see section 1.3.1.), is crucially dependent on the concentration of these growth factors and the timing of their addition and removal, respectively because each step in the process of mesoderm formation (and further mesoderm specification and cardiomyocyte formation) requires its own growth factor combination and concentration. However, the endogenous signalling can vary between different PSC lines or even between different passages of the same line so that the optimal concentration and timing of the added factors driving mesoderm formation may need to be determined for each individual PSC line (Paige et al., 2010; Kattman et al., 2011). Optimisation of the directed cardiac differentiation methods for the Shef3 line was not performed in this study due to time restraints of the project.

Another reason for the poor differentiation of Shef3 hESCs into cardiomyocytes may have been that the Shef3 cells lost their pluripotent state with prolonged culture on iMEFs and Matrigel, respectively. Although the pluripotency of Shef3 hESCs was confirmed at early culture passages via immunostaining against various pluripotency markers (Oct4, SSEA4 and Tra-1-60) it was not tested prior to the induction of cardiac differentiation. In future experiments a more rigorous testing of pluripotency markers at different Shef3 passages and immediately prior to cardiac differentiation is required.

Finally, it is noteworthy that it has been recently demonstrated that cardiac-like cells can be induced directly from mouse fibroblasts by overexpression of only three cardiac developmental transcription factors (Gata4, Mef2c, and Tbx5) (Ieda et al., 2010) or via brief overexpression of the reprogramming factors Oct4, Sox2, Klf4 and c-Myc (Takahashi and Yamanaka, 2006) and subsequent exposure to cardiogenic medium (includes BMP4) (Efe et al., 2011). These reprogramming strategies do not require the formation of iPSCs beforehand or the establishment of any further cardiac differentiation protocols and could represent an

alternative method for the generation of cardiomyocytes for therapeutic applications. However this approach is still in the early development stage and requires extensive characterisation.

6.4. The importance of hPSC-CM maturation *in vitro*

For initial characterisation of iCell-CMs and subsequently for the assessment of the effect of the maturation stimuli on the cells the molecular, structural and electrophysiological features of iCell-CMs were analysed using a broad spectrum of approaches including qRT-PCR, bright field and immunofluorescence microscopy, TEM, MEA and whole-cell patch-clamping technique. In addition, the characteristic features of the generated Shef3-CMs were analysed via immunostaining and MEA. In line with the studies, which describe early-stage hPSC-CMs as being largely immature (Snir et al., 2003; Liu et al., 2007a; Gherghiceanu et al., 2011), the characterisation studies conducted in this project showed that Shef3-CMs and iCell-CMs displayed principally immature, foetal-like structural features: They contracted spontaneously; were smaller and flatter relative to adult cardiomyocytes; showed poor ultrastructural organisation with low myofibril density and alignment; did not possess a TT network and the SR showed variable degrees of sarcomeric organisation. In addition, it was shown for iCell-CMs that they displayed relatively low expression of key ion channels (Kir2.1 and Kv4.3), Ca^{2+} cycling proteins (RyR2, IP3R2 and CSQ2) and genes implicated in TT formation or maintenance (Bin1, Cav3 and Tcap). Electrophysiological studies, however, demonstrated that the contraction of iCell-CMs seemed to depend on Ca^{2+} release from the SR stores as seen in adult cardiomyocytes although the presence of RyR2/Cav1.2 couplons in iCell-CMs could not be definitely confirmed via immunolabelling. We do not have a conclusive explanation for these contradictory results. However, as discussed in section 4.4.3.1. the difficulties to detect Cav1.2/RyR2 couplons may be due to the relatively weak fluorescent signal of the RyR2 staining and the punctate expression pattern of Cav1.2, which make the detection of any co-localisation of the two proteins difficult. Both the presence of Cav1.2/RyR2 calcium release units and the contribution of SR Ca^{2+} to cell contraction should be verified in additional experiments.

The double-labelling for RyR2 and Cav1.2 in iCell-CMs could be repeated at higher antibody concentrations and with a different set of antibodies. The role of SR Ca^{2+} may be further explored by measuring Ca^{2+} transient amplitudes in iCell-CMs treated with ryanodine, an inhibitor of RyR2, or nitrendipine, a specific inhibitor of L-type Ca^{2+} channels. If Cav1.2/ RyR2 couplons are present in iCell-CMs and the SR Ca^{2+} release contributes substantially to cell contraction the Ca^{2+} transient amplitudes should be significantly reduced by ryanodine and nitrendipine, respectively.

hPSC-CMs including iCell-CMs (Cellular Dynamics International, 2012a) are advocated as suitable test systems for drug discovery and drug toxicity investigations. However, their immature phenotype raises doubts about whether they represent an accurate cardiac cell model (Burridge et al., 2012). Furthermore, although it was reported that both hESC-CMs and hiPSC-CMs respond to a variety of pharmacological agents and are sensitive to β -adrenergic stimulation (Rajamohan et al., 2012) our group recently observed, that hESC-CMs and hiPSC-CMs (including Shef3-CMs and iCell-CMs) respond differently to hypertrophic stimuli (results in print). For example, treatment with the α -adrenergic receptor agonist phenylephrine increased the cell area and expression of the hypertrophic marker ANP in hESC-CMs but did not alter the cell area or ANP expression of hiPSC-CMs. This distinct responsiveness to phenylephrine may stem from different expression profiles of various G-proteins, which are downstream targets of adrenergic signalling.

With regard to potential applications of hPSC-CMs in regenerative medicine there are also concerns that the immature electrophysiology of the cells may lead to ineffective contractile force generation and induce arrhythmia when the cells are transplanted into diseased myocardium (Wah Siu et al., 2007). However, on the other hand immature cardiomyocytes, which rely on anaerobic glycolysis for their energy production (Porter et al., 2011), have been shown to be more resistant to hypoxic environment displaying better integration and survival potential than adult cardiomyocytes when engrafted into diseased heart (Boheler et al., 2011; Reinecke et al., 1999; Laflamme et al., 2005). Also, their maturation may occur *in situ* in the heart. Thus a fully mature phenotype of hPSC-CMs may not be advantageous for applications in cell-based therapies. In contrast, a high degree of maturity in hPSC-CMs is

desirable in pharmacological applications since it would increase the predictive potential of these cells in drug screening and toxicity assays.

6.5. Concluding remarks: What is the best maturation strategy?

This study has evaluated T3-treatment and forced expression of Bin1(-EGFP) as strategies to promote the maturation of iCell-CMs. The results indicate that both T3 and Bin1-EGFP promoted the maturation of the cells in some aspects.

Treatment with T3 shifted the expression of a number of ion channels (Cav3.1 and Kv4.3) and SERCA2a towards adult expression levels and seemed to induce hypertrophic growth as evident by an increase in ANP expression, although further analysis of the cell size is needed to confirm hypertrophy. T3 did not have a detectable effect on SR organisation and did not induce TTs.

Overexpression of Bin1-EGFP had a dramatic effect on the morphology of iCell-CMs by inducing numerous tubular invaginations in the sarcolemma. These were reminiscent of TT invaginations of adult cardiomyocytes but lacked continuous alignment to myofibrils and may have been linked to the SR only at few connecting points. Furthermore, although no clear co-localisation of Cav1.2 and the Bin1-induced tubules could be detected via immunofluorescence analysis, the measured increase in the Ca^{2+} current density in Bin1-EGFP transfected cells suggested that L-type Ca^{2+} channels may have been targeted to the Bin1-induced tubules as previously observed by Hong and colleagues (Hong et al., 2010). Overexpression of Bin1-EGFP was also shown to increase the expression of the ion channels Kir2.1 and Kv4.3, which are both preferentially localised in TT membranes (Clark et al., 2001; Deschenes et al., 2002), but surprisingly did not alter the expression of other TT associated genes Cav3, JP2 and Tcap. Generally, the expression levels of most genes after T3 administration or overexpression of Bin1-EGFP remained below adult heart controls and a number of genes (e.g. RyR2 and Kir2.1) remained also below expression levels in foetal heart cells.

One limitation of the transfection experiments in this study was the lack of alternative Bin1 expression vectors. In this study a vector was used, which allowed the expression of Bin1 as a fusion to the C-terminus of EGFP. However, it is possible that the function of the Bin1-protein was affected by the EGFP tag. In contrast, Hong and co-workers used in their study a fusion protein with Bin1 expressed to the N-terminus of EGFP or mCherry (Hong et al., 2010). In order to exclude that the EGFP fusion affected the function of Bin1, future transfection studies should also be conducted with control vectors containing untagged Bin1 or Bin1 expressed to the N-terminus of EGFP.

Overall this study represents a comprehensive characterisation of the commercially available hiPSC-CM line iCell-CMs (early-stage) and has expanded our knowledge on the effect of T3 and Bin1 in iCell-CMs. The answer to the question which of the presented strategies is the best approach to mature hPSC-CMs is probably that none of the strategies is better than the other. Similar to other published pro-maturation approaches (see section 1.6.1.; 3-D culturing, mechanical stretch, forced expression of Kir2.1 or CSQ2) T3-treatment and overexpression of Bin1-EGFP enhanced various cell features but none of the stimuli was sufficient to induce a fully mature phenotype in iCell-CMs. However, to our knowledge, the formation of TT-like structures, which are distinctive features of adult cardiomyocytes, has not been observed in hPSC-CMs in any other previous study.

However, a strong limitation of this study was that the exact differentiation conditions of iCell-CMs and the exact formulation of the iCell-CM Plating Medium (probably serum-containing medium) and the iCell-CMs Maintenance Medium are proprietary to CDI and not published. Thus it is not known whether the cells have not been already exposed to T3 or other factors, which may influence the development of the cell, during their differentiation or maintenance. Especially serum may contain a significant amount of T3. So, it is not clear whether this study has evaluated the full effect of T3 on hiPSC-CMs.

Cardiac maturation is a complex process, which depends on a variety of factors and is not completely understood. The best maturation strategy would probably be a combination of various already evaluated approaches, which provide crucial physiological cues, which may be missing during *in vitro* culture. Thus, such comprehensive pro-maturation approach may

include electrical stimuli, mechanical workload, hypertrophic signals and 3-D culturing conditions because two-dimensional culture on a rigid matrix does not represent the native environment in the heart.

Recently, the embedding of hPSC-CMs in 3-D constructs has been described (Caspi et al., 2007b; Tulloch et al., 2011; Schaaf et al., 2011). These constructs aim to mimic the microenvironment in the heart (Zimmermann et al., 2006). They comprise a 3-D scaffold made of synthetic polymers or protein components of the heart's ECM (e.g. fibronectin and collagen), in which hPSC-CMs can be co-cultured with other cell types such as fibroblasts, endothelial cells and stromal supporting cells, which are found in the heart.

Co-culture and co-transplantation of PSC-CMs with endothelial cells and fibroblasts (with or without a 3-D matrix) have primarily shown to induce vascularisation in PSC-CM transplants and to improve survival and engraftment of the cell transplants in diseased myocardium (Kolossoff et al., 2006; Caspi et al., 2007b; Tulloch et al., 2011). However, these cell types may play a bigger role in cardiomyocyte development, growth and maturation. Both endothelial cells and cardiac fibroblasts have been shown to secrete a variety of cytokines and growth factors (Narmonova et al., 2004; Hsieh et al., 2006; Ottaviano and Yee, 2011) and cardiac fibroblasts, which are the most prominent cell type in the heart, have also been shown to couple electrically with cardiomyocytes forming a functional syncytium (Ottaviano and Yee, 2011).

A novel strategy to engineer heart constructs is to repopulate decellularised heart tissue with neonatal cardiomyocytes or pluripotent stem cells and their derivatives, respectively (Ott et al., 2008; Ng et al., 2011; Lu et al., 2013). The main advantage of using decellularised heart scaffolds is that they preserve the 3-D native heart architecture including the complex ECM and various growth factors trapped within the ECM. Recently Lu et al. reported the engineering of functional human heart tissue by reseeding decellularised mouse hearts with hiPSC-derived multipotential cardiovascular progenitor cells (MCPs) (Lu et al., 2013). These early heart precursor cells were shown to proliferate and differentiate *in situ* into specialised cell types such as cardiomyocytes, smooth muscle and endothelial cells to reconstruct the decellularised hearts. As a result the repopulated mouse heart tissue generated mechanical

force by spontaneous contraction and was responsive to various pharmaceutical agents. However, the engineered heart constructs did not show full electrical synchronisation, the generated mechanical force was not sufficient to pump blood and the hearts exhibited a lack of fibroblasts. Lu and co-workers concluded that further studies will focus on enhancing the mechanical force and electrical synchronisation, which may be achieved by re-popularisation of the constructs with MCPs and fibroblasts.

In combination with stretch and electrical stimulation, which have been already described for 3-D constructs (Tulloch et al., 2011), synthesised or decellularised native heart scaffolds, which are populated with hPSC-derivates and other heart cell types including fibroblasts, may show to be the best strategy to obtain mature hPSC-CMs. Finally, such 3-D heart tissue constructs may reproduce the multicellular environment and even architecture of the heart and prove to be the most accurate cardiac models for pharmaceutical and clinical applications.

6.6. Future directions

The pro-maturation approaches T3-treatment and overexpression of Bin1-EGFP, which were evaluated in this study, were shown to promote the maturation of iCell-CMs, a hiPSC-CM line, to some measure but a fully mature adult-like cardiac phenotype was not reached. So, it is still an open question how to enhance the maturity of hPSC-CMs to release their full potential for applications in regenerative medicine and pharmacology.

In addition, a number of further experiments may extend the observations made in this study:

- Optimisation of directed cardiac differentiation protocols (according to the methods described by the Keller group) for Shef3 cultures on Matrigel may result in a more efficient cardiomyocyte differentiation.
- Optimisation of the immunogold labelling protocol for TEM sections may confirm that the membrane structures, which were observed in iCell-CMs transfected with Bin1-EGFP, were associated with the Bin1 protein.

- Assessment of the impact of T3 administration in other hiPSC-CM and hESC-CM lines generated and maintained in defined conditions without T3.
- Overexpression of Bin1 in other hiPSC and hESC lines to confirm its tubulating effects and analyse possible co-localisation of Cav1.2 and the Bin1 structures.
- Elucidate the signalling pathways regulating the transcription of Bin1, the overexpression of which was shown to be sufficient to induce TT-like structures. This may be beneficial for the development of future maturation strategies.
- Analysis of the protein profile of iCell-CMs for example via mass spectrometry for further characterisation of the cells.

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Appendix

Reagents for qRT-PCR

Tris borate EDTA (TBE) buffer

1 l of 10x stock: 109.3 g Tris-(hydroxymethyl) methylamine, 55.65 g Boric acid, 9.31 g EDTA.
10x stock was diluted 1:10 to 1x solution with dH₂O.

Reagents for transmission electron microscopy

Sodium cacodylate buffer

250 ml of 0.2 M stock solution, pH 7.4: 20.15 g sodium cacodylate trihydrate, 0.1 ml HCl (1 M) and 250 ml dH₂O.

Glut-fixative

20 ml containing 2.5 % glutaraldehyde in 0.1 M sodium cacodylate buffer: 10 ml glutaraldehyde (5 %), 5 ml sodium cacodylate buffer (0.2 M) and 5 ml dH₂O.

FA-fixative

10 ml containing 4 % formaldehyde, 0.05 % glutaraldehyde in 0.2 M HEPES: 5 ml formaldehyde (8 %), 2 ml HEPES (1 M), 62.5 µl glutaraldehyde (8 %) and 2.94 ml dH₂O.

2 % OsO₄ in 0.1 M phosphate buffer

0.1 g tablet of OsO₄ and 5 ml 0.1 M phosphate buffer.

1 % thiocarbohydrazide in dH₂O

0.01 g thiocarbohydrazide in 1 ml dH₂O.

2 % OsO₄ and 3 % potassium ferrocyanide mixture (1:1)

0.03 g potassium ferrocyanide in 1 ml dH₂O and 1 ml OsO₄ (2 %) solution in dH₂O

0.5 % uranyl acetate in dH₂O

50 µl (10 %) uranyl acetate in methanol and 950 µl dH₂O

Blocking/permeabilisation buffer (0.5 % BSA, 0.1 % saponin, 50 mM)

1.4 g NH₄Cl, 0.5 g saponin, 2.5 ml BSA and 497.5 ml dH₂O.