# Combining signal profiling with iPSC-derived "disease in a dish models" to study cardiovascular disease

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# Abstract

Dilated cardiomyopathy (DCM), a heart muscle disease, is characterized by leftor bi-ventricular enlargement and systolic dysfunction. DCM is a progressive cardiac disease, with a spectrum of etiologies, including idiopathic, genetic and environmental causes. Regardless of etiology, the left ventricle (LV) of the heart remodels, resulting in LV wall thinning, decreasing cardiac performance and output leading to morbidity and mortality. Since pathological remodelling of the heart cannot typically be reversed, heart failure (HF) is often inevitable for individuals diagnosed with this disease and many ultimately require heart transplantation. To reduce the severity of the symptoms associated with DCM, patients are routinely followed and prescribed pharmacotherapy that *largely* targets G protein-coupled receptors. With reasonable success, these drugs have been shown to reduce hospitalizations as well as patient mortality. However, as not all patients respond equally, more predictive, patient-specific cellular systems are needed for effective disease modelling to facilitate discovery of alternative therapeutic strategies.

As sympathetic overdrive is a classical feature of HF pathology and the inhibition the  $\beta$ -adrenergic system is a mainstay therapy for patients with DCM, this thesis describes the development of a panel of conformation-sensitive biosensors within the sequence of the human  $\beta_2$ -adrenergic receptor. These biosensors were designed for use in drug screening and enabled us to capture agonist-induced changes in  $\beta_2AR$  structure. While our biosensors were validated in heterologous HEK 293 cells, they are, in theory, portable for use in disease contexts. We then transitioned to working with primary rodent as well as induced pluripotent stem cells (iPSC) models of the cardiomyocyte, both providing a more relevant physiological context. Our comparative study, investigating the transcriptome and signaling networks of these two species, revealed differences between rat neonatal and iPSC-derived cardiomyocytes. These results further the premise that cellular context is a critical determinant dictating receptor function and needs to be considered when assessing the clinical impact of translational studies.

For a more patient-oriented approach and DCM disease significance, access to patients enabled us to generate iPSC-CMs to study the molecular complexities associated with DCM at the individual level. Combined with a single-cell high-content microscopy-based biosensor screening platform, protein kinase activation response profiles revealed patient- and biological sex-specific signaling patterns. Altogether, my work moves beyond the use of HEK 293 cells through the creation of a bedside to bench approach to improve cardiac care, focusing on individual patient outcomes, facilitating the development of tailored and effective drug regimens for patients diagnosed with DCM.

# Résumé

La cardiomyopathie dilatée (CMP), une maladie du muscle cardiaque, est caractérisée par l'élargissement ventriculaire gauche ou biventriculaire associée à un dysfonctionnement systolique. La CMP est une maladie cardiaque progressive comprenant un éventail d'étiologies incluant des causes idiopathiques, génétiques et environnementales. Indépendamment de l'étiologie, on observe un remodelage du ventricule gauche (VG), entraînant un amincissement de la paroi du VG en plus d'une diminution des performances et du débit cardiaques menant à une augmentation des risques de morbidité et de mortalité. Étant donné que le remodelage pathologique du cœur est généralement irréversible, l'insuffisance cardiaque (IC) est souvent inévitable chez les personnes diagnostiquées de cette maladie menant souvent à une transplantation cardiaque. Afin de réduire la gravité des symptômes associés au CMP, les patients sont régulièrement suivis médicalement et une pharmacothérapie ciblant en grande partie les récepteurs couplés aux protéines G (GPCR) leur est prescrits. Avec un succès raisonnable, ces médicaments sont connus pour réduire les hospitalisations ainsi que la mortalité chez ces patients. Toutefois, puisque chaque patient est unique, l'utilisation de systèmes cellulaires personnalisés à chaque patient permettra une modélisation plus performante de la maladie afin de faciliter le développement de stratégies thérapeutiques plus ciblées.

Comme la suractivité sympathique est classiquement associée à la pathologie d'IC et que l'inhibition du système β-adrénergique constitue la base thérapeutique chez ces patients atteints de la CMP, la présente thèse décrit le développement de biosenseurs sensibles aux changements conformationnels du récepteur  $\beta_2$ -adrénergique. Ces biosenseurs ont été conçus pour le criblage de médicaments et nous ont permis de mesurer ces changements de structure induits par une série d'agonistes spécifique du récepteur  $\beta_2$ AR. Bien que nos biosenseurs aient été validés dans des cellules HEK 293, ces mêmes protéines peuvent, en théorie, être exprimées dans des systèmes physiologiques beaucoup plus pertinent. C'est pour cette raison que nous avons commencé à travailler à la fois sur des lignées de cellules primaires de rongeurs ainsi que sur des cardiomyocytes provenant de cellules souches pluripotentes induites (CPSi-CM). Notre étude comparative du transcriptome et des réseaux de signalisation intracellulaires dans ces deux modèles, a ainsi révélée des différences marquées entre les cardiomyocytes provenant de ratons et ceux dérivés d'CPSi. Ces résultats supportent l'hypothèse que le contexte cellulaire influence la fonction du récepteur et doit donc être pris en compte lors de l'évaluation de l'impact clinique des études translationnelles.

Dans l'optique d'une approche ciblant spécifiquement le patient atteint de CMP, nous avons ainsi généré des CPSi-CM provenant de cellules de ces mêmes patients afin

de mieux comprendre la complexité moléculaire de la CMP au niveau de chaque individu. Avec l'aide de notre plateforme de dépistage basée sur l'utilisation de biosenseur sensible à l'activation de certaines protéines kinases combiné à un système d'imagerie cellulaire à haut-débit, les profils de réponse d'activation ainsi mesurés ont révélés des motifs spécifiques à chaque patient ainsi qu'au sexe de ces derniers. Dans l'ensemble, mes travaux m'ont permis de constater que les modèles cellulaires tels que les HEK293 ne donnent qu'une perspective limitée de la signalisation induite par les GPCRs et que l'étude de tels signaux dans des modèles plus représentatifs du système cardiovasculaire tels que les CPSi-CM provenant de patients atteints de CMP permettront le développement de stratégies thérapeutiques ciblées et donc plus efficace.

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#### Contribution to original knowledge

In its entirety, this body of work describes the generation of tools, including a physiological and disease relevant cellular model of the heart as well as associated methodologies for disease modelling applications. In Chapter 2, I will describe the engineering of conformation-sensitive biosensors to study the biology of the cardiac relevant  $\beta_2$ -adrenergic receptor. Understanding the conformational dynamics of this receptor is relevant in the context of heart disease as the biology of β-adrenergic receptors becomes dysfunctional in the failing heart. However, for these tools to report on  $\beta_2$ AR function, they must be emersed in the relevant cardiomyocyte cellular context. Thus, to understand the pluri-dimensional manifestations and etiologies underlying diseases of the heart such as dilated cardiomyopathy, better access to human-derived model systems was needed. In Chapter 3, I pioneered the use of hiPSCs and developed a standard operating procedure for the differentiation of hiPSCs into cardiomyocytes. Beyond the development of a reliable and robust protocol for our lab, I also compared neonatal rat and human iPSC-derived cardiomyocytes for research and disease modelling purposes as it is important to understand the limitations of the culture systems we work with. By performing our single cell biosensor analyses as well as bulk transcriptomics we were better able to appreciate the differences between two cardiomyocyte models. Finally, in Chapter 4, and in collaboration with my supervisor, and clinical colleagues from the MUHC Glen hospital centre, we were able to generate the 'Heart-in-a-Dish' project, which is unique in Canada. As part of this project, we created a biorepository of patient samples from those diagnosed with dilated cardiomyopathy. With patient access and the subsequent generation of hiPSCs, we have been able to perform phenotyping studies to better understand the molecular drivers of disease. It is our hope that we will be able to align and correlate patient clinical data with our experimental studies, to improve cardiac healthcare in Canada. Once complete, our biobank will be available to researchers throughout the world as collaboration fosters innovation. Overall, the summation of all three chapters provides value from multiple vantage points, further examined in the discussion section.

# **Author Contributions**

## Chapter 1

Literature review and original writing was performed by Kyla Bourque. Text was edited by Terry Hébert. Figures were either created by Kyla Bourque using BioRender or have been modified or adapted from published sources, as stated.

# Chapter 2

Kyla Bourque conducted experiments in all figures, analyzed data, produced figures, and wrote the original manuscript. Darlaine Pétrin designed and cloned the  $\beta_2$ -adrenergic biosensors used in the study. Rory Sleno designed and cloned, along with Alice Zhang, the conformation-sensitive biosensors engineered withing the F Prostanoid receptor used in the study. Dominic Devost designed and cloned the conformation-sensitive biosensors in human angiotensin II type 1 receptor used in the study. Terence E. Hébert contributed to conception of the project, provided supervisory support, contributed to experimental design and interpretation of results and edited the manuscript.

## Chapter 3

Kyla Bourque conducted experiments in all figures, analyzed data, produced figures, and wrote the original manuscript. Jace Jones-Tabah and Ryan D. Martin provided original computer code for analysis of single cell FRET data. Darlaine Pétrin performed several RNCM isolations. Terence E. Hébert contributed to conception of the project, provided supervisory support, contributed to experimental design and interpretation of results and edited the manuscript.

## Chapter 4

Kyla Bourque contributed to the conception of the project, conducted experiments in Figures 3, 5 and 6, analyzed data, assembled all figures, and wrote the original manuscript. Ida Derish, Kashif Khan, Jeremy Zwaig reprogrammed patient blood into induced pluripotent stem cells as shown in Figure 2B, C. Cara Hawey performed calcium experiments in Figures 4B-J. Alyson Jiang, Karima Alim and Hooman Sadighian provided support with iPSC culture and differentiations. Jace Jones-Tabah provided original computer code for analysis of single cell FRET data. Natalie Gendron is the project nurse and provided information for Figure 1. Nadia Giannetti and Renzo Cecere contributed to conception of the project. Terence E. Hébert contributed to conception of the project, provided supervisory support, contributed to experimental design and interpretation of results, and edited the manuscript.

## Chapter 5

Text was written by Kyla Bourque and edited by Terry Hébert.

# Abbreviations

AC	Adenylyl cyclase
ACE	Angiotensin converting enzyme
ACM	Arrhythmogenic cardiomyopathy
AKT	Protein kinase B
Ang II	Angiotensin II
ARB	Angiotensin II receptor blockers
AT₁R	Angiotensin II type I receptor
AT <sub>2</sub> R	Angiotensin II type II receptor
ATR	Angiotensin II receptor
AV node	Atrioventricular node
BAG3	BCL2-associated athanogene 3
BMD	Becker muscular dystrophy
BMP	Bone morphogenic protein
BNP	B-type natriuretic peptide
BRET	Bioluminescence resonance energy transfer
cAMP	Cyclic adenosine 3,5-monophosphate
CFP	Cyan fluorescent protein
CHIR99021	Chemical compound that inhibits GSK3
CM	Cardiomyocyte
c-myc	MYC Proto-Oncogene, BHLH Transcription Factor
cpFPs	Circular permutated fluorescent protein
CREB	Cyclic AMP-responsive element-binding protein
cTnT	Cardiac troponin T
DAG	Diacylglycerol
DCM	Dilated cardiomyopathy
DMD	Duchenne muscular dystrophy
EC	Endothelial cell
ECM	Extracellular matrix
Egr1	Early growth response protein 1
EHT	Engineered heart tissue
EOMES	Eomesodermin
ERK <sub>1/2</sub>	Extracellular signal regulated kinases 1/2
ET-1	Endothelin-1
ET <sub>A</sub> R	ET <sub>A</sub> receptor
ET <sub>B</sub> R	ET <sub>B</sub> receptor
ETR	Endothelin receptor
FAO	Fatty acid oxidation
FGF	Fibroblast growth factor
FIAsH	Fluorescein arsenical hairpin binder
FIAsH-EDT <sub>2</sub>	Fluorescein arsenical hairpin binder-ethanedithiol
FLNC	Filamin C
FP	F Prostanoid receptor
FRET	Fluorescent resonance energy transfer
GATA4	GATA binding protein 4

GDP	Guanosine diphosphate		
GECI	Genetically encoded calcium indicator		
GEVI	Genetically encoded voltage indicator		
GFP	Green fluorescent protein		
GPCR	G protein-coupled receptor		
GTP	Guanosine-5'-triphosphate		
GSK3	Glycogen synthase kinase 3		
HCM	Hypertrophic cardiomyopathy		
HEK 293	Human embryonic kidney 293 cell line		
HF	Heart failure		
ICL	Intracellular loop		
IP <sub>3</sub>	Inositol trisphosphate		
IP₃R	Inositol trisphosphate receptor		
iPSC	Induced pluripotent stem cell		
iPSC-CM	Induced pluripotent stem cell derived cardiomyocyte		
IWP2	Inhibitor of Wnt porcupine 2		
JNK	C-Jun N-terminal kinase		
Klf4	Krüppel-like factor 4		
LMNA	Lamin A/C		
LV	Left ventricle		
MAPK	Mitogen-activated protein kinase		
MEF2	Myocyte enhancer-factor 2		
MHC	Myosin heavy chain		
MI	Myocardial infarction		
MYPN	Myopalladin		
NES	Nuclear export sequence		
NFAT-Ca <sup>2+</sup>	Nuclear factor of activated T-cells-Ca <sup>2+</sup>		
NKX2-5	Homeobox-containing transcription factor NKX2-5		
NLS	Nuclear localization sequence		
Nppa	Natriuretic peptide A		
Nppb	Natriuretic peptide B		
Oct4	Octamer-binding transcription factor 4		
PBMCs	Peripheral blood mononuclear cell		
PI3K	Phosphoinositide 3-kinase		
PIP2	Phosphatidylinositol 4,5-bisphosphate		
PKA	Protein kinase A		
PKB	Protein kinase B		
PKC	Protein kinase C		
PLB	Phospholamban		
PMA	Phorbol myristyl acetate		
PPARα	Peroxisome proliferator-activated receptor alpha		
p38	p38 mitogen activated protein kinases (p38MAPK)		
R	Inactive receptor		
R*	Active receptor		
RAAS	Renin-angiotensin aldosterone system		
RALDH2+	Retinaldehyde dehydrogenase 2		

RBM20	RNA-binding Motif-20
RCM	Restrictive cardiomyopathy
RNCM	Rat neonatal cardiomyocytes
RyR2	Ryanodine receptors
SĂ node	Sinoatrial node
SERCA2a	Sarco-endoplasmic reticulum Ca <sup>2+</sup> -ATPase 2a
SCN5A	Sodium channel alpha unit
SI	SI ([Sar <sup>1</sup> ,Ile <sup>8</sup> ]AngII)
SII	SII ([Sar <sup>1</sup> ,IIe <sup>4</sup> ,IIe <sup>8</sup> ]AngII)
SMADs	Small mothers against decapentaplegic
Sox2	SRY-Box Transcription Factor 2
SR	Sarcoplasmic reticulum
ТМ	Transmembrane
TNNC1	Troponin C, slow skeletal and cardiac muscles
TPM1	Tropomyosin-α1 chain
TTN	Titin
TNNT	Troponin
TNNI3	Troponin I, cardiac muscle
VSMCs	Vascular smooth muscle cells
Wnt	Wingless-related integration site
YFP	Yellow fluorescent protein
α-MHC	Alpha myosin heavy chain
αıAR	α1-adrenergic receptor
β-ΜΗϹ	Beta myosin heavy chain
β-AR	β-adrenergic receptor
β₁AR	β <sub>1</sub> -adrenergic receptor
β2AR	β <sub>2</sub> -adrenergic receptor
β₃AR	β <sub>3</sub> -adrenergic receptor
2D	2-dimensional
3D	3-dimensional

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# Chapter 1 General introduction

The human body is comprised of numerous cells that coherently communicate as an ensemble, sustaining vital organ functions and life. After the formation of the primitive streak, around day 14 *in utero*, cells become increasingly specialized to carry out specific cellular and organ functions. The cardiovascular system is the first organ system to develop, as the heart is required to pump oxygen and nutrients throughout the growing embryo [1]. From the early embryo to old age, the human body sustains life through the coordinated efforts of numerous biological systems. However, in some instances, disease may arise from perturbations occurring at the cellular, subcellular and/or transcriptional levels. These disease-causing cellular disruptions may be a result of a variety of sources including, genetic, environmental, microbial, viral, or system-related failures.

Historically, drug discovery platforms combined animal models and phenotypic screening approaches to identify novel disease-modifying compounds [2, 3]. Briefly, once a disease phenotype was identified, compounds were tested on animals while scientists made observations on how these drugs altered disease-relevant phenotypes. This approach allowed drugs to be screened in "rich" tissue environments as they were conducted in living tissues or intact animals. In so doing, studies conducted in living animals provided useful mechanistic insight into how to treat disease and provided information regarding drug absorption, distribution, metabolism, and excretion. Animal models have thus proved critical for drug development as well as testing drug safety prior to placing drugs on the market [3]. However, there are always exceptions, as drug and safety testing in animal models is not without fault. Tragedies have occurred including the notorious thalidomide debacle which may have been avoidable with more rigorous animal testing, such as the testing in pregnant animals [4]. Importantly, this incident taught the scientific and medical communities the importance of testing drugs in multiple animal models as species differences can affect drug metabolism, clearance rates and placental transfer as observed in rodents versus rabbits, for instance [4-9]. Notably, not all animal models may be suitable equivalents of the human condition or of certain human diseases and should not be used interchangeably, without prior validation. In addition, in the 1950's

women were not included in clinical trials and so drug safety was only considered in men. Thus, there may be considerable species differences between animal *models* and humans as well as biological sex differences between males and females, topics that will be re-examined in later sections.

Meanwhile, considering that animal testing is, by definition, low throughput, to increase productivity, drug screening efforts transitioned towards the use of cell-based systems such as transformed immortalized lines or primary cultures. To automate drug discovery as well as reduce the number of animals used in research, for ethical reasons, cell-based assays grew increasingly trendy in industry. These cell-based assays commonly used heterologous expression systems such as HEK 293, HeLa, and COS-7 cells, which are immortalized cell lines that proliferate indefinitely. However, these cell lines often lack human physiological and disease relevance. With high-throughput automation as a goal, it was believed that thousands of compounds could be screened using these cells to identify lead compounds that would, with any luck, enter clinical trials. However, these efforts were unrewarding, as drugs frequently failed to perform as intended or demonstrated signs of toxicity when tested in animals in late-stage drug development. In the early 1990's, issues with drug discovery were related with efficacy and pharmacokinetic (ADME) properties while in the 2000's, efficacy as well as safety was an issue [10, 11]. The lack of safety was and still remains a great concern and is most likely attributed to using less biologically relevant cellular contexts. Issues encountered most likely relate to the notion that the model systems used to validate lead compounds are not predictive of human biology and the model system used does not translate well to human physiology when assessed in male and female patients. This highlights the importance of cellular context, a major theme of this thesis. If drug discovery campaigns are to be fruitful, it is important that drugs be tested in the cellular context that they are designed to target *in vivo*. For instance, drugs designed to treat heart failure should be tested in cardiomyocytes while also considering contributions of distinct myocardial linages such as ventricular versus atrial cardiomyocytes.

Based on observations and knowledge accumulated across this past decade, transformed cell lines like HEK 293 cells no longer suffice as they present with significant limitations. Yet, recently, in the mist of the COVID-19 pandemic, oversights, some being related to the use of inaccurate model systems, were still made. To elaborate, chloroquine, a widely used anti-malarial drug was thought to be an effective anti-viral drug against COVID-19, as when tested in vitro, in Vero E6 cells which are kidney epithelial cells extracted from an African green monkey, chloroquine appeared to inhibit viral infection [12]. Though, when tested in a randomized, double-blind, placebo-controlled trial, chloroquine was not demonstrated to prevent disease [13]. The use of chloroquine thus probably resulted in hospitalizations, that were otherwise preventable. Chloroquine's lack of efficacy was also demonstrated in intestinal organoids which represent a nontransformed human model system [14]. This example further highlights the importance of selecting the 'right' in vitro cellular or in vivo model system that accurately resembles the human condition or disease for translational studies. To move beyond HEK 293 or Vero E6 cells, for example, primary cultures isolated from rodents are often used. In addition, with recent advances in stem cell generation and the ability to reprogram adult somatic cells into induced pluripotent stem cells, phenotypic screening campaigns once again have access to "richer cellular environments" but this time, of human origin. Furthermore, biological materials sourced from both males and females are possible as is patient access, providing biological-sex specific information and pathological relevance when studying human diseases in a dish.

To address the lack of physiologically relevant cellular models used in biomedical research, this thesis aimed to develop a human-derived model system beyond the prototypical HEK 293 cell as means to study cardiovascular physiology and pathophysiology. In this thesis, I begin with performing experiments in the canonical HEK 293 cell line to understand G protein-coupled receptor (GPCR) biology. However, as mentioned above, to truly understand biological or pathobiological outcomes downstream pathway activation within live cells, it is imperative to use the appropriate cell type of interest. Here, we were interested in GPCRs that regulate cardiovascular function. Thus, I progressively transitioned to collecting data in cardiomyocytes as we were interested in

understanding how signaling transduction mechanisms were altered during the development of heart muscle disease. Initially, we used primary rat neonatal cardiomyocytes and then human iPSC-derived cardiomyocytes to accomplish this task. For disease relevance, we also engaged with colleagues at the McGill University Health Centre Glen campus, to gain access to samples from patients diagnosed with dilated cardiomyopathy. This provided us with the appropriate venue to explore how signaling modalities are altered in disease. To start, I will provide a general introduction to the main characters in this thesis, GPCRs and their effectors and discuss their relevance in regulating cardiac function while describing how cell models have evolved over time.

## 1.1 An introduction to G protein-coupled receptors

# 1.1.1 G protein-coupled receptors

G protein-coupled receptors (GPCRs) have seven transmembrane spanning domains and dynamically transduce extracellular stimuli into intracellular responses. To date, there are five distinct classes of GPCRs that have been identified in humans. These classes are based on their distinct sequences and structural characteristics and are referred to as; (1) class A or rhodopsin-like receptors, (2) class B secretin receptors, (3) class C metabotropic glutamate receptors, (4) the adhesion receptors, and (5) class F or the frizzled receptor family [15]. Mammalian species express all five of these receptor subfamilies. Other GPCR families have also been identified outside the human genome and include pheromone or class D receptors which are solely expressed in fungi and class E receptors that are only found in *Dictyostelium discoideum*; more commonly known as slime mold [16, 17]. As a significant fraction of GPCRs are part of the class A family, represented by 85% of GPCR genes, they will be the main class discussed from this point on.

Class A GPCRs have an extracellular amino terminus and ligand binding pocket as well as an intracellular carboxyl terminus with seven  $\alpha$ -helical transmembrane domains (see **Figure 1.1**). Most GPCRs also have a cytoplasmic eighth helix (H8) tethered to the

membrane. GPCRs are "allosteric machines" that are embedded within the plasma membrane integrating external cues into intracellular responses. The external signals are largely transferred to the inside of the cell through the binding at orthosteric or allosteric sites ensuing serial conformational rearrangements throughout the entirety of the receptor structure.



Figure 1.1 Structural features of G protein-coupled receptors (GPCRs).

(A)Cartoon depiction of a prototypical Class A GPCR. Class A GPCRs have an extracellular amino-terminus and intracellular carboxyl terminus. The plasma membrane position of a GPCR provides them with the ideal location to convert extracellular messages into intracellular responses. The extracellular loops (ECLs) can engage with ligands where ligand binding results in a conformational change within the overall receptor structure including the transmembrane (TM) domains and intracellular loops (ICLs). Figure was reproduced from Deganutti et al. 2019 [18], with permission. This Figure is licensed under the Creative Commons Attribution 4.0 License. To view a copy of this license, visit <u>http://creativecommons.org/licenses/by/4.0/</u>.

GPCRs are coupled to the heterotrimeric guanine nucleotide-binding proteins; G proteins,  $G\alpha\beta\gamma$ . There are four general classes of G $\alpha$  subunits,  $G\alpha_{i/0}$ ,  $G\alpha_s$ ,  $G\alpha_{q/11}$  and  $G\alpha_{12/13}$ , each with distinct cellular functions (see **Table 1.1**) [19]. The G $\alpha_i$  family, also includes  $G\alpha_z$ ,  $G\alpha_t$  and  $G\alpha_{gust}$ . Likewise, the  $G\alpha_s$  family also comprises  $G\alpha_{olf}$  while  $G\alpha_{11}$ , and  $G\alpha_{14,15,16}$  belong to the  $G\alpha_q$  family [20]. Thus far, there are 16 G $\alpha$  subunits that have been identified in mammals [20, 21]. Historically,  $G\alpha$  was believed to be the main effector

orchestrating downstream signaling events while the GBy obligate dimer was believed to act as negative regulator of  $G\alpha$ . However, it has now become widely accepted that  $G\beta\gamma$ subunits are also important signaling effectors, in their own right (reviewed here [22, 23]). There are 5 G $\beta$  and 12 G $\gamma$  subunits which can pair to generate a myriad of unique combinations with their own distinct cellular functions. The activation cycle of  $G\alpha\beta\gamma$ essentially primes the serial activation of a cascade of second messengers and downstream effector molecules regulating a wide array of cellular responses. Experimental data has indicated that GPCRs, regardless of their activation state, can interact with Ga proteins [24-26]. In the inactive G protein conformation, Ga is tightly bound to GDP and the constitutive GBy dimer. When GPCRs interact with external stimuli, this prompts a conformational change in the receptor which catalyses the release of GDP on the Gα protein [27, 28]. Evidence suggests that the release of GDP is triggered through the interaction of the C-terminus of Ga with the GPCR via allosteric changes but Gβγ has also been reported to play an active role (reviewed in [29]). Upon release of GDP, a transient GPCR-Gα-GTP complex forms as GTP is more abundant in the cell than GDP. This event triggers the dissociation of G $\beta\gamma$  from G $\alpha$ , and both can engage with other downstream effectors triggering cellular responses. This activation cycle ends as Ga exhibits intrinsic GTPase activity and hydrolyses GTP into GDP thus reverting to the inactive state (see Figure 1.2A). However, this is a simplification of the G protein activation cycle as regulators of G protein signaling, RGS proteins, are also involved in regulating GTPase activity, influencing GPCR signaling kinetics and playing a part in cardiac diseases (discussed in [30, 31]).

**Table 1.1** Cellular effects of Ga proteins

			A
G protein	α-subunit subfamily	Effect of activation	
Gs	Gas, Gaolf	Stimulation of adenylyl cyclase	GEF activity
Gi	Gα <sub>i1-3</sub> , Gα₀, Gα₂α	Inhibition of adenylyl cyclase	× Contraction of the second se
	Gat	Activation of cGMP phosphodiesterase (specific for retinal phototransduction)	
Gq	Gα <sub>q</sub> , Gα <sub>11</sub> , Gα <sub>14</sub> , Gα <sub>18</sub>	Activation of phospholipase C $\beta$ (PLC $\beta$ )	
<b>G</b> <sub>12</sub>	Gα <sub>12</sub> , Gα <sub>13</sub>	Activation of RhoA signalling; activation of PLCe	
Neubig, R., S drug targets.	iderovski, D. Re Nat Rev Drug D	gulators of G-Protein signalling as new central nervous system iscov 1, 187–197 (2002). https://doi.org/10.1038/nrd747	CAP activity

#### Figure 1.2 Schematic illustration of the GPCR activation cycle.

In the inactive G protein conformation, G $\alpha$  is tightly bound to GDP and the constitutive G $\beta\gamma$  dimer. Upon ligand binding, the association event triggers a conformational change in the receptor which catalyses the release of GDP on the G $\alpha$  protein. This event prompts the dissociation of G $\beta\gamma$  from G $\alpha$ , and both molecules, being signaling competent can engage with other downstream effectors initiating a plethora of cellular responses. This activation cycle terminates as G $\alpha$  exhibits intrinsic GTPase activity and hydrolyses GTP into GDP thus reverting to the inactive state. RGS proteins also participate in the termination cycle as they behave as GTPase-accelerating proteins (GAPs) for G $\alpha$  subunit. Table and figure were from Neubig & Siderovski et al. 2002, used with permission [32].

## 1.1.2 Basic principles guiding GPCR pharmacology and signaling

#### 1.1.2.1 Ligand classes

Ligands carry the chemical information that is required to initiate signal transduction. Ligand efficacy is defined as the ability of a ligand to activate a signaling pathway producing a physiological effect. Depending on the biological action they ensue, downstream GPCR activation, ligands can be sub-divided into four *general* categories; full, partial, or inverse agonists and neutral antagonists [33]. In the classical sense, full agonists maximally activate a signaling pathway whereas partial agonists result in receptor activation, although, to a lesser degree. Neutral antagonists bind to the

orthosteric binding pocket and block/inhibit the binding of other ligands, preventing signal transduction. Lastly, inverse agonists decrease the constitutive activity of the receptor reducing its ligand-independent basal activity. As simplistic as this may seem, ligand and GPCR pharmacology is quite complex. This classification scheme is not '*etched in stone*' in the sense that an individual ligand can behave as an agonist, antagonist, full, partial or inverse agonist depending on the signaling pathway or cell type being assessed [34-37]. These broad classes depend on several system parameters including, but not limited to, the expression levels of receptors, G protein partners and other signaling effectors [38]. Hence, depending on the cellular and disease context in which a GPCR is expressed, activation by the same ligand can elicit distinct cellular phenotypes. However, prior to discussing the biological outcomes of GPCRs, it is important to review how the signal ligand propagates its information through a GPCR via conformational rearrangements.

#### 1.1.2.2 Receptor activation and the ternary complex

Historically, GPCRs were viewed as rigid entities. Based on this assumption, signal transduction was proposed to occur through a the 'lock-and-key' mechanism (see Figure 1.3A). This theory stated that a ligand chooses or binds a receptor based on structure complementarity. Upon ligand binding and fitting into the metaphorical 'keyhole', the receptor 'unlocks' and adopts the active conformation. Based on this dogma, a receptor can exist in two conformational states, either in an inactive or active state conformer (see Figure 1.4A). However, with accumulated experimental evidence, this dogma was modified and in attempt to describe the dynamic molecular mechanisms underlying GPCR activation, other hypotheses were put forth. It was suggested that a ligand could either induce the receptor to adopt the 'right' conformation based on its agonist/antagonist properties, termed "induced fit", or recognize a specific conformation known as "conformational selection" (see Figure 1.3B, 3C) [39, 40]. Induced fit refers to the ligands ability to incite a 'shape-shifting' event in the receptor post-binding, allowing the receptor to 'fit' the ligand. In the induced fit scenario, a GPCR can exist in two states, open, unbound or closed and ligand bound [39]. Alternatively, the "conformational-selection"

theory states that all plausible receptor conformations may exist at any given moment in time. The ligand can essentially *sample* different receptor shapes until it recognizes its *'matching'* conformation, thereby stabilizing it and initiating signal transduction. This binding event also induces a population shift, changing the equilibrium and favoring the conformation recognized by the ligand. As GPCRs are highly complex allosteric macromolecules, it is increasingly plausible that the conformational selection theory more accurately describes the molecular interaction occurring between a GPCR and a given ligand. In this setting, a full agonist would induce a population shift where more GPCRs can engage their cognate G protein partner while an antagonist or inverse agonist would incite a 'shape shift' favoring the stabilization of other distinct states [39].



# Figure 1.3 Various postulates that have been put forth to elucidate the mode of ligand induced GPCR activation.

(A) The lock and key hypothesis. The lock and key mechanism suggested that GPCRs could only exist in two states: a locked- inactive or an unlocked- active conformational state. With time, other assumptions were theorized. (B) Induced fit hypothesis. Induced fit describes a ligands ability to provoke a 'shape-shifting' event in the receptor postbinding, allowing the receptor to 'fit' the ligand. In the induced fit scenario, a GPCR can exist in two states, open, unbound or closed and ligand bound. (C) Conformational selection hypothesis. The conformational-selection theory states that all plausible GPCR conformations exist at any given moment in time. Basically, a ligand can sample different receptor conformations until it recognizes its complementary conformation where it binds initiating signal transduction. The figure was created with BioRender.com

Besides the ligand, there are two other biological partners required to initiate signal transduction: a G protein and a GPCR. When assembled, these three elements compose the ternary complex. Based on the activation model which follows the concept of conformational selection, GPCRs exists in an equilibrium continuously interchanging from the inactive state (R) to the active state (R\*) (see Figure 1.4B). In accordance, GPCR activation can be further explained by integrating the concept of free energy landscapes (reviewed in [40, 41]). In absence of ligand, GPCRs exist in a low energy state. At baseline, it can be assumed that GPCRs fluctuate between various low energy states as low energy states are essentially the most stable. These low energy states can represent inactive or active conformations that are signaling competent as some GPCRs exhibit constitutive activity [40]. The energy stored in ligands can be transmitted to receptors via a binding event altering the energy landscape. This binding can either decrease or increase the energy level required or energy *barrier* to attain the next conformation (see Figure 1.4C-E) [41]. Essentially, a receptor can only 'shape shift' and adopt a new conformation if it can overcome the energy cost. For example, binding of an agonist to the receptor would decrease the energy required or energy barrier to reach the active R\* conformation. In principle, the conformational landscape within the membrane would redistribute by increasing the population of the active state conformer. In this example, the equilibrium shifts favoring the active state conformer. An inverse agonist, on the other hand would increase the energy barrier constraining the population in an inactive state [41].



Figure 1.4 Two- to unlimited-state model of G protein-coupled receptor states.

(A) The two-state GPCR model. Historically, GPCRs were believed to be able to adopt two-states; an inactive state and an active state that was signaling competent. This hypothesis was later demonstrated to be an oversimplified depiction of the dynamic nature of GPCRs. (B) GPCRs are dynamic macromolecular entities and exist in an ensemble of conformational states. Decades of research has demonstrated the ability of GPCRs to 'shape-shift' into an ensemble of various active and inactive conformer states. Ligand binding has been determined to stabilize a given GPCRs conformation dictating the downstream pathway engaged. (C-E) Energy landscapes influence the overall conformational population of GPCRs. Unliganded GPCRs exists in a low energy state. The transition to active states requires agonist and G protein binding events to alter the energy barriers that cause changes in GPCR conformation resulting in activation. Panels A & B of this figure were created with BioRender.com and panels C-E were taken from the Latorraca et al. 2017 [42], https://pubs.acs.org/doi/10.1021/acs.chemrev.6b00177, with permission.

GPCR conformational dynamics are of paramount importance when attempting to understand cellular signaling and its downstream physiological outcomes. In theory, conformation dictates a given GPCR's function. Different ligands can stabilize distinct conformational states by either breaking intermolecular interactions or creating new interactions within the receptor, both actions acting to stabilize new receptor conformations [41]. The stabilized receptor state then dictates the downstream pathways that become activated as the chemical information within the ligand propagates into the GPCR structure through to the intracellular surface (see Figure 1.4B). GPCR activation results from the disruption of different microswitches that alter GPCR conformation. There are four main GPCR microswitches, reviewed in ([43-46]). Microswitches within GPCRs are non-covalent interactions between amino acid residues that need to 'unlock' in order for a GPCR to achieve a fully activated state. Undisturbed microswitches essentially keep a GPCR in a 'locked', inactive state. For instance, the ionic lock is an interaction occurring on the cytoplasmic side between TM3 and TM6 [47, 48]. This interaction occurs between glutamic acid (E), aspartic acid (D), arginine (R), and tyrosine (Y) residues and is commonly referred to as the E(D)RY motif. This motif is highly conserved among the class A GPCR family. In the intact conformation, TM3 and TM6 are in close proximity thus constraining the GPCR in an *inactive* state. This was originally based on mutagenesis studies, where mutations disrupting the interaction between glutamic (TM6) and aspartic (TM3) acid increased the constitutive activity of the receptor. GPCR activation also results in the disruption of the 3-7 lock switch, a salt bridge that connects TM3 and TM7. This lock mechanism was identified in rhodopsin [49] and may also be present in other GPCRs. It is, however, not as conserved as the ionic lock. Further, the transmission switch also known as the Trp rotamer toggle switch, involves the rearrangement of interactions occurring between TM3-5-6. Similarly, the tyrosine toggle switch, or the nPxxY motif also constraints the conformational dynamics of GPCRs.

Further, it is important to recognize that GPCR activation is not vertical or linear (see **Figure 1.5A, B**). A ligand can bind a receptor's orthosteric site initiating conformational rearrangement in the receptor which results in the activation of downstream effector molecules and cellular responses. However, this three-step activation paradigm is overly simplistic. As a GPCR sits within the plasma membrane, other molecules can interact with it in a *horizontal* manner (see **Figure 1.5B**). As such, molecules that interact with GPCRs at topographically distinct sites other than the canonical orthosteric site, are known as allosteric molecules and can influence GPCR function; introduced below.



Figure 1.5 Schematic representation of the allosteric partners that may influence GPCR conformation and function.

(A) Traditional GPCR signal transduction requires three signaling partners: a ligand, receptor and G protein partner. Even if this signaling cascade appears to be linear, GPCR activation is intricately more complex. (B) GPCR activation can be influenced by the presence of effectors and allosteric modulators. GPCR activation may be influenced by its immediate 360-degree cell- and tissue-specific environment. The figure was created with BioRender.com.

## 1.1.2.3 The theory of GPCR allosterism

Countless cellular parameters may elicit allosteric control over GPCRs fine tuning their functions *in cellulo* [50, 51]. Allosteric regulation occurs at sites that are topographically distinct from the orthosteric site where the endogenous agonist binds. Depending on how allosteric molecules influence the cellular system, they can be classified as positive allosteric modulators (PAMs) that enhance the affinity and/or efficacy of the endogenous/allosteric ligand or as negative allosteric modulators (NAMs) which behave in an opposing manner by reducing agonist affinity and/or efficacy (reviewed in [52-56]). Some allosteric agonists may have intrinsic activity and are referred to as ago-PAMs. Receptor allosterism is believed to occur via conformational rearrangements/stablization of the GPCR structure which are translated as adjustments in ligand affinity and efficacy [57]. In theory, when an allosteric compound binds a GPCRs, it can stabilize a distinct conformational state, altering the conformational landscape and

thus shifting the conformer's equilibrium. By so doing, an endogenous ligand can behave differently when compared to the absence of allosteric modulator.

GPCR drug discovery efforts have been interested in the development of allosteric compounds considering that allosteric sites are potentially more numerous compared to the conserved orthosteric sites across GPCR subtypes [58, 59]. However, chemical compounds are not the only allosteric modulators that regulate GPCR conformation and corresponding function. In principle, any protein that encounters a GPCR may, in some way, modulate its activity. Acknowledging the transmembrane location of this receptor family, the candidate list of potential allosteric modulators may be perceived innumerable. Briefly, GPCRs can be influenced by cholesterol molecules [60], phospholipids, ions [61] (Na<sup>+</sup> [62, 63], Mg<sup>2+</sup> [64]), pH, stoichiometry of signaling effectors, receptor dimerization partners [65] etc. (see Figure 1.5B). As a direct consequence of allosterism, GPCR biology and signaling can be significantly altered depending on the cellular and subcellular location being assayed [66]. Therefore, the discovery of GPCR allostery created a push to study these receptors in more physiologically relevant cellular and sub-cellular contexts. The theory of allostery essentially set up one main theme/rationale for this thesis, as the body of work presented here will attempt to demonstrate that the impact of cellular context, defined as the differential availability of putative receptor partners, G proteins and effectors, should not be ignored when conducting cell-based assays for disease modelling in vitro. Second to allostery, another "hot" topic relevant to GPCR conformation and function is the concept of functional selectivity, discussed hereunder.

# 1.1.2.4 $\beta$ -arrestins and the theory of functional selectivity or biased agonism

GPCRs are promiscuous proteins that can couple to multiple G protein partners as well as  $\beta$ -arrestins.  $\beta$ -arrestins are intracellular proteins that were initially discovered as they "*arrested*" GPCR signaling by preventing G protein coupling through steric hindrance [67]. More recently,  $\beta$ -arrestins are widely accepted to be involved in GPCR internalization, endocytosis and participate in the activation of signaling pathways that are

"independent" from G protein cascades (reviewed in [68-70]). In the classical sense, after ligand binding and G protein activation, G protein receptor kinases (GRKs) are recruited and phosphorylate the carboxyl terminus of the receptor. This provides an anchoring site for  $\beta$ -arrestin binding and results in receptor desensitization.  $\beta$ -arrestins lead to the endocytosis of GPCRs via clathrin-coated pits allowing receptors to either be recycled back to the cell surface or degraded. Depending on how GPCRs interact with  $\beta$ -arrestins, these receptors are further sub-categorized in two distinct (alphabetized) classes [71]. Class A GPCRs such as the  $\beta_2$ -adrenergic receptor transiently interact with arrestins and quickly recycle back to the cell surface [71]. In contrast, Class B GPCRs stably interact with arrestins and the receptor co-internalizes alongside arrestin. This is exemplified by the angiotensin II type I receptor (AT<sub>1</sub>R) which takes a much longer time to recycle back to the cell surface. Furthermore, there are two  $\beta$ -arrestin isoforms which have different roles and cellular functions and GPCRs have been shown to have distinct affinity and interactions with specific isoforms. For example,  $\beta_2$ -adrenergic receptors internalize through interactions with  $\beta$ -arrestin2 while the AT<sub>1</sub>R, for instance, can interact with either isoform [71]. Besides their role in receptor desensitization,  $\beta$ -arrestins are scaffolding proteins that can recruit effectors and activate downstream signaling. The mitogenactivated protein kinases (MAPKs) for instance can be activated through β-arrestin specific interactions. Aside from these above-mentioned roles,  $\beta$ -arrestins are most famously known for their involvement in biased signaling, as described below.

Biased agonism is the ability of an agonist to selectively activate a subset of signaling pathways among all the pathways modulated by a single receptor (see **Figure 1.6A**). Biased signaling can occur because of intrinsic ligand or receptor properties, and/or the cellular system (reviewed in [72-74]). Ligand bias refers to a ligands ability to stabilize distinct receptor states that can specifically couple to different G proteins or  $\beta$ -arrestin isoforms conferring a signaling *bias*. Naturally occurring variants in receptor sequence can also result in biased responses by changing the strength of interaction with either ligands, G protein partners or other signaling partners [75-77]. Lastly, system bias is arguably the most important type of bias. A ligand cannot generate the same biased response if the receptor and associated effector is under- or over-represented in the

cellular system used for the assay [78]. Besides cell type, species origin can also contribute to system bias as receptor, G protein and  $\beta$ -arrestins may be differentially expressed in human versus rodent cellular models, for example [79]. Thus, it can be quite challenging to quantify bias as responses can change depending on the *in vitro* system parameters. In theory, ligand bias is encoded in the ligand's properties and should elicit biased responses independent of the cellular or tissue context. However, this may not always be the case as assays are hardly conducted in isolated systems but rather in various cellular and tissue contexts which can equally create or abolish a potential biased response [73]. In addition, bias is rarely assessed with the presence of the endogenous ligand which would be the most representative of the *in vivo* situation.

The concept of biased signaling is important in the cardiovascular field (see Figure **1.6B**) [80]. For instance, it has been suggested that  $G\alpha_q$ -mediated signaling in the vasculature downstream the angiotensin II type I (AT<sub>1</sub>) receptor is ultimately detrimental to the myocardium whereas the activation of  $\beta$ -arrestin signaling is cardioprotective [81]. In the heart, the activation of the AT<sub>1</sub>R results in an increase in arterial pressure. Elevated AT<sub>1</sub>R activity has been reported in heart failure, and chronic activation of this receptor is well known to cause maladaptive remodelling in the myocardium. On the other hand, activation of β-arrestins enhances cardiac contractility as well as preserves myocyte viability [82-84]. Accordingly, drug companies have attempted to develop  $\beta$ -arrestinselective drugs that target the AT<sub>1</sub>R with intent on promoting these positive myocardial effects while preventing the negative  $G\alpha_q$ -mediated effects. Currently marketed angiotensin receptor blockers are referred to as 'balanced' antagonists as they inhibit all downstream AT<sub>1</sub>R pathways including both  $G\alpha_q$ - and  $\beta$ -arrestin-mediated signaling. The need for more selective drugs prompted several pharmaceutical and academic labs to invest time towards the development of  $G\alpha_q$  selective AT<sub>1</sub>R inhibitors that maintain the  $\beta$ arrestin mediated increase in cardiomyocyte viability and contractility (discussed in later sections) [85]. Yet, experimental evidence points to a more complex picture when discussing  $\beta$ -arrestin-mediated biased responses. Not only does receptor conformation come into play but the conformation of effectors including  $\beta$ -arrestin and the selectivity of GRKs are equally important [86]. To further complicate the matter,  $\beta$ -arrestin itself can

adopt numerous active conformations [87]. Conflicting evidence serves to suggest that  $\beta$ arrestin conformations may, in part, be dictated by the GRK phosphorylation 'barcode' indicating that numerous effectors should be factored in when studying biased agonists and receptor responses [87, 88]. Thus, identifying the effector conformation while integrating the multifaceted molecular mechanisms that induces the  $\beta$ -arrestin mediated cardioprotective benefits still need to be elucidated.

Beyond the AT<sub>1</sub>R, biased agonism has also been a topic of interest for the development of next-generation drugs that target  $\beta$ -adrenergic receptors. Chronic and heightened activation of the adrenergic system also leads to cardiomyocyte apoptosis and maladaptive cardiac remodelling seen in heart failure [89]. This negative outcome has been shown to be associated with Ga<sub>s</sub>-mediated signaling. Several drugs have consequently been tested to explore their potential to selectively activate  $\beta$ -arrestins. Carvedilol treatment has been shown to result in the activation of cardioprotective ERK<sub>1/2</sub> signaling through interactions with  $\beta$ -arrestin [35]. In clinical trials, carvedilol was shown to reduce mortality and the risk of cardiovascular-related hospitalizations [90, 91]. Carvedilol ameliorates patient outcomes by improving left ventricular ejection fraction through enhancements in chamber contractility and has also been shown to improve cardiac endpoints in patients with dilated cardiomyopathy [92-94].

It is clear that GPCRs have a rich pharmacology and are important drug targets in various diseases. With this in mind, below, we briefly review GPCR targeting therapeutic agents in the drug discovery pipeline.



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#### Figure 1.6 Biased GPCR signaling in the heart.

(A)Schematic illustration of the concept of functional selectivity or biased agonism. Biased ligands (pink or cyan drug molecules) can stabilize specific GPCR conformations (yellow or cyan GPCRs) that preferentially engage with specific signaling partners ensuing preferred pathway activation. This concept is currently being taken advantage of to design and identify specialized compounds such as for the development of more refined agents for heart failure. (B)  $\beta$ -arrestin biased agonism results in beneficial cellular outcomes in the myocardium. The human AT<sub>1</sub>R and  $\beta_2$ AR represent significant therapeutic targets within the cardiovascular system. Biased agonism at these receptors has been a subject of therapeutic innovation to discover novels drug candidates to treat heart failure. Since G protein-mediated signaling in the myocardium has been demonstrated to be associated with negative prognostic indicators,  $\beta$ -arrestin biased therapeutic agents are being pushed forward in the drug development pipeline. This figure was created with BioRender.com.

#### 1.1.2.5 G protein-coupled receptors and drug discovery

As hinted above, GPCRs represent a large fraction of current drug targets. It is estimated that 30% of all drugs currently on the market target GPCRs. Drug discovery efforts essentially take advantage of assays that measure various endpoints of GPCR signal transduction such as calcium, cAMP accumulation or protein kinase activity for instance. Large compound libraries are often screened for activation or inhibition of the desired endpoint. Finally, 'lead' molecules are further engineered to optimize their pharmacodynamic and pharmacokinetic properties. Unfortunately, the GPCR drug discovery arena has, in some ways, come to a standstill as fewer candidates are reaching the market. This may be in consequence to the use of physiologically irrelevant models being used in assays aimed at identifying novel drug candidates. Until now, immortalized cell lines have been a popular choice for large-scale compound screening campaigns since they are easy to culture and can be grown at scale in short timeframes making them seem like ideal venues for assay automation and high-throughput screening. However, several issues may arise when using heterologous cell systems, especially in drug discovery (discussed in [95]). Concerns arise regarding artificial systems generated by overexpression of non-native amounts of receptors and their partner proteins [95]. As a
direct consequence of overexpression, GPCRs may not behave as their *in vivo* tissue residing equivalents. For instance, drugs may act as partial agonists *in vivo* but when tested *in vitro* using overexpression systems, the same compounds may act as full agonists. This has been observed for the  $\mu$ -opioid [96] and well as serotonin receptors [97]. Relatedly, overexpression studies may produce artifacts such as constitutively active GPCRs and can alter the balance of homo- or heterodimer species [95]. Further, altering the expression of G proteins can also influence interactions between agonists of GPCRs through the stabilization of alternate states [95].

In clinical trials, the success rates decrease as drugs move from phase I to II and ultimately phase III, reported as 78%, 39% and 29% respectively [98]. Phase I trials are mostly concerned with safety while phases II and III are concerned with safety, efficacy, effectiveness, as well as side effects. It is important to highlight that numerous drugs fail due to their cardiotoxic effects such as the unwanted occurrence of arrhythmias. Even when considering that drugs are already screened in a series of toxicity assays, several reports show that drugs still often fail due to lack of safety as well as efficacy [99]. It is thought that these high attrition rates may result as a consequence of the use of unpredictive model systems, increasingly complex diseases, better standards of care compared to past decades and stricter regulatory oversight [99]. In parallel, drugs aimed at women's health are subject to high attrition rates estimated at 42% [99]. Thus, model systems, including male rats and heterologous cell systems, can be unpredictive when extrapolating data designed for the typical 70 kg white male. This unpredictableness can worsen when generalizing data to a population that also includes women and various ethnicities. To address this, a new approach is required in the healthcare system that takes into consideration biological sex as well as inter-patient individuality. To resolve this, novel patient-derived models should be used in GPCR drug discovery platforms while conducting clinical trials in a dish. Perhaps using human derived iPSCs and their differentiated derivatives may help rescue attrition rates. This thesis will attempt to demonstrate the importance of using human iPSC-based as well as patient-derived models for disease modelling purposes, with a focus on heart failure and dilated cardiomyopathy, introduced in the upcoming sections.

#### 1.2 Physiology, pathophysiology and regulators of the adult human heart

#### 1.2.1 G protein-coupled receptors are central regulators of cardiac function

Broadly speaking, the cardiomyocytes in the heart can do three things. They can beat faster/slower, enlarge, or die in response to sustained stimulation by different neurohormonal modulators. These processes are all controlled, in part, by signals transduced through GPCRs. For instance, parasympathetic activation through the muscarinic M2 receptor slows down heart rate. Briefly, acetylcholine binding to M2 muscarinic receptors prompts the activation of  $G\alpha_i$  and the partial or complete release of G $\beta\gamma$  subunits. The latter, which subsequently activate G protein-coupled inwardly rectifying potassium channels (GIRK, Kir3), results in hyperpolarization of the cardiomyocyte [100, 101]. These coordinated signals facilitated by G $\beta\gamma$  lead to decreased heart rate and conduction speed orchestrated mainly by the SA and AV nodes [100, 101]. On the other hand, sympathetic input driven by the  $\beta$ -adrenergic receptor family results in increased heart rate and contractility. This thesis focuses on four main GPCR systems that will be examined in detail below starting with the hormones that target them.

#### 1.2.2 Endogenous hormones and peptides that modulate cardiac function

#### 1.2.2.1 Epinephrine and norepinephrine

Epinephrine and norepinephrine (synonymous with adrenaline and noradrenaline, respectively) are circulating catecholamines released by the sympathetic nervous system that regulate cardiovascular function. These two hormones belong to the catecholamine class since they have a benzene ring, two hydroxyl side chains and an ethylamine side chain. Both these hormones are synthesized in the chromaffin cells of the adrenal medulla [102]. Essentially, the amino acid tyrosine is converted into dihydroxyphenylalanine (DOPA) by tyrosine hydroxylase. Then, DOPA decarboxylase catalyses the production of dopamine followed by dopamine- $\beta$ -hydroxylase catalysing production of norepinephrine. Norepinephrine converted into epinephrine the gets through actions of

phenylethanolamine-N-methyltransferase [102, 103]. Epinephrine acts on the  $\alpha_1$ -  $\alpha_2$ - as well as  $\beta_1$ -,  $\beta_2$ - and  $\beta_3$ -adrenergic receptors while norepinephrine can bind preferentially to  $\alpha_1$ -  $\alpha_2$ - as well as  $\beta_1$ -adrenergic receptors (see **Figure 1.7**, **Table 1.2 & 1.3**). Thus, epinephrine has larger biological effects on the  $\beta$ -adrenergic system. These hormones are involved in the "fight-or-flight response" and in stressful circumstances are released into the blood stream. Once in the blood, epinephrine and norepinephrine act to modulate heart rate and blood pressure [104]. These hormones have short half-lives, acting for a few minutes before being degraded to inactive products: normetanephrine and metanephrine [103]. These hormone levels have been detected to be elevated in heart failure [105], contributing to the negative phenotypes observed in the clinic.





Figure 1.7 Cardiac and vascular effects of inotropes and vasopressors.

adrenergic receptor subtype with greater affinity than phenylephrine (PE) which specifically targets the  $\alpha_1$ -adrenergic receptors. Administration of phenylephrine, norepinephrine (NE) and epinephrine (Epi) result in vasoconstriction. Lower doses of norepinephrine affects cardiac output. Table 1.2 was modified from Senz, Nunnink, 2009 [106] and Figure 1.7 was modified from Hollenberg et al 2009 [107] (https://litfl.com/inotropes-vasopressors-and-other-vasoactive-agents/), both with permission.

In terms of pharmacology, epinephrine is most known for its treatment of anaphylaxis (i.e. Epipen) [108]. Epinephrine is also used to reverse cardiac arrest and in instances of cardiogenic shock. On the other hand, norepinephrine, is prescribed to treat low blood pressure acting as a vasoconstrictor [109, 110]. Although, the vasoconstricting effects of norepinephrine are nuanced in a sense as low doses impact cardiac output by stimulating  $\beta_1$ ARs while higher doses lead to vasoconstriction through actions of the  $\alpha_1$ ARs (*see* **Table 1.2**) [111]. Norepinephrine can also be prescribed as a treatment for depression [112] in the form of serotonin-norepinephrine reuptake inhibitors (SNRIs).

### 1.2.2.2 Endothelin-1

Endothelins are peptides with potent vasoconstrictor effects. There are three endothelin isoforms, ET-1, ET-2, and ET-3. Endothelins bind  $ET_A$  and  $ET_B$  receptors with different affinities. For example, ET-1 and ET-2 bind  $ET_A$  with higher affinity than ET-3 whereas all three have equal affinity for  $ET_B$  (reviewed in [113]). The site of synthesis of endothelin depends on the isoform. For instance, ET-1 is mainly synthesized in endothelial cells but can also be synthesized in the cardiomyocytes while ET-2 and ET-3 are synthesized in kidney and intestinal cells [114]. Endothelin-1 begins as a 212 residue pre-pro-peptide which is cleaved to become a 38-residue big endothelin-1 pro-peptide. Then, endothelin converting enzyme (ECE) converts big endothelin-1 into the active, 21-amino acid peptide, endothelin-1 [114].

ET-1 behaves in an autocrine manner but also displays paracrine actions. Endothelial cells as well as cardiomyocytes have the enzymes required to produce ET-1 and the peptide can act at receptors on these cells or other cardiac resident cells to elicit vasoconstrictor effects. ET-1 can also influence levels of epinephrine [115], angiotensin II [116], aldosterone [117], behaving in a paracrine fashion [114]. This evidence essentially suggests that ET-1 may play a role in heart failure due to the interplay with these other cardiac relevant modulators. Circulating levels of ET-1 have been suggested as a negative prognostic indicator of heart failure as increased levels have been reported in instances of myocardial infarction [118], hypertension [119], idiopathic dilated cardiomyopathy [120] and congestive heart failure [121]. Similarly, mRNA levels of endothelin converting enzyme are increased in cardiomyocytes in patients with ischemic cardiomyopathy [122]. The ET-1 peptide has also been reported to play a role in the

development of the cardiovascular system as mice lacking ET-1 have been shown to have cardiovascular defects [123]. Thus, studying the cardiovascular effects of ET-1 are of interest to this thesis.

Lastly, with relevance to pharmacology, endothelin receptor antagonists are not commonly prescribed in heart failure as clinical trials have not show benefit over placebo [124, 125]. ET-1 antagonists are however used to treat pulmonary artery hypertension.

# 1.2.2.3 Angiotensin II

Angiotensin II is an octapeptide peptide hormone and is part of the reninangiotensin aldosterone system (RAAS). In this system, angiotensinogen, which is secreted by the liver into the circulatory system is cleaved by renin, an enzyme secreted by the kidneys, to generate angiotensin I in plasma. Then, angiotensin converting enzyme (ACE), located on the cell surface, further processes angiotensin I into the active hormone, angiotensin II (Ang II). Ang II elicits actions in the cardiovascular system modulating vascular tone [126]. In addition, angiotensin II acts at the level of the kidneys and the adrenal cortex to promote sodium reabsorption resulting in an increase in blood pressure. In the same vein, Ang II binds the AT<sub>1</sub>R in the vasculature resulting in signal transduction events that result in vasoconstriction which increases peripheral resistance, consequently resulting in an increase in blood pressure. Ang II can be processed further into Ang III and Ang 1-7 through the actions of various other enzymes. Interestingly, Ang 1-7 behaves as a vasodilator, binding the Mas receptor [127, 128]). Ang II can bind the AT<sub>1</sub> as well as the AT<sub>2</sub> receptors.

The Ang II system has been shown to act locally, with all the necessary components present in cardiomyocytes to produce Ang II. As such, several reports have shown that cardiomyocytes have the enzymes required to process angiotensinogen into angiotensin II [129]. Elevated levels of Ang II have been associated with cardiac disease [130]. In rats, levels of Ang II have been reported to increase post-myocardial infarction [131]. Human studies have also corroborated these findings [132, 133]. Ang II signaling

is a common target in heart failure where ACE inhibitors act to lower levels of Ang II peptide which have been shown to be detrimental to heart function. Angiotensin receptor blockers (ARBs) are also routinely prescribed to heart failure patients and will be described below.

	αıAR	α <sub>2</sub> AR	βıAR	β <sub>2</sub> AR	AT₁R	EΤ <sub>A</sub>	EΤ <sub>B</sub>
Epinephrine	Х	Х	Х	Х			
Norepinephrine	Х	Х	Х				
Isoproterenol			Х	Х			
Phenylephrine	Х						
Angiotensin II					Х		
SI ([Sar <sup>1</sup> ,Ile <sup>8</sup> ]AngII)					X		
SII ([Sar <sup>1</sup> ,Ile <sup>4</sup> ,Ile <sup>8</sup> ]AngII)					X		
ET-1						Х	Х

**Table 1.3** Summary of receptor targets for cardiac relevant hormones and synthetic drugs relevant to this dissertation.

## 1.2.3 Cardiac-relevant G protein-coupled receptors

## 1.2.3.1 $\alpha$ -adrenergic receptors

α-adrenergic receptors were first identified in rat myocardial tissue using radioligand binding studies [134]. The α-adrenergic family of receptors can be subdivided into two subtypes,  $\alpha_1$ - and  $\alpha_2$ -adrenergic receptors. Of relevance to this thesis,  $\alpha_1$ -adrenergic receptors are coupled to  $G\alpha_{q\backslash11}$  and are subdivided in three isoforms  $\alpha_{1A}$ ,  $\alpha_{1B}$  and  $\alpha_{1D}$  [135]. There is no  $\alpha_{1C}$  as it was later identified as the pharmacological equivalent of the  $\alpha_{1A}$  receptor [136, 137]. While both the  $\alpha_{1A}$  and  $\alpha_{1B}$  are found in the heart muscle,  $\alpha_{1D}$  is expressed in coronary arteries [138]. The expression levels of the  $\alpha_1$ AR are similar in human as well as guinea-pig, mouse, pig and calf models [139]. The  $\alpha_{1B}$ AR isoform has been demonstrated to be present on most ventricular myocytes in mice while the  $\alpha_{1A}$ AR subtype represents a smaller population [140]. However, based on radioligand studies using the tritiated antagonist prazosin, the expression of  $\alpha_1$ AR has been

demonstrated to be 5-8 times higher in rats [139]. This marks an important species difference [141] that cannot be ignored when performing cell-based assays exploring  $\alpha_1AR$  signaling in rat for human translation. Based on this data, mice models may be a better alternative compared to rat neonatal cardiomyocytes, the most commonly used *in vitro* system to study this receptor subtype.  $\alpha_1AR$  subtype differences have also been observed across species as humans have higher abundance of  $\alpha_{1A}AR$  while rat  $\alpha_{1B}AR$  [135]. However, differences between these two receptor subtypes have not been fully elucidated due to the limited subtype-specific pharmacological toolbox. Nevertheless, differences in receptor desensitization and  $\beta$ -arrestin interactions have been reported [142].

α<sub>1</sub>AR are located on the plasma membrane but evidence has also suggested their functional expression on nuclear membranes of cardiomyocytes [143-146]. In the myocardium, the  $\alpha_1$ -adrenergic receptor family functions are primarily mediated by  $G\alpha_{q/11}$ and enhance cardiac contractility through calcium release from the sarcoplasmic reticulum.  $G\alpha_q$  signaling leads to the activity of the membrane-associated enzyme phospholipase C (PLC) which catalyses the production of inositol [1,4,5]-trisphosphate (IP<sub>3</sub>) and 2-diacylglycerol (DAG) [147]. Activation of α<sub>1</sub>AR results in a positive ionotropic effect in the heart (see Figure 1.8). This physiological effect holds true in several model species including human [148-150], rodent and canine hearts [151-153]. This positive inotropy is speculated to occur via the activation of secondary messengers which liberate internal calcium stores from the sarcoplasmic reticulum thereby increasing contractions. Other reports have demonstrated that positive inotropy is mediated via increased Ca<sup>2+</sup> sensitivity of the myofilaments [151]. α<sub>1</sub>AR mRNA levels in the left ventricle have been correlated with left ejection fraction [154]. More recently, the  $\alpha_1$ -adrenergic receptors have been reported to also couple to  $G\alpha_s$  as phenylephrine stimulation has been observed to lead to nuclear PKA activity in neonatal rat cardiomyocytes [155]. Activation of the α<sub>1</sub>AR has been demonstrated to activate immediate early genes, c-fos, Egr1, and c-jun [135]. Longer stimulation with  $\alpha_1AR$  agonists such as phenylephrine result in the activation of natriuretic peptides, ANP and BNP often resulting in a hypertrophic cardiomyocyte phenotype [135]. For more comprehensive reviews of the α<sub>1</sub>-adrenergic system, readers are referred to [156, 157].

# 1.2.2.1.1 α-adrenergic receptors: disease and attendant pharmacology

Generally, the  $\alpha$ -adrenergic receptor family represents a small portion of the overall cardiac adrenergic population as  $\beta$ -adrenergic receptors are the main players modulating cardiomyocyte function and are discussed in depth below [158, 159]. Reports have shown that the expression levels of human ventricular  $\alpha_1$ -AR are not significantly altered in heart failure [158, 160]. However, some evidence may indicate small increases in expression [160]. This contrasts with  $\beta_1$ -adrenergic receptors which are downregulated in human failing hearts [161]. Several clinical trials have tested  $\alpha_1$ -antagonists in the context of heart failure and have failed to show significant patient benefit [162]. For this reason, antagonists for the  $\alpha_1$ -adrenergic receptor are not commonly used in instances of heart failure but rather in the treatment of benign prostatic hypertrophy and hypertension [163].

Certain lines of evidence have also suggested a protective role of the  $\alpha_1$ adrenergic receptor. For instance, phenylephrine stimulation has been observed to reduce apoptosis in neonatal rat cardiomyocytes by modulating the expression of the cell death regulator, Bcl-2 [164]. In the myocardium,  $\alpha_1$ -adrenergic signaling has been shown to be important for survival signaling through the activation of ERK<sub>1/2</sub> [165]. This may be a reason for the lack of success for  $\alpha_1$ -antagonists in heart failure. Even if accumulated experimental evidence suggests that  $G\alpha_q$  signaling in the myocardium is cardiotoxic, most accounts mostly investigated canonical  $G\alpha_q$  signaling originating from the plasma membrane. One publication proposed that nuclear  $G\alpha_q$  signaling downstream  $\alpha_1AR$ activation may be cardioprotective thus this overall belief of the negative effects of  $G\alpha_q$ signaling may need to be revaluated [166]. Receptor compartmentalization is thus highly important biological consideration when assessing receptor function based on whole cell assays. Moreover, a pressure overload model has demonstrated that  $\alpha_{1a}AR$  and  $\alpha_{1b}AR$  null mice have aggravated dilated cardiomyopathy phenotypes [167]. Activation of  $\alpha$ -adrenergic receptors has also been shown to promote cardiomyocyte hypertrophy in various model systems in mice [168], rat [169-171], cat [172] and human models. Both subtypes have been linked with hypertrophic phenotypes as it has been observed in response to the activation of the  $\alpha_{1A}$ -adrenergic receptor [173] as well as in transgenic mice overexpressing the  $\alpha_{1B}$ -adrenergic receptor [174].  $\alpha_1$ -AR are thus involved in the maladaptive remodelling of the heart in the context of disease. It is thus speculated that  $\alpha_1$ -adrenergic receptors may have a duplicitous role modulating compensatory and decompensatory mechanisms in heart failure and is important for maintaining core heart functions.

## $1.2.3.2 \beta$ -adrenergic receptors

The  $\beta$ -adrenergic receptor ( $\beta$ AR) subtype represents approximately 90% of all adrenergic receptors in the heart. The  $\beta$ -adrenergic family of receptors can be subdivided into three receptor subtypes specifically, the  $\beta_1$ -,  $\beta_2$ -, and  $\beta_3$ -adrenergic receptors. In the healthy human heart, the  $\beta_1$ AR represents an estimated 75% of total  $\beta$ ARs while  $\beta_2$ AR correspond to 15% and  $\beta_3$ AR approximately 2-3% [175, 176]. In the neonatal rat heart, the  $\beta_2$ AR is estimated to represent 64% of the  $\beta$ AR receptor pool as the  $\beta_1$ AR comprises 36% [177]. This contrasts with the adult rat heart where the  $\beta_1$ AR prevails with an approximate expression of 59% while the expression of the  $\beta_2$ AR has been estimated at 41% [177, 178].

The  $\beta_1$ - and  $\beta_2$ -adrenergic receptors are central regulators of cardiac function whereas the  $\beta_3$ -adrenergic receptor is mostly present in adipose tissue. However, some evidence may serve to suggest that there are small pools of  $\beta_3AR$  in the heart that may become more important in disease such as ischemic and dilated cardiomyopathy [179]. The  $\beta_1AR$  is present in the kidneys and adipose tissue while the  $\beta_2AR$  also functions in the airway smooth muscle, brain, and skeletal muscles [180].  $\beta_1$ - and  $\beta_2$ - adrenergic subtypes are found in both atrial and ventricular human tissue [181, 182] as well as the sinoatrial node [183]. The  $\beta_1AR$  and  $\beta_2ARs$  can be activated by endogenous hormones epinephrine and norepinephrine. These receptors are also the target of the synthetic isopropylamine analog of epinephrine, isoproterenol (*see Table 1.3*). Of note, norepinephrine targets the  $\beta_1AR$  with higher affinity than the  $\beta_2AR$ .

The  $\beta_1$ -adrenergic receptors are coupled to the stimulatory G protein,  $G\alpha_s$ , and regulate cardiac contractility, growth and survival (see **Figure 1.8**). Activation of  $G\alpha_s$  leads to the activation of adenylyl cyclase (AC) and production of the second messenger cyclic adenosine monophosphate (cAMP). Serial activation of these effectors leads to the downstream activation of protein kinase A (PKA) which phosphorylates proteins involved in the contractile machinery such as L-type calcium channels thereby initiating cardiac contractility. β-adrenergic receptors exhibit positive ionotropic and chronotropic effects in the heart [184-186]. The  $\beta_2$ -adrenergic receptor is coupled to both  $G\alpha_s$  and  $G\alpha_i$ . Activation of the inhibitory G protein,  $G\alpha_i$ , inhibits cAMP accumulation and typically leads to the activation of mitogen-activated proteins kinases (MAPKs). The activation of  $G\alpha_i$  is commonly believed to result in cardioprotective effects as it activates ERK<sub>1/2</sub> which reduces cardiomyocyte apoptosis while promoting cell survival [187]. β-adrenergic receptors also signal independently from G proteins via the  $\beta$ -arrestin pathway [188]. Activated receptors can be phosphorylated by G protein-coupled receptor kinases (GRKs) permitting recruitment of  $\beta$ -arrestins. As stated above,  $\beta$ -arrestin signaling is thought to be cardioprotective but may be more nuanced than initially anticipated [189]. In primary adult murine cardiomyocytes, lipitated peptides known as pepducins that produce a bias towards the  $\beta$ -arrestin signaling axis, have been shown to induce contractility through a G protein independent pathway [190]. Further, knock-out arrestin mouse models have been observed to have increased isoproterenol-induced ejection fractions [191].

Beyond the cell surface,  $\beta$ -adrenergic receptors have also been demonstrated to signal from intracellular compartments. For instance,  $\beta_1$ - and  $\beta_3$ -adrenergic receptors have been shown to be present, eliciting signals from the nuclear membrane of adult rat and mouse ventricular cardiomyocytes [192]. Associated effectors have also been

demonstrated at nuclear sites [193, 194].  $\beta_1$ - and  $\beta_2$  adrenergic receptor-mediated signaling can also be distinguished through spatial regulation. For example, in cardiomyocytes isolated from mice, stimulation of β1ARs with isoproterenol was shown to result in diffuse cAMP signals while  $\beta_2$ AR-mediated cAMP signaling is thought to be more compartmentalized or restricted to lipid rafts, caveolae or T-tubules [195-198]. Further, the may also influence β2AR compartmentalization Gαi coupling [199]. Compartmentalization can allow to parse out signals emanating from different receptor pools. Signaling competent  $\beta_1$ - and  $\beta_2$ -ARs have also been demonstrated in endosomes [200] and in the Golgi apparatus [201]. In summary, compartmentalized signaling is important as disease has been shown to alter receptor expression, localization and signaling in subcellular microdomains [202].

## 1.2.3.2.1 $\beta$ -adrenergic receptors: disease and attendant pharmacology

As mentioned above, the  $\beta_1$ -adrenergic receptor predominates in healthy cardiac tissue representing an estimated 70-80% of  $\beta$ -AR. However, in heart failure, these ratios change, with a 2-fold increase in the expression levels of the  $\beta_2AR$ , from an initial 20% to 40% [203, 204]. Chronic activation of  $\beta_1$ - and  $\beta_2$ -adrenergic receptors has been shown to result in cardiomyocyte hypertrophy. This has been shown in vivo as well as in vitro in mice [205], rats [177] as well as in human model systems. These detrimental effects are most often believed to occur via the  $G\alpha_s$ -AC-cAMP-PKA signaling axis but other pathways have also been found culprit. Activation of the  $\beta_1AR$  subtype has been associated with cardiomyocyte apoptosis while activation of  $\beta_2 AR$  is believed to be cardioprotective [187, 206-208]. The anti-apoptotic effect of the  $\beta_2AR$  is believed to be mediated by  $G\alpha_i$ . Coincidently, in human failing hearts,  $G\alpha_i$  levels have been observed to increase [209-211]. Yet, in some rodent models,  $\beta_2$ AR overexpression has been shown to result in heart failure and hypertrophy. However, the degree of overexpression needs to be considered as excessively high levels have been shown to be detrimental while lower levels can be beneficial [212]. Addedly, transgenic models have demonstrated that the overexpression of the β-adrenergic receptors may have a transient-lived benefit to myocardial contractile

function but overtime result in heart failure [213, 214] A  $\beta_1$ AR transgenic mouse model was also shown to be associated with ECM remodelling and ventricular dilatation [215].

To mitigate the symptoms associated with heart failure and the increase in circulating catecholamines which negatively impact heart tissue,  $\beta$ -receptor antagonists or  $\beta$ -blockers are often prescribed to patients. In general,  $\beta$ -blockers are not "disease modifying drugs" as they are not curative and do not reverse cardiac damage *per se*.  $\beta$ -blockers, however, do reduce the strain on the heart by inhibiting signal transduction through  $\beta$ -adrenergic receptors by preventing the binding of the endogenous agonist. There are several  $\beta$ -blockers that can be administered to heart failure patients with distinct chemical and signaling properties (reviewed in [216]). Numerous clinical trials: BEST [217], CIBIS-II [218], MERIT-HF [219], COMET [220], CAPRICORN [221], COPERNICUS [222] and SENIORS [223], have demonstrated the benefit of this drug class for treating heart failure with reduced ejection fraction [224]. Essentially, they have been shown to decrease mortality and rate of hospitalizations.  $\beta$ -blockers are first-line drugs for patients suffering from not only heart failure but also dilated cardiomyopathy.  $\beta$ -blockers can also be prescribed to hypertensive patients as the administration of these agents decrease heart rate. When treating essential hypertension,  $\beta$ -blockers are second line drugs.

Three generations of  $\beta$ -blockers have been developed (reviewed in [225, 226]). The first-generation refers to the generation of non-selective antagonists like propranolol that exerted effects in the heart but also undesired effects in the lungs. Second generation blockers were more "cardio-selective" with increased selectivity for  $\beta_1$ -adrenergic activity (atenolol, bisoprolol, celiprolol, and metoprolol). Third generation agents, carvedilol, nevibolol, have demonstrated a vasodilatory mode of action [227, 228]. For instance, carvedilol is classified as a third generation  $\beta$ -blocker as it is mildly selective for the  $\beta_1$ AR, shown to be dose dependent but also acts by inhibiting the  $\alpha_1$ -ARs which explains its vasodilatory effects [229]. Nevibolol also exhibits vasodilatory effect through nitric oxide production [230, 231]. Antioxidant and antiproliferative effects have also been reported for carvedilol.

It has also been speculated that  $\beta$ -blockers may exert their positive outcomes on the myocardium based on the 'resensitization hypothesis'. The latter suggests that  $\beta$ blockers act not only by blocking adrenergic signaling but also by upregulating the population of  $\beta$ -adrenergic receptors on the cardiomyocyte membrane. This may seem counterintuitive, yet rational explanations have been put forth. Essentially, in heart failure, the density of  $\beta$ -adrenergic receptors is downregulated therefore, in theory, by reducing the downregulation of β-adrenergic receptors on cardiomyocyte membranes, it should help improve contractility. As it happens, one study has shown that long-term metoprolol administration increased expression of  $\beta$ -adrenergic receptors in dilated cardiomyopathy patients and was associated with positive outcomes [232]. β-blocker therapy has also been observed to alter gene expression patterns of calcium handling and contractilerelated genes in DCM [233]. For instance, β-blockers have been demonstrated to upregulate mRNA encoding the sarcoplasmic reticulum calcium ATPase as well as αmyosin heavy chain while reducing levels of  $\beta$ -myosin heavy chain [233, 234]. Therefore, with higher β-adrenergic density, there are more receptors available to signal through Gαs leading to improved cardiac function in heart failure.

Beyond the adrenergic system, angiotensin receptors are also critical players regulating cardiovascular function; described below.

## 1.2.3.3 Angiotensin II type I receptors

There are two types of angiotensin receptors in the heart:  $AT_1R$  and  $AT_2R$ . There is no doubt that the  $AT_1R$  is a member of the GPCR superfamily however there is some speculation suggesting that the  $AT_2R$  may not be G protein-coupled [235]. The controversy surrounding the  $AT_2R$  stems from the conflicting data demonstrating  $G\alpha_i$ protein coupling, and the absence of agonist induced internalization, two features of prototypical GPCRs [236, 237]. The interested reader can refer to the following review of  $AT_2R$  signaling [238]. Of interest to this thesis, the  $AT_1R$  has two subtypes  $AT1_A$  and  $AT1_B$ found in rodents and in humans [239]. In the rat, the  $AT_{1A}$  and  $AT_{1B}$  are pharmacologically equivalent as they activate calcium signaling with the same efficacy [240]. Evidence from mouse models has demonstrated that the  $AT_{1B}$  receptor is mainly involved in regulating contraction in smooth muscle cells of the vasculature while the AT1A is located in the kidneys [241, 242]. AT<sub>1</sub>Rs are also expressed in cardiac fibroblasts and endothelial cells with lower expression in cardiomyocytes [243, 244]. In rat cardiomyocyte cultures, the  $AT_1$  has been observed to be expressed on the sarcolemma and T-tubules [245]. AT\_1 and AT<sub>2</sub> receptors have also been observed on the surface of nuclear membranes of adult rat cardiomyocytes [245, 246]. AT1Rs are functional at both cell surface and nuclear locations within cardiomyocytes and are signaling competent. The stimulation of nuclear  $AT_1$ receptors has been shown to augment *de novo* RNA synthesis, nuclear calcium levels and regulate transcription [246]. Relatedly, pathological relevance of nuclear localized AT<sub>1</sub> receptors has been demonstrated in canine atrial fibroblasts where nuclear AT<sub>1</sub>Rs were observed to be increased in a heart failure model [247]. Future studies are required to parse out how these nuclear receptor pools regulate cardiomyocyte function and further elucidate their role in heart failure (reviewed in [248]). To this end, the stimulation of patient-derived iPSC-CMs with caged compounds that deliver Ang II to the nucleus, without activating cell surface receptors, should be able to expose the physiological and pathological significance of nuclear AT<sub>1</sub> receptors [249, 250].

Angiotensin II type-I (AT<sub>1</sub>) receptors are coupled to  $G\alpha_{q/11}$ ,  $G\alpha_i$  and  $G\alpha_{12/13}$ . In the heart, activation of  $G\alpha_q$  downstream of the AT<sub>1</sub>R results in the activation of  $\beta$ -type phospholipase C which hydrolyzes phosphatidylinositol 4,5-bisphospate (PIP<sub>2</sub>) into diacyl glycerol (DAG) and inositol triphosphate (IP<sub>3</sub>). IP<sub>3</sub> is a second messenger that can bind to the specific receptors located on the sarcoplasmic reticulum (SR), resulting in calcium release that facilitates contractility through calmodulin binding and activation of myosin light chain kinase. On the other hand, both calcium and DAG can trigger activation of protein kinase C (PKC) which phosphorylates several substrates, provoking biological events such as cell growth, vasoconstriction and cell migration (see **Figure 1.8**) [251]. The AT<sub>1</sub>R is also known as a 'stretch sensor' and can be activated via mechanical stretch in absence of endogenous Ang II [252]. In adrenal glomerulosa cells, Ang II has been demonstrated to activate G $\alpha_i$  and stimulate the activity of T-type calcium channels [253, 254]. Calcium entry promoted aldosterone release which acts to regulate blood pressure

[253]. Ang II has also been shown to inhibit current of L-type calcium channels, a mechanism postulated as means to control cytosolic calcium levels [255]. In vascular smooth muscle cells, Ang II can also result in the activation of  $G\alpha_{12/13}$  which activates Rho kinase, a signaling cascade involved in vascular remodelling [256].

### 1.2.3.3.1 Angiotensin II type I receptors: disease and attendant pharmacology

In non-failing human hearts, the expression ratio of AT<sub>1</sub>R to AT<sub>2</sub>R is approximately 60%-40% [257]. In DCM hearts, expression of AT<sub>1</sub>R has been reported to be reduced while AT<sub>2</sub>R levels are elevated [257]. At baseline, in rat and in human, Ang II plasma concentrations are low, estimated at 10-60 pM [254, 258]. In a rat model, plasma levels of Ang II have been shown to increase in malignant hypertension [254, 259]. Interestingly, plasma Ang II levels in normotensive versus hypertensive humans have not been shown to be significantly different [260].

Animal models have helped elucidate the role of the ATRs in the heart and vasculature. Transgenic mice with overexpression of AT<sub>2</sub>R have been observed to develop dilated cardiomyopathy [261]. In a mouse model, the cardiac specific overexpression of the AT<sub>1</sub>R was demonstrated to result in cardiac hypertrophy and premature death due to heart failure [262]. Further, AT<sub>1</sub>R overexpressing mice have been demonstrated to have an arrhythmogenic phenotype due to delayed repolarization [263] and altered cardiac contractility [264]. In a mouse model of dilated cardiomyopathy, female mice were shown to be more vulnerable to the negative myocardial effects of Ang II [265]. Likewise, Ang II has been observed to result in cardiomyocyte hypertrophy via autocrine and paracrine effects stimulating the release of TGF-B1 and ET-1 from fibroblasts or cardiomyocytes [243, 266, 267]. Ang II has also previously been linked with stretch-induced hypertrophic response in neonatal rat ventricular cardiomyocytes [268]. Certain AT<sub>1</sub>R variants have been shown to contribute to cardiovascular disease, including hypertrophy, fibrosis and atheroslerosis [269]. Several of the maladaptive effects of the angiotensin system have been correlated with  $G\alpha_q$  signaling [270]. The cardiac-specific overexpression of  $G\alpha_q$  in mice has also been associated with a hypertrophic phenotype [271]. These mice were also observed to develop a form of dilated cardiomyopathy [271]. Another transgenic mouse model with the tamoxifen inducible expression of  $G\alpha_q$ , showed a dilated cardiomyopathy phenotype linked to the activation of PLC $\beta$  [272].

Due to its involvement in numerous cardiac pathologies; heart failure, hypertrophy, hypertension as well as atherosclerosis, the  $AT_1R$  is an important drug target. Further, the AT<sub>1</sub>R is an attractive candidate due to its biased signaling capacity, as mentioned in an earlier section. Some drug companies have thus pushed for the development of  $\beta$ arrestin biased drugs due to their beneficial activity in the heart. Trevena, a biopharmaceutical company developed several iterations of biased AT<sub>1</sub>R targeting drugs. Initial preclinical studies revealed the promise of  $\beta$ -arrestin biased compounds [273, 274]. However, in the BLAST-AHF human trial, TRV027 was tested in an acute heart failure setting and no significant benefit was reported compared to placebo [275, 276]. It would be interesting to re-test this drug in a chronic, long term, heart failure trial, as done in animal models. Experimental evidence also serves to suggest that TRV023, TRV026, TRV034 and TRV067 also display β-arrestin biased potential [277]. TRV023 has been shown to improve cardiac contractility, cardiomyocyte survival and prevents hypertrophy [278-280]. In a mouse model of dilated cardiomyopathy, TRV067 has been shown to improve left ventricular function as well as dysregulated signaling through the activation of the ERK<sub>1/2</sub> pathway [281]. In an animal model, chronic biased signaling has been shown as a viable treatment strategy for dilated cardiomyopathy [281]. Long-term treatment of mice with β-arrestin biased compound, TRV120067, has revealed improvements in cardiac architecture and function through the activation of ERK-RSK3 [281]. Relatedly, a β-arrestin knock-out mouse model has also revealed that β-arrestins are important mediators of cardiac function during aging [282]. Even if an AT<sub>1</sub>R targeted β-arrestin biased drug has yet to make it to the market with a cardiovascular indication, the search remains promising.

Regardless, the angiotensin II type I receptor has rich pharmacology. The AT<sub>1</sub>R is an important target in heart failure with ACE inhibitors and ARBs being commonly

prescribed. Angiotensin converting enzyme (ACE) inhibitors are often prescribed to patients diagnosed with DCM even after having progressed to heart failure. ACE inhibitors work by reducing the circulating levels of the peptide hormone, angiotensin II, which contributes to maladaptive cardiac remodelling. Several clinical trials have reported improvement in patients with failing hearts who have been administered ACE inhibitors (reviewed in [283]). However, some patients develop a cough with the continuous use of ACE inhibitors prompting physicians to prescribe angiotensin receptor blockers (ARBs) instead. Inhibition of RAAS using angiotensin receptor blockers such as losartan has been observed to reverse left ventricular hypertrophy in hypertensive patients [284].

Dual targeting therapies have more recently been demonstrated to be effective in DCM and heart failure. One such example is Entresto, a combined medication composed of an angiotensin receptor blocker, valsartan and a neprilysin inhibitor, sacubitril [285, 286]. Neprilysin is a zinc-dependent metalloprotease that cleaves peptides and hormones including natriuretic peptides, bradykinin and angiotensin II. The natriuretic peptide system essentially counters the harmful effects caused by the hyperactivation of the RAAS and other detrimental signals that damage the heart. In these instances, natriuretic peptides like B-type natriuretic peptide (BNP) are released and act to promote natriuresis and vasodilatation thus reducing blood pressure and the strain on the heart. When developed as a single drug, neprilysin inhibitors did not outperform placebo in lowering blood pressure for hypertension [287]. It has been reported that part of this failure could be attributed to the overlooked detail that neprilysin proteases also result in an increase in Ang II [288-291]. To address this, Entresto was developed as a single medication with dual action, combining a neprilysin inhibitor and angiotensin receptor blocker. In the PARADIGM-HF clinical trial, this drug formulation demonstrated a reduced risk of mortality and hospitalization for heart failure and is currently being used to treat dilated cardiomyopathy [292].

Several other GPCRs that couple to  $G\alpha_q$  have been associated with maladaptive cardiac remodelling and the development of hypertrophy such as the Prostanoid F receptor [293] and the endothelin receptor [294]. The latter is discussed below.

#### 1.2.3.4 Endothelin receptors

There are two endothelin receptor subtypes expressed in the myocardium: ET<sub>A</sub> and ET<sub>B</sub> [295-297]. The relative distribution of either isoform has been relatively well studied demonstrating a higher abundance of ET<sub>A</sub> receptors in human vascular smooth muscle and ET<sub>B</sub> in endothelial cells, fibroblasts, kidneys and non-vascular tissues [297]. In the myocardium, ET<sub>A</sub> is most abundant, representing 90% of endothelin receptors [298, 299]. Evidence suggests that endothelin receptors are localized at the cell surface as well as nuclear membrane of ventricular myocytes [300]. A larger fraction of ET<sub>B</sub> receptors are localized to the nucleus compared to ET<sub>A</sub> subtype [300, 301]. ET-1, the peptide which binds endothelin receptors has been reported to exert ionotropic effects in the human and rat myocardium [302-304]. Endothelin receptors couple to G $\alpha_{q/11}$  and result in the activation of calcium dependent pathways. It has been proposed that this peptide increases the force of contraction of the heart via enhanced calcium sensitivity [305]. Endothelins have also been demonstrated to influence heart rate. In guinea pig atria, a dose-dependent effect has been shown where low doses of ET-1 increase heart rate while higher concentrations reduced heart rate [306].

#### 1.2.3.4.1 Endothelin receptors: disease and attendant pharmacology

To help elucidate pathological functions of ET-1 and its cognate receptors, numerous animal models as well as transgenic lines have been used. For example, in a rat model of congestive heart failure, levels of ET-1 were higher than in healthy counterparts while the level of ECE-1 was unchanged [307]. ET<sub>A</sub> and ET<sub>B</sub> levels were increased in the diseased rat model [307]. Changes in endothelin receptor density at both cytoplasmic and nuclear locations may also be relevant in the context of disease [308, 309]. The activation of nuclear endothelin receptors can influence calcium mobilization and overall cardiomyocyte function and possibly also gene expression [310] (reviewed in [248, 311, 312]). Further, a cardiac-specific ET-1 transgenic mouse model also served to demonstrate the negative role of endothelin's in heart failure. Mice overexpressing ET-

1 displayed increased myocardial inflammation and left ventricular dilation and overall diminished cardiac function [313]. In neonatal rat cultures, ET-1 has been shown to repeatedly result in cardiac hypertrophy [294]. In comparison, ET<sub>A</sub> receptor density of DCM patients was shown to be increased compared to non-failing human hearts [314]. A diminished ionotropic effect was also reported [314]. Relatedly, levels of the ET-1 peptide have been observed to be a poor prognostic indicator post-myocardial infarction as it was associated with reduced survival [315].

Like RAAS inhibitors and β-blockers, endothelin blockers have been investigated as therapy for heart failure. Unfortunately, contraindicatory data has been reported thus far. In a rat model of chronic heart failure, endothelin antagonists initially showed promise through observations of reduced hypertrophy, fibrosis and ventricular dilation [316]. Left ventricular function had also been shown to be improved in a dog model of heart failure [317]. However, other reports have shown the ineffectiveness of this type of receptor inhibition [318]. Focusing on human trials, the HEAT, Heart Failure ETA Receptor Blockade Trial, short-term (3-weeks) antagonism of the ET<sub>A</sub> receptor was shown to be beneficial to heart failure patients [319]. Considering that patients typically use heart failure medication on a long-term basis, trials should be designed to recapitulate this frequency as well. However, long-term trials did not prove successful. In the EARTH (24 weeks) [124] and ENABLE (9 months) [320], ET blockade was not shown to improve clinical patient outcomes. In the ENABLE trial, for instance, bosentan, a non-selective endothelin antagonist resulted in edema which caused increased hospitalizations. Thus, endothelin antagonists are not mainstay therapy for DCM or heart failure. However, compartmentalized antagonism may be of interest, targeting nuclear pools of ETRs, representing a new therapeutic avenue for the future development of endothelin receptor inhibitors. ET-1 inhibitors, on the other hand, are used to combat pulmonary hypertension.



## Figure 1.8 Effect of select GPCRs in the cardiovascular

Cardiac homeostasis is regulated by the same receptors that regulate cardiac diseases. Dysregulated signaling of the  $\alpha_1$ - and  $\beta_1$ -and  $\beta_2$ -adrenergic receptors have been linked with cardiac pathologies as their enhanced signaling leads to maladaptive remodelling and failure due to the heart muscle over-exerting itself. Angiotensin as well as endothelin systems modulate cardiovascular stasis and their disruptive enhanced activation also result in hypertrophy and heart failure. The figure was created with BioRender.com.

Once GPCRs are activated, a plethora of downstream effector molecules are set in motion and act to regulate various aspects of cardiac physiology. One important class of effectors are protein kinases which can phosphorylate target substrates in the cytosol and nuclear compartments and are differentially active in cardiac health and disease.

## 1.2.4. Protein kinases downstream GPCR activation

# 1.2.4.1 Protein kinase A (PKA)

Protein kinase A (PKA) or cAMP-dependent protein kinase is most often activated downstream the activation of  $G\alpha_s$ -coupled receptors. The activation of  $G\alpha_s$  triggers the activity of adenylyl cyclase (AC) which catalyses the production of cAMP from adenosine triphosphate (ATP). PKA is a tetrameric holoenzyme composed of two regulatory and two catalytic subunits [321]. Free cAMP can bind the regulatory subunits of PKA leading to a conformational change and subsequent dissociation of the catalytic subunits (*see* **Figure** 

**1.9A**). PKA is a serine threonine kinase and once activated can phosphorylate target substrates in the cytosol and nucleus. PKA activity is controlled by levels of cAMP in the cell which are regulated by the activity of ACs and phosphodiesterases (PDEs) which degrade cAMP where PDE2, PDE3 and PDE4 are well expressed in cardiomyocytes [322, 323]. Furthermore, PKA functions are also compartmentalized through association with AKAPs or A kinase anchoring proteins (discussed in [324]). PKA activation has been associated with the control of numerous homeostatic processes including cell growth, division and differentiation, metabolism, regulation of ion fluxes as well as gene transcription [321, 325]. In terms of metabolism, for instance, PKA can phosphorylate members of the glycogenesis pathway such as glycogen synthase. This PKA induced phosphorylation event impedes the conversion of glucose into glycogen thereby providing glucose molecules for the actively contracting heart muscle [325]. Besides metabolism, PKA also regulates gene transcription by phosphorylating transcription factors such as cAMP regulatory element binding proteins (CREB) modulating transcription in health and disease (see Figure 1.9B). PKA phosphorylates numerous substrate proteins that regulate cardiac contractility including but not limited to cardiac troponin I (cTnI), cardiac myosin binding protein C (cMyBPC), phospholamban (PLB), L-type calcium channel, the ryanodine receptor (RyR2) among others [325]. As PKA is involved in regulating various aspects of cardiac homeostasis, it comes as no surprise that dysregulation in PKA activity have been associated with heart pathologies.

To understand the role of PKA in the healthy versus diseased heart, various model systems have been used including animal models and end-stage human failing heart tissue. For instance, transgenic mice expressing the catalytic subunit of PKA have been observed to develop dilated cardiomyopathy [326]. These mice were also shown to have impaired contractile phenotypes, arrythmias and vulnerability to sudden death [326]. In a hamster model of genetic cardiomyopathy, PKA levels were shown to be elevated [327] whereas in human dilated cardiomyopathy, the expression of PKA regulatory subunits were observed to be decreased as well as basal PKA-dependent Tnl phosphorylation [328]. Yet, in the failing heart, PKA has been reported to hyperphosphorylate RyR2 receptors causing calcium to leak out of the sarcoplasmic reticulum, ultimately resulting

in diminished cardiac contractility [329-331]. Using tissue extracts from failing human myocardium, compromised SR function has been linked with a decrease in cAMP-dependent phosphorylation of phospholamban [332]. These results were echoed in another study where decreased calcium sensitivity of SERCA2a was associated with decreased cAMP-dependent phosphorylation of phospholamban in membrane preparations sourced from patients diagnosed with idiopathic dilated cardiomyopathy [333]. Morphological changes have also been demonstrated as cardiomyocytes lose their T-tubule networks in heart failure, an observation that may also affect cAMP and PKA compartmentalization [334-336]. In line with this, a proteomic study conducted using human patient and control heart tissue hinted that the compartmentalization of PKA may be altered in failing hearts due to differences in PKA association with AKAPs [337, 338]. Thus, studying cytosolic, nuclear and myofilament activation of PKA may provide insight on how kinase activity is altered in disease.

Aberrant PKA activity has also been correlated with cardiac hypertrophy. More specifically, PKA activation has been shown to result in CREB- or NFAT-mediated transcription of hypertrophic gene programs (reviewed in [339]). PKA has also been linked with cardiotoxic effects causes by chemotherapeutic agents including anthracyclines [340, 341]. The use of  $\beta$ -blockers have been shown to be helpful in these patients [342] as has levosimendan, a calcium sensitizer [343]. Evidence has suggested that levosimendan functions through a cAMP-PKA-PLN pathway as inhibition of PKA diminished the beneficial effects in the heart [343]. Differential PKA activities may also be sex-dependent and should be considered when assessing cardiac pathologies in men versus women [339, 344-349].



### Figure 1.9 Protein kinase A in the heart.

A) Activation mechanism of PKA, a tetrameric holoenzyme composed of two regulatory and two catalytic subunits. The binding of four cAMP molecules to two regulatory subunits catalyses the dissociation of the catalytic subunits. Phosphodiesterase can terminate the PKA activation cycle by decreasing the pools of cAMP thereby attenuating PKA activity. (B) Activation of Gas-couped GPCRs results in accumulation of cAMP that bind the regulatory subunits of PKA. Dissociated catalytic PKA subunits can then phosphorylate cytosolic and nuclear substrates including cytosolic substrates or transcription factors, influencing gene expression. Panel (A) is sourced from Moorthy et al. 2011 [350]. Figure panel (B) is sourced from London et al. 2020 [351], with permission.

## 1.2.4.2 Extracellular signal-regulated kinase (ERK)

Mitogen-activated protein kinases regulate cellular proliferation, differentiation, and survival, among other processes [352]. ERK1 and ERK2 are used interchangeably as they are similar in terms of sequence and functions and are also referred to as p44/42 as ERK1 is known as p44 MAPK while ERK2, p42 MAPK. Serial phosphorylation events result in the activation of ERK<sub>1/2</sub> beginning with the activation of the small GTPase Ras followed by the activation of Raf and MEK<sub>1/2</sub> (*see* **Figure 1.10A**). ERK<sub>1/2</sub> kinases are activated through tyrosine and threonine phosphorylation in their TEY domains. Hormones, growth factors, neurotransmitters are common activators of the ERK cascade. Both ERK<sub>1/2</sub> are ubiquitously expressed in numerous cell types and ERK signaling has

been well studied with effects ranging from the cell surface, to the cytosol and in the nucleus [353]. Under basal conditions, ERK remains in the cytosol [354] through interaction with MEK1 and other scaffolding proteins [355-357]. Phosphorylation of ERK<sub>1/2</sub> by MEK<sub>1/2</sub> leads to a conformational change that releases ERK from its scaffolds (see **Figure 1.10B**). Cytosolic ERK can interact with  $\beta$ -arrestin while G protein-mediated ERK activation has commonly been known to be associated with nuclear ERK translocation and transcription [358, 359]. Evidence serves to suggest that the activation of the ERK- $\beta$ -arrestin pathway is slow and long lasting (60 minutes) while G protein-ERK activation works on shorter timescales (discussed in [360, 361]).

In the cytoplasm, ERK has been reported to phosphorylate substrates involved in cell survival including proteins involved in the apoptosis pathway and proteins involved in regulation of the cell cycle. For example, ERK can phosphorylate and inhibit the proapoptotic BH3-only protein, BAD as seen in human ovarian cancer cell lines [362]. In neuronal PC12 cells, ERK has also been shown to phosphorylate the pro-apoptotic protein, Bim, rendering it inactive and conferring protection against cell death [363]. Similarly, the ERK cascade has been reported to interfere with caspase-9 apoptotic actions through phosphorylation, thereby promoting cell survival [364]. In cardiomyocyte cultures, a link has been suggested correlating ERK-mediated phosphorylation of GATA4 to cell survival effects [365, 366]. However, the cell survival roles of ERK in the heart have not been fully elucidated and further research is required.

In the myocardium, the effect of ERK is somewhat controversial as conflicting evidence has been reported in animal models. Canonically, ERK is often associated with a cardioprotective effect as it is thought to protect the heart from stress-induced events. Accordingly, ERK has been reported to enter the nucleus through interactions with importin 7, a nuclear pore protein, allowing it to phosphorylate transcription factors associated with cell proliferation and survival. Yet, in cardiomyocyte cultures, ERK<sub>1/2</sub> has been reported to regulate cell growth and result in cardiomyocyte hypertrophy [366, 367]. Further, the activity of ERK is regulated by cytosolic and nuclear phosphatases [368, 369].

Phosphatases dephosphorylate ERK<sub>1/2</sub> turning off the kinase activity and play a role in the maintenance of cellular homeostasis and well as in cardiac disease (reviewed in [370]). Dual-specificity phosphatases have been shown to be protective against cardiac hypertrophy [371-373]. In a mouse model, the overexpression of DUSP7 led to ERK<sub>1/2</sub> inhibition and led to a dilated cardiomyopathic phenotype [374]. Mutant versions of phosphatase 2A (PP2A) have also been associated with heart dilatation in mice [375]. In LMNA cardiomyopathy, the deletion of DUSP4 in mice demonstrated improved cardiac phenotypes through an ERK<sub>1/2</sub>-mediated mechanism [376]. These studies reveal the importance of ERK signaling for the maintenance of cardiac health yet inhibiting ERK may prevent cardiac hypertrophy. Altogether, ERK activity is nuanced and studies should transition to using hiPSC-CMs to further discern its role in the heart.



Figure 1.10 Activation of extracellular signal-regulated kinase (ERK).

(A) ERK activation cascade. Serial phosphorylation events, Raf, Ras, MEK result in the activation of ERK<sub>1/2</sub>. Signals coming from activated ERK influence cellular proliferation, migration, survival, and differentiation among other cellular outcomes. (B) Cytosolic ERK activity and nuclear translocation. ERK substrates are found in the cytosol as well in the nucleus. ERK is initially anchored in the cytosol by anchoring proteins. MEK phosphorylation of ERK releases it for its cytosolic anchors allowing ERK to translocate into the nucleus through interactions with importin7. Figure panel (A) figure is from Wen et al. 2022 [377] and panel (B) from Flores, Seger, 2013 [378], both with permission.

In failing human hearts with A-type nuclear lamin (LMNA) associated cardiomyopathy, increased phosphorylated ERK was observed by immunoblot [379]. Mice with this mutation were also shown to develop dilated cardiomyopathy [379]. Treatment with MEK<sub>1/2</sub> inhibitor, selumetinib prevented ERK<sub>1/2</sub> activation and diminished signs of fibrosis and improved heart function [379]. SOS1, son of sevenless homolog 1, mutations have also been correlated with the DCM phenotype along with an enhanced ERK<sub>1/2</sub> activation profile [380]. Relatedly, in a mouse model of DCM induced by gene deletion of caveolin-3, elevated ERK<sub>1/2</sub> phosphorylation was observed [381]. The loss of this gene may explain the hypertrophic and dilated phenotypes reported [381]. Different MAP kinases have also been shown to be differentially expressed in the failing heart [382]. For instance, in human DCM hearts, the use of phospho-specific antibodies revealed increased ERK<sub>1/2</sub> activation while p38 kinase was reduced and JNK was similar to control hearts [382]. Differences in the activation profiles of MAPKs and Akt have been reported in hypertrophic hearts compared to advanced heart failure [383]. Increased ERK signals were observed in failing compared to hypertrophic hearts [383]. These data suggest that therapies may need to be tailored based on the nature and progression of the disease moving from compensated towards decompensated phenotypes.

In terms of pharmacotherapies that target ERK<sub>1/2</sub> signaling, inhibiting the pathway may be beneficial as reported with selumetinib [379], however, tyrosine kinase inhibitors (TKIs) have also been shown to result in cardiomyopathic phenotypes [384, 385]. A balance may be required to fine tune the cardiac actions of ERK which have been demonstrated to be both beneficial and harmful depending on the intensity, location and longevity of the signals.

In summary, there are several GPCRs and protein kinases that regulate cardiac function, although here I focused on two that are of particular relevance to my thesis. Chronic and heightened stimulation by the very peptides and catecholamines that target these receptor systems and aberrant activation of downstream effectors such as protein kinases as described, eventually result in maladaptive cardiac remodelling and heart failure, discussed below.

#### 1.2.5 Heart failure

Heart failure (HF) is an ever-increasing burden in the Western world. Based on a 2016 report of the Canadian Heart and Stroke Foundation on the health of Canadians, it was estimated that 50,000 Canadians are diagnosed with heart failure every year. This number is currently on the rise with 600,000 Canadians currently living with heart failure. Based on these numbers, 1 in 2 Canadians and their families have been touched by heart failure. Patients living with heart failure present with dyspnea (shortness of breath), edema/fluid retention (peripheral swelling) and are easily fatigued and overexerted due to the inability of the heart to supply enough oxygenated blood to meet the body's demands. Over time, since the heart no longer adequately pumps oxygenated blood throughout the body, organ systems begin to fail causing the kidneys, liver and other essential organ systems to shut down. Advanced heart failure is a life-threatening condition and necessitates immediate medical intervention.

Heart failure is sometimes also referred to as congestive heart failure since the weakened heart is less efficient in pumping blood out of the left ventricle causing blood returning to the heart from the venous system to be retained in the lungs or the peripheral tissues [386]. This causes fluid retention and "congestion" in the lungs and explains why patients experience shortness of breath. Heart failure can occur suddenly or develop gradually overtime. Acute or *de novo* heart failure is defined as symptoms that occur in patients without a prior history of a cardiac condition [387]. For example, this may occur in direct consequence of a myocardial infarction (MI) [387]. After an MI, cardiomyocytes frequently undergo apoptosis due to a lack of oxygenation and the heart subsequently remodels in such a way that reduces the heart's pumping efficacy. On the other hand, hypertension or different types of cardiomyopathies increase the strain on the heart, resulting in maladaptive remodelling and over time this can cause heart failure. Heart failure is diagnosed through numerous medical tests. Clinicians begin by investigating the level of natriuretic peptides in the blood [388]. High levels of natriuretic peptides, ANP, BNP, are poor prognostic indicators for heart failure. Electrocardiograms (ECGs) are also performed. Most effectively, cardiac echocardiography is the best method to diagnose

heart failure. This test provides useful evidence on chamber volumes, ventricular systolic and diastolic function, wall thickness, valve function and pulmonary hypertension as explained in [388-391]. This data collection, used for diagnostic, disease tracking and therapeutic purposes is currently underused as deep within lies information that can improve patient care [392, 393], discussed in Chapter 5.

The pathophysiology resulting in heart failure can begin either in the left or right side of the heart. In the clinical setting, heart failure is classified based on the left ventricular ejection fraction (LVEF). There are three categories of heart failure which have been described to help inform physicians for diagnostic and therapeutic purposes as well as for clinical trial inclusion [394]. Heart failure can occur with reduced left ventricular ejection fraction (<40%), mid-range (between 41-49%) or preserved ejection fraction (≥50%) [388]. Diastolic heart failure has no effect on ejection fraction but rather the heart muscle is stiff and, in some cases, can thicken [395]. These structural features are most often associated with hypertrophic cardiomyopathy. In comparison, systolic heart failure often implies left heart failure with a reduction in the ejection fraction. The latter which is often associated with structural features such as enlargement or dilatation of the left ventricle. Dilated cardiomyopathy is a common cause of heart failure and is defined as systolic dysfunction with dilated left ventricle (reviewed in [396, 397]). Heart failure can also develop due to longstanding cardiac conditions that progress overtime due to maladaptive remodelling such as cardiac hypertrophy or other cardiomyopathies and are described below.



Figure 1.11 Features of a healthy versus diseased heart.

In response to chronic pathological stimuli such as enhanced neurohormonal stimulation or pressure/volume overload, the heart adapts and remodels its structure as means to maintain cardiac output. Pressure overload often results in concentric hypertrophy where sarcomeres are added in parallel resulting in a thicker muscle mass and narrower chamber. In contrast, volume overload has been associated with eccentric hypertrophy with sarcomeres being added in series resulting in lengthier cardiomyocytes as seen in dilated cardiomyopathy. Overall, these types of maladaptive remodelling weaken the heart and often progresses towards heart failure. The figure was created with BioRender.com.

## 1.2.5.1 Cardiac hypertrophy

In certain instances of heart failure, the heart is less effective at pumping blood and requires '*compensatory adaptation*' to accomplish its task. Since the heart is a plastic organ, it can remodel its architecture as means to compensate for reduced left ventricular function and cardiac output. One type of cardiac adaptation or remodelling implicates the enlargement or hypertrophy of individual cardiomyocytes. Since the overall purpose is to increase cardiac output, the heart increases mass through hypertrophy of individual cardiomyocytes to increase contractile force. The hypertrophied growth of cardiomyocytes can occur in two ways: eccentric or concentric growth. Eccentric hypertrophy refers to the addition of sarcomeres in series increasing the length of cardiomyocytes while concentric hypertrophy results in sarcomeres being added in parallel thus widening the cell. By adding sarcomeres, the basic contractile unit of the cardiomyocyte, it enhances the contractile force. These two types of hypertrophy are not mutually exclusive as concentric hypertrophy can become eccentric [398]. Depending on the stress or injury, the overall heart structure and geometry can remodel in distinct ways. For instance, concentric hypertrophy is associated with thickened wall mass whereas eccentric hypertrophy often leads to dilated left ventricle (*see* **Figure 1.11**).

However, cardiac hypertrophy is not necessarily always associated with cardiac pathologies. Cardiac hypertrophy is initially an adaptive response to increased workload or stress. In essence, the heart has one task; to supply blood to the rest of our body. Thus, the heart will pump more frequently or with greater force if need be. Evidently, the heart needs to work harder if an individual is jogging as opposed leisure walking. Thus, the heart adapts to our daily and routine needs by pumping blood faster or slower to meet our demands. When exercising, the heart needs to pump more oxygenated blood to the actively contracting skeletal muscles, increasing its workload. Continuous and rigorous exercise results in a hypertrophied heart and this is frequently seen in athletes. This is referred to as physiological hypertrophy which is reversible and is also observed in pregnant women [399, 400]. However, the heart can also hypertrophy in result to chronic pathophysiological stimuli commonly seen in heart failure patients. Adverse cardiac events include pressure or volume overload which commonly result in the hypertrophied phenotype. Pressure overload occurs in instances of hypertension and is often associated with concentric hypertrophy. Volume overload because of mitral or aortic valve insufficiency tends to result in eccentric hypertrophy [401].

Owing to the complex nature of the molecular mechanisms underlying pathological cardiac hypertrophy, they are still the subject of active investigations. Accumulated evidence has demonstrated altered calcium handling, a switch towards fetal gene expression programs, dysregulated mitochondrial function, and metabolism as well as the occurrence of fibrosis and apoptosis (reviewed in [402]). For example, intracellular

calcium transients in hypertrophied hearts have been demonstrated to be prolonged in various animal [403, 404] and human models [405]. Calcium handling has been shown to be irregular in dilated cardiomyopathy hearts [406]. In part, abnormal calcium signaling has been attributed to reduced expression of SR proteins such as ryanodine receptors and SERCA2A [407-409]. A hallmark of cardiac hypertrophy is the reactivation of fetal gene expression programs [410]. Myosin isoforms, particularly an increase in  $\beta$ -MHC (neonatal) and decrease in  $\alpha$ -MHC (adult) has been reported in hypertrophy and heart failure. Titin isoforms have also been demonstrated to be altered in heart failure. The neonatal and more compliant N2BA isoform is more abundant in the hypertrophied heart compared to the stiffer adult N2B isoform [411, 412]. An increase in natriuretic peptides, NPPA and NPPB, are also key features of the hypertrophied heart [413]. Cardiac hypertrophy has been determined to involve the dysregulation of several core signaling pathways including G proteins [414], MAPKs, ERK<sub>1/2</sub> [415], p38, JNK, NFAT-Ca<sup>2+</sup>, AKT or Protein kinase B, PKA and PKC (reviewed in [416]). Transgenic mice expressing MEK, the upstream kinase in ERK<sub>1/2</sub> cascade have been observed to develop cardiac hypertrophy [417]. The activation of ERK<sub>1/2</sub> has also been shown to be an important cell survival signal in hypertrophy and heart failure [418, 419]. To note, there is conflicting data in the literature as ERK<sub>1/2</sub> has been reported to be a negative and positive regulator in the heart. More research is required to understand the exact role of ERK in the healthy, hypertrophied and failing hearts.

Some forms of hypertrophy are inherited due to genetic mutations. These cases often fall under the umbrella of hypertrophic cardiomyopathy (HCM) and are examined below along with other forms of cardiomyopathies.

## 1.2.5.2 Cardiomyopathies

Cardiomyopathies are diseases that affect the heart muscle. There are five different types of cardiomyopathies depending on how the disease manifests itself in terms of changes in the heart's architecture and function. These classes are as follows; dilated cardiomyopathy (DCM), hypertrophic cardiomyopathy (HCM), restrictive cardiomyopathy (RCM), arrhythmogenic cardiomyopathy (AC) and unclassified which includes Takotsubo cardiomyopathy. In all five instances of the disease, the heart muscle remodels in attempt to compensate for reduced cardiac output where the heart muscle can either dilate, thicken or become more rigid. Besides these five *general* classes, there are also other types of cardiomyopathies such as left ventricular non-compaction (LVNC) [420], or cardiomyopathy induced by chemotherapeutic agents such as anthracyclines [421, 422]. Cardiomyopathies can be acquired or inherited and affect children as well as adults. Cardiomyopathies can manifest as the primary driver of disease, but it can also occur as secondary phenotypes to other diseases. For instance, in Duchenne muscular dystrophy, some patients develop dilated cardiomyopathy due to mutations or deletions in the dystrophin gene. Dilated cardiomyopathy is of particular importance in this thesis and will be further discussed, in detail, below.

### 1.2.5.2.1 Dilated cardiomyopathy

Dilated cardiomyopathy is clinically defined as systolic dysfunction with dilated left ventricle in absence of ischemia or coronary artery disease [423]. Most patients diagnosed with DCM have a left ventricular ejection fraction less than 40%. Cardiac output and stroke volume are thus reduced in DCM. The disease is also accompanied by diminished ventricular filling and a rise in end-diastolic pressure [396]. This leads to a dilated phenotype as there is more blood in the ventricle prior to contraction [396]. In dilated cardiomyopathy, the heart stretches and thins decreasing cardiac performance [424]. In this case, sarcomeres are added in series, lengthening individual cardiomyocytes and the left ventricle also increases in size. The precise mechanism of dilation has yet to be fully elucidated but may be caused by cardiomyocyte apoptosis as well as increased blood volume in the left ventricle. In DCM, the heart attempts to compensate for reduced cardiac output by activating the sympathetic nervous system, releasing catecholamines such as epinephrine and norepinephrine [424]. Norepinephrine stimulation can also result in vasoconstriction which increases preload and afterload. Acute compensation is benign however if the adrenergic system is chronically in overdrive, adverse remodelling occurs and can lead to the dilation as well as hypertrophy

of the left ventricle [424]. Even if the underlying etiology may not be known, the mechanism of wall stretching, thinning and compensation has been speculated to follow the Frank and Starling mechanism. Briefly, in the late 19<sup>th</sup> century, seminal discoveries from Otto Frank and Ernest Starling revealed that an increase in pressure in the left ventricle caused an increase in contractile force and stroke volume, up to a certain point [425, 426] (see Figure 1.12). This increase in filling pressure in the left ventricle at the end of diastole is known as preload. During preload, cardiomyocytes are stretched allowing for left ventricular filling, prior to contraction. Afterload is the pressure that the heart needs to pump against to eject blood during systole. For efficient cardiac contraction, there is a precise relationship between the tension and length of sarcomeres, the individual units responsible for contractions determining the force they can emit [427]. Intuitively, if the muscle fiber is too short or too long, it results in suboptimal force generation [425, 427]. With short sarcomere lengths, there is a decrease in Ca<sup>2+</sup> sensitivity while excessively long sarcomeres lead to a decrease in overlap between thick and thin myofilaments [428]. In both these instances, cardiac contraction is suboptimal (see Figure 1.12C, D). In systolic dysfunction, as seen in DCM, sarcomeres are too extended resulting in a reduction in cardiac contractility and a subsequent decrease in cardiac output. This is essentially visualized by a downshift of the Frank-Starling curve where stroke volume is decrease due to an increase in ventricle end diastolic volume [427].



# Figure 1.12 *Mechanisms and phenotypes of DCM and heart failure.*

(A)Compensatory and decompensatory mechanisms in the heart. In pathophysiological conditions the heart attempts to compensate for the reduced cardiac output through innervation of the sympathetic nervous system. Secretion of epinephrine and norepinephrine innervate adrenergic receptors leading to an increase in heart rate and enhanced cardiac output. Chronic sympathetic input eventually becomes decompensatory resulting in adverse remodelling of the heart muscle including chamber dilatation and hypertrophy. (B) Remodelling of the heart in DCM. Maladaptive ventricular remodelling leads to a dilated ventricle as a consequence of eccentric growth. sarcomeres being added in series, widening the chamber volume. (C) The Frank Starling mechanism depicting left ventricular performance in normal versus diseased hearts. In the normal heart, cardiac output increases as preload or ventricular end-diastolic volume increases. (D) In the failing heart, cardiac output decreases for a given preload as is related to the stretch of sarcomeres [426]. Essentially, in DCM, sarcomeres are over-stretched which leads to reduced contractility and consequent cardiac output. The same is true if sarcomeres are too short. Thus, there is an optimal relationship, described by the Frank Starling Law that states that optimal sarcomeres stretch, which is related to preload, the volume of the LV before contraction, results in optimal cardiac output. Figure adapted from [427] and [424] and created with BioRender.com.

### 1.2.5.2.1.2 Prevalence and etiology of dilated cardiomyopathy

Dilated cardiomyopathy is estimated to affect 1 in 250 individuals and the majority end up progressing towards heart failure [429]. DCM is often diagnosed late as some individuals are asymptomatic [430]. DCM is not necessarily an 'old age' condition as there are several pediatric forms of the disease. As it happens, DCM is the most common cause of pediatric cardiomyopathy [431]. Pediatric DCM is also the most common disease requiring a heart transplant [432]. Generally, there are several triggers that can lead to the development of dilated cardiomyopathy including genetic, environmental, inflammatory, infection (viral or bacterial), connective tissues disorders, etc. (see **Table 1.4**). It is estimated that 50% of DCM cases are clinically labelled as idiopathic with no easily identifiable underlying cause. This is based on the patient's family history as the patient must be the first person in their family to be diagnosed with DCM for it to be regarded as idiopathic. However, this may simply be an overestimation owing to inadequate genetic testing. Genome or exome sequencing is costly thus not readily accessible. In the province of Quebec, for instance, genetic testing is only covered if another family member has already been diagnosed with dilated cardiomyopathy.

When DCM is inherited, it is referred to as familial DCM defined when two or more family members are diagnosed with the disease [433, 434]. Familial DCM represents approximately 30-50% of the DCM cases. The main drivers of familial DCM are genetic defects in sarcomere-related proteins. Mutations have been identified in titin (TTN), troponin T (TNNT), myosin heavy chain 7 (MYH7), Lamin A/C (LMNA), RNA-binding motif 20 (RBM20) among several other genes [435]. Drugs can also result in dilated cardiomyopathy including antineoplastic drugs (doxorubicin, tyrosine kinase inhibitors, etc.) and psychiatric drugs (tricyclic antidepressants, lithium etc.). DCM can also manifest itself due to excess alcohol exposure, cocaine use, or exposure to toxins such as arsenic and cobalt. Nutritional deficiencies have also been reported to be linked with the occurrence of DCM. For a complete list of potential causes of DCM, refer to **Table 1.4**.

**Table 1.4** List of etiologies that have been associated with the development of dilated cardiomyopathy. Table adapted from Pinto et al 2016 [436], Schultheiss et al 2019 [396] and Seferović et al. 2019 [397], with permission.

Familial	Titin (TTN), Troponin T (TNNT2), Myosin heavy chain (MYH7), Lamin A/C (LMNA), RNA-binding Motif-20 (RBM20), Myopalladin (MYPN), Sodium channel alpha unit (SCN5A), BaCl2-associated athanogene 3 (BAG3), Phospholamban (PLN), Troponin C, slow skeletal and cardiac muscles (TNNC1), Troponin I, cardiac muscle (TNNI3), Tropomyosin-α1 chain (TPM1) and Filamin C (FLNC).		
Neuromuscular	Duchenne muscular dystrophy (DMD), Becker muscular		
disorders	dystrophy (BMD), Myotonic dystrophy or Steinert (MD).		
Syndromic diseases	Mitochondrial diseases, Tatazin (TAZ/G4.5).		
Drugs Toxic and overload Nutritional deficiency	Antineoplastic drugs (Anthracyclines; antimetabolites; alkylating agents; Taxol; hypomethylating agent; monoclonal antibodies; tyrosine kinase inhibitors; immunomodulating agents); Psychiatric drugs (Clozapine, olanzapine; chlorpromazine, risperidone, lithium; methylphenidate; tricyclic antidepressants); Other drugs (Chloroquine; all-trans retinoic acid; antiretroviral agents; phenothiazines). Ethanol, Cocaine, amphetamines, ecstasy, Other toxins (Arsenic; cobalt; anabolic/androgenic steroids), Iron overload (Transfusions; haemochromatosis). Selenium deficiency, Thiamine deficiency (Beri-Beri), Zinc and copper deficiency,		
	Carnitine deficiency.		
Electrolyte disturbance	Hypocalcemia, nypopnosphatemia.		
Endocrinology	Phaeocromocytoma, Acromegaly, Diabetes mellitus.		
Infection	Viral (including HIV), bacterial (including Lyme disease), mycobacterial, fungal, parasitic (Chagas disease).		
Auto-immune diseases	Organ-specific (Giant-cell myocarditis (GCM), Inflammatory DCM) Not organ specific (Polymyositis/dermatomyositis; Churg–Strauss syndrome; Wegener's granulomatosis; systemic lupus erythematosus, sarcoidosis).		
Peripartum	HFrEF occurring during late pregnancy or months following childbirth.		
#### 1.2.5.2.1.3 Sex differences in dilated cardiomyopathy and heart failure

With time, a greater appreciation has developed concerning the fact that men and women are differentially susceptible to heart failure and DCM and experience the diseases differently. Considering that some cardiomyopathies are X-linked recessive, men are more greatly affected. These include Duchenne muscular dystrophy, Dannon's, Becker's and Fabry's diseases [437, 438]. Familial DCM is commonly inherited in an autosomal dominant fashion, suggesting that, at baseline, there should not be sexspecific differences [437]. However, men carrying mutations in the titin gene [439] or the lamin A/C gene [440] have been reported to encounter adverse events earlier than women and with a worse prognosis, respectively. Similarly, men diagnosed with DCM have worse long-term prognosis than women [441, 442]. Also, hypertrophic, and dilated cardiomyopathy manifest more often in men than women. In idiopathic DCM, women have been reported to have better outcomes [443]. Sex differences have also been observed in long QT syndrome which has been linked to sex hormones [444, 445]. For excellent reviews examining sex differences in heart disease, readers are redirected to the following [437, 446, 447].

Furthermore, in the human heart, men have been reported to have enhanced cardiomyocyte loss due to aging compared to women [448, 449]. Cardiomyocyte death measured as necrosis and apoptosis was shown to be reduced in females [449]. In parallel, similar observations have been seen in animal models. For example, in a cardiac injury model, the control of pro- and anti-apoptotic proteins has been shown to be different, with higher levels of pro-apoptotic proteins in males compared to female rats [450]. Further, cardiac remodelling has been observed to be different in men versus women [451, 452]. In instances of aortic valve stenosis often due to pressure overload, women displayed concentric hypertrophy with reduced fibrosis and recovered faster than men after surgery [453, 454]. Moreover, there are differences between men and women at the molecular level that may explain why certain cardiovascular diseases manifest differently in men and women in the clinic. For instance, sex differences have been reported in terms of calcium handling in mice [347], rat [455, 456] as well as in rabbit

hearts [457]. In mice, the estrous cycle has also been shown to influence cardiac contractility and calcium dynamics [458]. Similar findings have been found with testosterone [459, 460]. Relatedly, male cardiomyocytes have been observed to have higher  $\beta$ -adrenergic densities compared to females [456]. Beyond cardiomyocytes, sex-differences have also been reported in endothelial cells for instance [461].

Regarding the pharmacotherapy of heart disease, sex-differences have also been reported. The pharmacological management of cardiac conditions is not as well tolerated in women compared to men [462-464]. This may be correlated with the underrepresentation of women in clinical trials and the reality that middle-aged men are overrepresented (see Table 1.5). This pushed several groups to re-evaluate the data collected in clinical trials and assess outcomes on the basis of sex (see Table 1.5). In a post-hoc analysis, digoxin therapy has been demonstrated to lead to a higher risk of mortality in women compared to men [465]. When re-examining the data of the PARAGON-HF trial, it was revealed that women may have beneficial outcomes when prescribed sacubitril-valsartan compared to men with heart failure with preserved ejection fraction [466]. In a secondary analysis of the CIBIS II trial, women were shown to have reduced all-cause mortality when treated with bisoprolol compared to men [467]. Drugs dosage may also need to be titrated to different optimal doses dependent on biological sex. A post-hoc assessment of the BIOSTAT-CHF study demonstrated that optimal doses of  $\beta$ -blockers and angiotensin inhibitors are typically lower for women than those for men [468]. Several of these differences may be attributed to pharmacokinetics and pharmacodynamics parameters between sexes [469]. Women may benefit from lower doses of CYP2D6-dependent β-blockers as they experience more adverse reactions when taking these drugs [470]. The undesired outcomes are due to pharmacokinetics as women metabolize these drugs quicker than men resulting in higher plasma concentrations of the drug [470]. CYP2D6 dependent β-blockers include carvedilol, metoprolol, nebivolol and propranolol while atenolol, sotalol, and bisoprolol are CYP2D6independent. Since there are contradictory results, the scientific community needs to better control for biological sex when designing and conducting clinical trials for heart failure medications. One review article highlighted that 23 clinical trials revealed to have

sex differences, underscoring the urgent need to consider sex in clinical trials but also in basic science research [447]. *Individualizing* the dose may offer better patient outcomes as opposed to identifying an optimal population-wide dose. Overall, it is quite evident that better therapeutic approaches are required. Below we briefly examine current pharmacotherapies that are available to heart failure and DCM patients.

Drug class	Study	Drug	Published (year)	LVEF	% women	No. of women	Primary endpoint	Sex-specific outcome
ACE-I	SOLVD- Treatment	Enalapril	1991	≤35%	20	505	Mortality	Significant benefit in men, trend towards benefit in women
	CONSENSUS	Enalapril	1987	≤35%	30	74	Mortality	Significant benefit in men, not in women
ARB	CHARM	Candesartan	2004	≤40%	26	1188	Cardiovascular death or HF hospitalization	No sex difference in primary endpoint, p for interaction 0.95
	Val-HeFT	Valsartan	2001	<40%	20	1003	Mortality or HF hospitalization/ED presentation	Significant benefit in men, trend towards benefit in women
B-blocker	CIBIS II	Bisoprolol	1999	≤35%	19	515	Mortality	Significant benefit in men and women
	COPERNICUS	Carvedilol	2001	<25%	20	465	Mortality	Significant benefit in men, trend towards benefit in women
	MERIT-HF	Metoprolol	1999	≤40%	23	898	Mortality or all-cause hospitalization	Significant benefit in men, not in women
	SENIORS	Nebivolol	2005	≤35%	37	785	Mortality or cardiovascular hospital admission	HR 0.93 (0.78- 1.11) in men HR 0.72 (0.55- 0.93) in women p for interaction 0.11
	U.S. Carvedilol HF	Carvedilol	1996	≤35%	23	256	Mortality	HR 0.41 (0.22- 0.80) in men

 Table 1.5 Perceived sex-differences in HFrEF clinical trials.

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MRA	RALES	Spironolactone	1999	≤35%	27	446	Mortality	HR 0.23 (0.07- 0.69) in women No sex difference in primary endpoint No sex
	EMPHASIS-HF	Eplerenone	2011	≤35%	22	610	Cardiovascular death or HF hospitalization	difference in primary endpoint, p for interaction 0.36
ARNI	PARADIGM-HF	Sacubitril/ valsartan	2014	≤40%	22	1832	Cardiovascular death or HF hospitalization	No sex difference in primary endpoint, p for interaction 0.63 HR 0.84 (0.76-
lvabradine	SHIFT	Ivabradine	2010	≤35%	24	1535	Cardiovascular death or HF hospitalization	0.70- 0.94) in men HR 0.74 (0.60- 0.91) in women p for interaction p=0.26

Table sourced from Carolyn S P Lam, Clare Arnott, Anna L Beale, Chanchal Chandramouli, Denise Hilfiker-Kleiner, David M Kaye, Bonnie Ky, Bernadet T Santema, Karen Sliwa, Adriaan A Voors, Sex differences in heart failure, *European Heart Journal*, Volume 40, Issue 47, 14 December 2019, Pages 3859– 3868c, <u>https://doi.org/10.1093/eurheartj/ehz835</u> [464].

#### 1.2.5.3 Pharmacotherapy for dilated cardiomyopathy and heart failure

There is no magic bullet when managing dilated cardiomyopathy or heart failure. Combination approaches are common, and patients often need to be switched to several different drug regimens before finding the combination that is best suited for their condition [471]. By definition a "foundational therapy" for patients diagnosed with heart failure associated with reduced ejection fraction is one that has been demonstrated to decrease cardiovascular mortality and also decrease the risk of hospitalization associated with heart failure in large scale clinical trials [472]. It has been reported that physicians currently prescribe drugs based on the sequence published in clinical trials. Based on this approach, angiotensin receptor blockers or ACE inhibitors are generally prescribed first, followed by  $\beta$ -blockers and mineralocorticoid antagonists. Finally, a neprilysin inhibitor is prescribed, followed by an SGLT2 inhibitor. This approach is not based on personalised or rational patient observations, yet as discussed by John McMurray and Milton Packer,

it presumes that the first drug developed is the most efficacious for all patients with reduced ejection fraction [472, 473]. Physicians are also instructed to titrate the dose until they reach the target dose. If the first drug class fails to improve patient symptoms, then the patient is switched to the next drug class. If a given patient were to respond to the more recent marketed drug class, then it is estimated that it would take 6 months to arrive to this class. This approach most likely results in needless hospitalizations. Since this is scientifically unfounded, a new sequence of foundational drugs has been proposed in the literature. This new therapeutic strategy is, in part, based by the notion that beneficial patient outcomes are seen within a 30-day period thus patients should receive treatment from all four classes within the first month post-consultation. It has been proposed that it is unnecessary to titrate drugs until the target dose is reached. In theory, low doses of each drug are clinically efficient and different drug classes should be prioritized in contrast to reaching the target dose of each drug class. Also, it has been argued that since patients who were enrolled in clinical trials for the  $\beta$ -blockers and ACE inhibitors, were prescribed digoxin, this drug is no longer being prescribed even if it has been historically been used for the treatment of heart failure [472]. The "accelerate new approach" recommends patients to first be co-prescribed  $\beta$ -blockers as well as a sodium–glucose co-transporter 2 (SGLT2) inhibitor [472, 473]. One to two weeks later, patients can then receive sacubitril/valsartan, followed by mineralocorticoid receptor antagonist another one to two weeks later. These decision should be based on drug efficacy, safety and ease-of-use as opposed to clinical trial dates [472]. However, considering that this "accelerate new approach" favours newer drugs, it is difficult to know if this suggested new approach is truly founded by science or if it is confounded as a result of ties with pharmaceutical industries trying to push "newer" drugs onto the market. Several drugs prescribed to DCM patients have been discussed in earlier sections as they target GPCRs. Below, non-GPCR targeted pharmacological treatment strategies are discussed.

#### 1.2.5.3.1 Mineralocorticoid receptor antagonists

Mineralocorticoid receptor antagonists (MRAs) like spironolactone are prescribed to HF and DCM patients. As described above, angiotensin II results in the secretion of aldosterone which binds mineralocorticoid receptors in the kidneys promoting Na<sup>+</sup> and water reabsorption resulting in water retention, thereby increasing blood volume and pressure [474]. Elevated aldosterone levels are detrimental to the heart and can cause maladaptive cardiac remodelling including hypertrophy, fibrosis and apoptosis [475]. The administration of mineralocorticoid receptor antagonists have been shown to improve the survival of HF patients in clinical trials [476, 477]. Spironlactone is effective at reducing mortality in HF with reduced ejection fraction, but less benefit is seen in patients with preserved ejection fraction [478, 479]. Mineralocorticoid antagonists have also been shown to be beneficial to DCM patient populations [480]. With improved patient outcomes, spironolactone withdrawal should be evaluated on a case-by-case basis as evidence serves to suggest that patients will relapse if they stop taking the medication [481].

# 1.2.5.3.2 Sodium-glucose co-transporter 2 (SGLT2) inhibitors

Since heart disease is often associated with co-morbidities including diabetes mellitus (DM), certain therapies have been designed to address glucose related issues observed in heart failure patients [482]. This particular pharmacotherapy acts to lower high glucose levels via inhibition of the sodium glucose co-transporter 2. The SGLT-2 transporter is found in the renal tubules of the kidneys and functions to reabsorb glucose back into the blood stream (reviewed in [483]). This glucose-lowering drug works independent from insulin while lessening glucose blood levels. DAPA-HF [484] and EMPEROR-Reduced [485] trials have shown positive outcomes of SGLT2 inhibitor therapy for heart failure reducing mortality rates [486]. In addition, a meta-analysis examining 15 clinical trials, revealed that SGLT2 inhibitors reduce all-cause and cardiovascular mortality providing substantial benefit for heart failure patients [487]. Remarkably, this drug class may also be beneficial to patients without diabetes. As such,

several mechanisms of actions have been proposed. For instance, evidence suggests that this drug class may improve ventricular filling, reduce fibrosis and remodelling, improve endothelial function among other hypotheses [488-493].

# 1.2.5.3.3 IF current inhibitors

The funny (f) channel is present in cardiomyocytes of the sino-atrial (SA) node, often referred to as pacemaker cells which are specialized cells responsible for maintaining cardiac rhythm. The funny channel is a member of the hyperpolarization-activated cyclic nucleotide-gated (HCN) ion channel family that permits the inward flow of sodium and outward flow of potassium ions [494]. By doing so, this channel regulates the spontaneous diastolic depolarization in the SA node thereby controlling heart rate [495]. Essentially the ion channel adopts an open configuration at hyperpolarized potentials when cAMP is bound [496]. Pharmacological agents that inhibit current through this channel results in prolonged diastolic depolarization thus slowing down heart rate and are classified as negative chronotropic drugs. Ivabradine is a first in class agent that acts to inhibit the funny channel. Inhibiting the funny channel slows down heart rate without affecting cardiac contractility.

Initial clinical trials did not show benefit in patients with coronary artery disease and left-ventricular systolic dysfunction. Interestingly, in this randomized, double-blind placebo-controlled study, the investigators noticed that there may be a clinical benefit in patient with heart rate 70 beats per minute or higher. This prompted another trial, SHIFT-HF where ivabradine was shown to reduce the risk of mortality and hospitalizations in patients with heart rate greater than 70 bpm [497]. In addition, supplementing standard care with a funny (I<sub>f</sub>) channel inhibitor has been shown to improve the quality of life of patients diagnosed with DCM [498, 499]. This therapy has also been tested in children with DCM showing safety albeit it was also mentioned that titrating the dose is important to achieve an optimal dose-response relationship [500].

#### 1.2.5.3.4 Diuretics

Diuretics are often used in heart failure to treat water retention and pulmonary and/or peripheral edema. There are multiple classes of diuretics based on their mode of action and are reviewed elsewhere [501]. Diuretics help the body clear itself of excess water/fluid and work at the level of the kidneys by releasing sodium. This, in turn, lowers blood volume and blood pressure. In congestive heart failure, the use of diuretics often helps patients' breath better by removing fluid in patient lungs and chest area. Diuretics are often prescribed with ACE inhibitors or  $\beta$ -blockers as alone, they are not sufficient to significantly improve patient outcomes. Several clinical trials have shown that diuretics decrease patient hospitalizations and mortality [502].

#### 1.2.5.3.5 Digoxin

Digoxin is a member of the cardiac glycoside family. Digoxin is a "modern-day" derivative of digitalis, the oldest known agent to treat heart failure, initially reported in 1785 [503-505]. Mechanistically, digoxin acts by inhibiting the Na<sup>+</sup>-K<sup>+</sup> ATPase. Inhibiting this pump prevents sodium ions from leaving the cell thus increasing the amount of intracellular sodium. With increased Na<sup>+</sup>, this alters the electrochemical gradient, and affects the Na<sup>+</sup>/Ca<sup>2+</sup> exchanger. Essentially, the elevated increase in cytosolic sodium reduces the efflux of calcium, thereby increasing cytosolic calcium levels and increasing cardiac contractility. Digoxin also slows down the heart rate through actions on the AV node and lowers plasma norepinephrine levels [506, 507]. In the Digoxin Investigation Group, digoxin was shown to reduce the hospitalization of HF patients with reduced ejection fraction but had no effect on mortality [508]. Nowadays, digoxin is prescribed if DCM manifests with irregular heart rhythm such as atrial fibrillation.

Currently, there are several drug classes that can be prescribed to help patients suffering from dilated cardiomyopathy and heart failure, lead a better quality of life. However, with important sex differences highlighted above and other adverse effects associated with the use of heart failure medications, improved therapies are still necessary. To accomplish this, drug discovery efforts need better model systems to test candidate drugs. Below, various model systems are introduced followed by a discussion of how human stem cells and their derivatives ought to aid in the quest to develop better, more personalized, therapeutic regimens for DCM.

### 1.3 Cellular models to study GPCR conformation and function (in the heart)

#### 1.3.1 The HEK 293 cell and other heterologous systems

For years, the prototypical human embryonic kidney (HEK) 293 cell has been the cell of choice when studying GPCR biology. These cells were generated in the early 1970's and are still routinely used four decades later [509]. These cells were generated by integration of adenoviral DNA into chromosome 19 which effectively altered the normal cell cycle within these cells rendering them indefinitely proliferative [510-512]. Considering that there is some uncertainty of the origin of these cells, derived either from kidney, adrenal or neural sources, these cells are not used as a model of kidney cells per se but are instead viewed as heterologous expression systems that can be used to carry out experimental studies. For instance, HEK 293 cells have been used to study GPCR ontogeny, signal transduction, protein-protein interactions among several other biological phenomena. HEK 293 cells endogenously express several GPCRs and signaling effectors making them ideal vehicles to study GPCR biology in vitro. For example, HEK 293 cells express the mRNA of numerous Class A GPCRs such as adrenergic, acetylcholine, adenosine, chemokine, endothelin, dopamine and serotonin receptors, to name a few [513]. In parallel, they also express many members of the GPCR signaling machinery such as  $G\alpha\beta\gamma$  subunits, adenylyl cyclases, GRKs, PKA, PKC as well as  $\beta$ arrestins [513]. If the receptor under investigation is not expressed endogenously in HEK 293 cells, it is possible to overexpress the receptor by transfecting plasmid DNA that encodes the receptor. For a more detailed overview of immortalized cells like the HEK 293 cell, readers are directed to the following reviews [95, 514].

HEK 293 cells have also been combined with CRISPR-Cas9 technology to better understand how certain genes function in a '*human*' context. Of particular interest, one laboratory has generated several useful cellular tools to better comprehend GPCR signaling using CRISPR-Cas9 mediated deletion of various G $\alpha$  isoforms or  $\beta$ -arrestin proteins [515, 516]. These cells have been extensively used to better inform how G proteins or arrestins influence signaling cascades especially when trying to address questions related to biased signaling. There are also different variants of the HEK 293 cells that have been engineered [517]. For instance, HEK 293 T cells have an SV40 origin of replication thus they produce high copy numbers of plasmid DNA when transfected with vectors containing the SV40 origin site [518]. These cells are commonly used as vehicle to generate viruses such as lenti-virus or adeno-associated viruses for *in vitro* or *in vivo* research use. Spiking HEK cells, stably expressing NaV 1.3 and KIR 2.1, have also been published as vehicle to optimize voltage-based biosensors demonstrating their use for biosensor validation studies [519, 520].

Normal, healthy human cells carry 23 pairs of chromosomes for a total of 46 whereas some HEK 293 cell lines have been reported to have 64 chromosomes. Thus, the genome of HEK 293 cells is karyotypically abnormal [521]. Different karyotype analyses have documented different numbers of chromosomes ranging from 56 to 78, thus HEK 293 cells are aneuploid: presenting with missing or extra chromosomes [522-524]. Over time, with bi-weekly passages, HEK 293 cells tend to develop more karyotypic abnormalities and two HEK 293 cells passaged in different laboratories may drift and diverge into distinct cell lines. To address this and ensure labs are using good quality cells in research, guidelines have been published to ensure uniformity between research programs [525]. However, even if *H*EK 293 cells begin with the word *human*, it is unlikely that they reflect human biology, in all its complexities.

Generally, HEK 293 cells are used to study common biological processes and are suboptimal for studies of human disease as they lack physiological and disease relevance. To address this, researchers have transfected mutated receptors and have generated gene knockouts or knock-ins as means to model various human diseases. However, this is not ideal as diseases are often multifaceted. Not to mention, the study of idiopathic disease is not possible in these contexts. Further, HEK 293 cells do not express T-tubules, a well-organized sarcoplasmic reticulum or proteins of the contractile machinery and present with significant limitations when interested in studying human heart disorders. Besides, the HEK 293 cell, HeLa [526, 527] and COS-7 cells are other examples of heterologous cell systems. Neither of these cells can accurately model the human heart, thus alternatives were developed and are discussed below.

# 1.3.2 Immortalized cardiomyocyte cell lines

To advance one step closer to modelling the human myocardium, immortalized cardiomyocytes have been established for in vitro research applications. These are attractive models as they are proliferative in nature and are thus easier to maintain than primary cardiomyocytes derived from neonatal or adult rodents. These cell lines have been derived from both rodent and human cardiomyocyte sources. For instance, HL-1 cells were derived from an AT-1 atrial cardiomyocyte tumor lineage excised from an adult female Jackson Laboratory-inbred C57BL/6J mouse [528]. These cells can be maintained in culture for long periods of time and routinely passaged. HL-1 cells are also contractile and spontaneously beat in culture. HL-1 cells have been used to model cardiomyocyte hypertrophy as well as atrial fibrillation [529]. H9C2 cells represent another immortalized cell line derived from embryonic rat ventricular tissue [530]. Unlike HL-1 cells, they do not beat but retain certain aspects of their cardiomyocyte identity. H9C2 cells are initially proliferative and the addition of all-trans-retinoic acid with low serum concentrations has been shown to further differentiate them into a cardiac-like phenotype [531]. H9C2 cells express relevant cardiac markers such as sarcomeric proteins and calcium channels. Similarly, atrial conditionally immortalized cardiomyocyte cell lines have also been established. For example, neonatal Wistar rat atrial cardiomyocytes, iAM-1 cells, have been immortalized and with slight alterations to the culture media, either the addition or removal of doxycycline, these cells can transition from a proliferative to a contractile nature [532]. They are excitable cells and can be used to model atrial fibrillation as well as other electrophysiological properties within cardiomyocytes in an *in vitro* setting.

An alternative to using rodent-derived lines is the AC16 cell which represents a human cardiomyocyte immortalized cell line [533]. AC16 cells were derived from human ventricular tissue and were fused with SV40 transformed, uridine auxotroph human fibroblasts, devoid of mitochondrial DNA [533]. These cells express relevant cardiac transcription factors as well as contractile and gap junction proteins. AC16 cells can be cryopreserved, thawed and can proliferate to provide an indefinite source of *humanized* cardiac cells. AC16 cells can be further differentiated by switching media formulations rending them more quiescent in nature, a phenotype shared with adult human cardiomyocytes. These cells respond to GPCR agonists and have been shown to recapitulate aspects of the hypertrophic phenotype in response to known inducers.

# 1.3.3 Primary rodent-derived cardiomyocytes & large mammalian animal models

Echoing once more that immortalized cell lines lack certain physiological aspects, especially considering their proliferative nature while attempting to mimic terminally differentiated cells, primary cells isolated from rodents have been used extensively. It has been almost 60 years since the first report of cardiomyocytes being isolated from neonatal rats was published in 1963 [534, 535]. Currently, neonatal cardiomyocytes isolated from either mouse or rats are most frequently used since it is more challenging to isolate and culture adult primary cardiomyocyte's for more than a couple of days. Primary neonatal cardiomyocytes have been successfully used to model cardiomyocyte hypertrophy, atrial fibrillation, amid numerous cardiac diseases. Primary neonatal cardiomyocytes can be maintained in serum-free conditions as not to create confounds in experiments. However, there is still a significant barrier when attempting to translate results obtained in rodent models for human application. For instance, at baseline, the rodent heart beats between 4-10 times faster than the human heart; mice 500-600, rats 260-450 and human 60-70 beats per minute (see **Table 1.6**) [536]. Interestingly, heart rate is species dependent and is not based on species size as elephants heart beats approximately 35 beats per minute [537].

	Rody Woight	Hoart Pato	Systolic	Diastolic
Species			Pressure	Pressure
	(K <u>g</u> )	(opin)	(mmHg)	(mmHg)
Mouse	0.02-0.063	310–840	113–160	81–110
Rat	0.225–0.52	250–493	84–184	58–145
Rabbit	1–6	130–300	90–130	60–90
Canine	7–16	70–160	95–136	43–66
Sheep	20–160	60–120	91–116	102
Pig	200–300	50–116	135–150	-
Human	50–86	72	120	80

**Table 1.6** Summary of cardiac parameters of human and animal models of heart research.

Table as summarized in Ostergaard et al. 2010 [538] and published in Milani-Nejad et al. 2014 [539]

There is no single animal model that can be considered as the best to model the human cardiovascular system. In fact, there is no perfect model system. It is thus important to examine the advantages and disadvantages of each system prior to selecting one for experimental studies. Importantly, there are also financial aspects that do weigh into these types of decisions. For instance, rodent models are widely used as they have short gestational periods, large litters and are relatively easy to handle and are relatively inexpensive to house. Large animal models (canine, ovine, porcine) are more costly and often require technicians to maintain colonies. In studies interested in genetics, mice are often used due to the wealth of publicly available data and for the ease of manipulating their genomes. However, mouse models of human disease are flawed [540]. For instance, the mdx mouse, developed to model Duchenne muscular dystrophy manifests a mild cardiac phenotype whereas DMD causes dilated cardiomyopathy and significantly decreases life expectancy in humans [541, 542]. In the same vein, phospholamban (PLB) deficient mice display increased contractility whereas the absence of PLB in humans causes severe dilated cardiomyopathy [543]. Mice are also poor models when investigating calcium fluxes due to differences in the balance of SERCA2a and NCX activity [544]. There are several reports that have exemplified differences between rodent and human myocardium (reviewed in depth elsewhere [540]). Briefly, differences have been reported at the level of action potential, myofilaments, isoforms of certain proteins and their phosphorylation status [540].

Besides rodent models, canine, ovine, guinea pigs, porcine and non-human primates have been used to model the human heart. Advantages and disadvantages of select mammalian model systems is described in the following review [539]. Since adult rat cardiomyocytes dedifferentiate in culture and become rounded as opposed to elongated within days post isolation, guinea pig CMs have been evaluated as an alternative [545]. Correspondingly, cardiomyocytes isolated from adult guinea pigs have been reported to represent a better option as they have been shown to maintain shape and function for up to 5 weeks in culture [545]. Further, canine, ovine and porcine hearts resemble human cardiac anatomy more closely (see Figure 1.13). For instance, canines with DMD more closely resemble the human dilated cardiomyopathy phenotype compared to mice models [546]. Research performed on the canine model led to the development of the electrical defibrillator to restore normal heart rhythm [547]. The canine heart shares several similarities with the human especially in terms of its electrical conduction system making them good models to study atrial fibrillation. However, the coronary anatomy in pigs is more similar to human than dogs which have a more extensive collateral coronary circulation [548]. Porcine models are thus useful to study myocardial infarction considering that the artificial generation of an MI is much more difficult to create in canine via the occlusion of coronary arteries as a result of too much collateral flow (see Figure 1.13 B,C) [549, 550]. Since porcine hearts are anatomically similar to human hearts, they are being investigated as surrogate hearts for cross-species xenotransplantation due to the shortage of human hearts. The first transplant of a genetically-modified *humanized* heart actually occurred in early 2022 but unfortunately was not successful [551]. It is also important to consider that animal models of heart disease are generated through transverse aortic constriction, aortic banding or the genetic modification of otherwise healthy animals [552, 553]. This, in itself, does not accurately model the progressive and long-term development of heart failure as observed in humans.



Figure 1.13 Comparative analysis of human, canine, ovine and swine hearts.

(A) Plastinated hearts of four mammalian species. The overall morphology of the canine heart appears to be more rounded while ovine/sheep hearts are more conical in shape. Differences are also observed in the trabeculae carneae shown as finer and more abundant in human compared to the other three models. (B) Species differences in terms of collateral flow. Bar graph represents the percentage of collateral flow after the occlusion of a significant branch of the left anterior descending artery. (C) Collateral flow in canine versus porcine hearts. As depicted in the image, canine hearts have a more extensive collateral network compared to porcine hearts. Figure panel (A) and (C) source: Hill & laizzo et al. 2015 [549] and www.vhlab.umn.edu/atlas, Comparative Anatomy Tutorial. Figure panel (B) was reproduced from Hearse et al. 2000 [550].

Overall, the above-mentioned issues and several other disadvantages paved the way towards the need of physiologically relevant human models of the myocardium which was made possible due to advancements in the field of stem cell research.

# 1.3.4 Induced pluripotent stem cells

Prior to 2007, *in vitro* cultures of human cardiomyocytes were quite limited. Access to human cardiomyocytes was restricted in a sense since cardiac materials could only be obtained from biopsies, non-transplantable hearts or through access to post-mortem

tissue. These tissue samples are most often obtained from diseased patients making it difficult to access healthy cardiac materials to study cellular signaling in physiologically relevant tissue and cell contexts. Further, human primary adult cardiomyocytes are notoriously difficult to maintain in culture. However, recently, a new protocol, with optimized culture conditions has been published citing the ability to keep these cells in culture for 7 days [554]. In this study, the human primary CMs were successfully cryopreserved and thawed. Yet, this development still does not address the bottleneck of cardiomyocyte access, in large numbers.

Significant advances have now made it possible to generate millions of cardiomyocytes derived from human adult somatic cells. In 2007, it was discovered that induced pluripotent stem cells (iPSCs) can be generated by the overexpression of pluripotency specific transcription factors such as the four Yamanaka factors: Oct4, Sox2, Klf4 and c-myc [555, 556]. These factors were shown to be sufficient to induce adult somatic cells to transform into pluripotent stem cells with the *potential* to differentiate into the three germ layers: endoderm, mesoderm and ectoderm [556]. Nowadays, c-myc is often omitted from the cocktail due to its oncogenic potential. iPSCs have also been shown to be generated without the need of Oct4, as it has been suggested that Oct4 may have a negative impact on iPSC guality [557]. Improvements in reprograming strategies and culture conditions such as the adapted use of defined feeder-free conditions has led to major advancements in the field. Induced pluripotent stem cells were initially generated using dermal fibroblasts [558, 559]. More recently, it is more common to use peripheral blood mononuclear cells (PBMCs) due to the minimal invasiveness of the venipuncture extraction method. iPSCs have also been generated from T cells [560, 561], B cells [562], pancreatic beta cells, dental pulp [563, 564], gastric epithelial cells [565], hepatocytes, keratinocytes [566], neural progenitor cells [567, 568] and human renal epithelial cells [569]. The latter most useful when establishing iPS cell lines for the study of neurodevelopmental disorders [570, 571].

Somatic cells have been reprogrammed using multiple delivery techniques including viral infection and episomal plasmids. For example, retroviruses and lenti-

viruses have been used however they are now considerably less popular due to their integrating nature. Sendai viruses are preferred in this regard as they do not integrate into the host genome, are not pathogenic to humans and require fewer starting cells compared to other techniques [572, 573]. More recently, episomal plasmids have become the standard in several laboratories since they do not require the generation of viruses and represent a non-integrative technique. DNA-free alternatives have also been used such as protein or RNA delivery systems in addition to the use of chemical reprogramming or small molecules [574]. An overview of the methods available to generate pluripotent stem cells are available in the following selected references ([575, 576]).

Once generated, iPSCs provide an inexhaustible source of human cells with the potential to become virtually any cell type but also offer access to patient cells with disease relevance [577]. Since 2007, various laboratories have been able to model long QT syndrome [578], hypertrophic cardiomyopathy [579], dilated cardiomyopathy [580], right arrhythmogenic cardiomyopathy [581], as well as the cardiac phenotypes of Duchenne muscular dystrophy [582] among other human diseases, in a petri dish. Phenotypes associated with these diseases have been relatively well recapitulated using hiPSCs. For instance, the cardiotoxic effects of doxorubicin has been recapitulated in iPSC-cardiomyocytes [583]. Patients that exhibited cardiotoxicity post doxorubicin administration displayed similar features in a dish while patients that were spared of this toxicity did not show signs of a toxic phenotype when modeled using their iPSCs [583].

However, considering that no technology is without flaw, there are limitations to consider when modelling diseases using hiPSCs. For instance, when reprogramming adult somatic cells for the generation of iPSCs, the technique has the possibility of altering the epigenetic landscapes unintentionally changing the cellular properties. During the reprogramming stage, it has been demonstrated that the promoter regions of pluripotency genes need to be demethylated to reach a *pluripotent* state. In line with this, the methylation status of pluripotency genes increases for gene silencing during differentiation [584, 585]. During the reprogramming stage, epigenetic aberrations have been observed to occur. As such, the efficiency of reprogramming has been shown to be

inversely related to the epigenetic and methylation changes needed during reprogramming [586]. Relatedly, some iPS cell lines have been reported to be aneuploid [587, 588]. iPS cells need to be frequently karyotyped to ensure their chromosomes are intact.

Epigenetic memory has also been a topic of active investigations. It has been reported that fibroblasts derived from the cardiac niche results in better conversion into cardiomyocyte-like cells [589]. In keeping with this, iPSCs from cardiac progenitor cells (CPCs) or dermal fibroblasts (dFs) from the same fetal or adult donor have been differentiated into CMs using the 3D embryoid body (EB) method [590]. A higher number of CPC-EB-CM exhibited spontaneous beating and after their dissociation into single cells, FACS analysis revealed that CPC-iPSC-CM had a higher proportion of cells expressing cTNT compared to cells originating from fibroblasts of the same donor [590]. These results were independent from the EB method as monolayer approaches have yielded similar results. Methylation was also increased upstream of the transcription start site of NKX2-5 in fibroblast cultures compared to CPCs [590]. Yet, no difference was revealed at the functional level when investigating electrophysiology or calcium handling of iPSC-CMs derived from either cell source. Epigenetic memory also seemed to affect other cardiac progenitor cells as the differentiation towards endothelial cells or vascular smooth muscle cells was more robust using CPC than dermal fibroblasts [590].

Somatic cell memory has also been linked to the maturation status of iPSC-derived cardiomyocytes. When differentiating iPSCs derived from three different somatic cell sources, cardiac derived mesenchymal progenitor cells, bone marrow derived mesenchymal stem cells and dermal fibroblasts, functional differences were observed post-differentiation [591]. When differentiating from a cardiac cell source, beating cells were observed earlier compared to other sources and had a larger proportion of cells that responded to caffeine induced Ca<sup>2+</sup> release [591]. CPC lines also had higher expression of potassium channels KCNQ1 and KCNE1 and displayed a more mature electrophysiology profile compared to non-cardiac sources (longer EFP duration when treating cells with a  $I_{Ks}$  blocker after a  $I_{Kr}$  blocker) [591]. Methylation patterns upstream of

the transcriptional start site of cardiac NKX2-5 exhibited lowered trends in CPC compared to other cell types at the somatic and iPSC stage [591].

Moreover, cardiomyocytes generated either by direct reprogramming or those going through an extra iPSC stage have been compared [592]. Gene ontology of iPSC-CMs was suggestive of a hyperdynamic epigenetic regulation with enrichment in "mRNA transport", "RNA splicing", and "covalent chromatin modification" for instance [592]. A deeper analysis of the maturation status of iPSC-CMs versus iCMs revealed that gene sets associated with iCMs were representative of mature cardiomyocytes while iPSC-CMs were correlated with neonatal gene sets [592]. Prolonged culture of iPSC-CMs improved their "maturity" and revealed more aligned sarcomere pattern compared to iPSC-CMs cultured for a shorter period of time [592]. iCMs preferentially utilized fatty acid oxidation as metabolic pathway, a characteristic associated with adult CMs while iPSC-CMs used glycolysis [592]. iCMs cell cycle related genes were downregulated, an observation that was suggestive of a quiescent state, while iPSC-CMs were enriched for characteristics of a more mature status in iPSC-CMs [592].

In terms of iPSC-based disease modelling, when using female-derived iPS cells to model Lesch-Nyhan syndrome, a disease caused by mutations in the X-linked *HPRT* gene, it has been demonstrated that the long-term passage of these cells leads to the reactivation of the repressed X chromosome leading to the expression of a functional *HPRT* gene [593]. It was reported that early passages of female iPSC-neurons recapitulated the disease phenotype *in vitro* while long term passage did not due to the transcriptional de-repression of the X chromosome [593]. Another paper essentially warns scientists that female iPSCs may be more variable as X-linked reactivation of the X chromosome may lead to epigenetic differences overtime such as the expression of X-linked oncogenes [594]. Sex differences may need to be examined not only at the differentiated cell state but also at the iPSC state. This may be one method to be able to ascertain novel disease phenotypes observed *in vitro*. Other concerns regarding

epigenetic modifications and their potential impact on iPSC-cultures and their differentiated derivatives are examined in ([595, 596]).

# 1.3.4.1. Differentiation of iPSCs into functional cardiomyocytes

Prior to discussing signaling and transcriptional networks involved in the differentiation of iPSCs into cardiomyocytes, the basics of cardiac development will be summarized. In the early embryo, gastrulation occurs at day 15 leading to the formation of the three germ layers: endoderm, ectoderm, and mesoderm [597, 598]. The heart is derived from the anterior splanchnic mesoderm. Cardiac progenitor cells are arranged as two crescent-shaped endocardial plates. These then fuse at the midline to form the linear heart tube with anterior-posterior specification for future atrial and ventricular development. The heart then progressively changes its structure through a process known as cardiac looping and chamber formation. Various heart configurations are adopted during this process, from an initial linear tube to a "C"-shaped helical loop followed by an "S"-shaped two-handed helical loop [599, 600]. Cardiac septation then occurs involving the remodelling of the heart from a single-channel peristaltic pump to a dual-channel, synchronously contracting device with 1-way valves [601]. Heart specification also involves the temporal regulation of several signaling and transcriptional networks [602]. Evidence accumulated from mice, chicken and zebrafish models have shown the importance of BMP-4, Wnt, activin, nodal and, FGF signaling [603]. These seminal findings have contributed to the differentiation protocols used today.

# 1.3.4.2 Signaling pathways involved in differentiating iPSCs into cardiomyocytes

A brief review of Wnt signaling is also required prior to exploring the role of Wnt in cardiogenic programs (for comprehensive reviews on Wnt signaling, please refer to [604-607]). Wnt ligands bind to the class F or Frizzled family of G protein-coupled receptors. Canonical Wnt signaling involves the stabilization of  $\beta$ -catenin, a molecule involved with the transcriptional co-activation of transcription factors and the activation of Wnt-associated gene expression programs. In the absence of Wnt, cytoplasmic  $\beta$ -catenin gets

phosphorylated by casein kinase  $1\alpha$  (CK1) and glycogen synthase kinase  $3\beta$  (GSK3 $\beta$ ). CK1 $\alpha$  and GSK3 are part of the destruction complex along with axin, adenomatosis polyposis coli and protein phospahatse 2A. With these modifications,  $\beta$ -catenin then gets recognized by a ubiquitin ligase, essentially leading to its ubiquitination and consequent degradation. In contrast, in the presence of the Wnt ligand, Wnt binds to frizzled receptor and lipoprotein receptor related protein, LRP5/6, leading to the inactivation of the destruction complex. Essentially, LRP5/6 becomes phosphorylated by GSK3ß as well as CK1y. These phosphorylation events permit axin to bind rendering it and the destruction complex inactive. This permits the accumulation of  $\beta$ -catenin in the cytoplasm which then get translocated into the nucleus and binds transcription factors resulting the activation of Wnt-associated gene expression programs. Generally, canonical Wnt signaling is involved in the modulation of cell fate decisions such as proliferation, differentiation and axis specification [608]. In contrast, non-canonical Wnt signaling, are  $\beta$ -catenin independent and involve two pathways known as the planar cell polarity and Wnt-Calcium pathway. Both pathways have been experimentally determined to be involved in cardiac differentiation and is examined below.

There have been several protocols that have described the generation of functional, spontaneously beating cardiomyocytes from human iPSCs (see **Table 1.8**). The temporal modulation of Wnt signaling has been repeatedly shown to modulate the differentiation of the early and late cardiac mesoderm. Wnt signaling has also been observed to be important for primitive streak as well as mesodermal formation [609]. Experimental evidence has indicated that  $\beta$ -catenin null mouse embryos do not to form mesodermal tissue [610]. Wnt signals have also been demonstrated to be important for the development of the second heart field [611]. Conflicting evidence in regard to the role of Wnt signaling in cardiogenic programs led to the notion that temporal regulation may be a key factor [612, 613]. Using wildtype and hsDKK1 transgenic zebrafish embryos, it has been shown that the activation of Wnt/ $\beta$ -catenin pre-gastrula is a positive regulator of cardiomyocyte differentiation while negatively impacting cardiogenic programs during gastrulation [614]. Similar results have been demonstrated in mouse ES cells as well [614].

Wnt as well as BMP signals have been reported to repress the expression of pluripotency factor Sox2 [615]. The silencing of Sox2 has been shown to be important for cardiac fate determination. Sox2 has also been reported to bind the EOMES locus and Wnt has been demonstrated to repress Sox2 promoting EOMES and mesodermal expression [615, 616]. Progenitor cells within the primitive streak positive for EOMES, the T-box transcription factor, have been shown to lead to the cardiac mesoderm [617]. EOMES activates the bHLH transcription factor, Mesp1, one of the earliest cardiogenic markers [618, 619]. In EOMES null hESCs, the differentiation into cardiomyocytes has reportedly been unsuccessful [620].

Distinct and sequential roles for canonical versus non-canonical Wnt signaling has been observed in cardiac differentiation programs. WNT3 and WNT8A are established to signal via the canonical Wnt pathway and have been detected to be expressed during mesodermal induction [621]. WNT5A, WNT5B, and WNT11 are members of the noncanonical Wnt pathway and have been shown to be involved in the early cardiogenic mesoderm [621]. Experimental evidence also suggests that canonical Wnt activity leads to brachyury expression while non-canonical Wnts are involved in the regulation of Mesp1 expression [621]. Others have also identified the need of Wnt signaling for Mesp1 expression [622].

Milestone	Cellular model	Culture substrate	Pluripotency media	Dissociation reagent	Differentiation format	Growth factors, small molecules	Efficiency	Reference
Spontaneous differentiation	hESC	MEF	KO-DMEM, 20% FBS	Collagenase	EB	FBS	8% OF EBs	Kehat et al, 2001
Co-culture differentiation	hESC	MEF	DMEM, 20% FBS	Dispase	Colonies	FBS, END-2 cells	N/S	Mummery et al., 2003
Growth-factor EBs	hESC	Matrigel	MEF-CM	Trypsin	EB	Activin A, FGF2, FBS	24 % EB of EBs	Burridge et al., 2007
Growth factor monolayer	hESC	Matrigel	MEF-CM	Collagenase IV or EDTA	Monolayer	Activin A, BMP4	30% MYH7+	Laflamme et al., 2007
First application of a small molecule (SB203580)	hESC	HF	KO-DMEM, 20% KSR, FGF2	Collagenase IV	Monolayer	SB203580	22% MYH6+	Graichen et al., 2008
First chemically- defined medium, insulin has negative effects early in differentiation	hESC	MEF	KO-DMEM, 20% KSR, FGF2	Collagenase IV	EB	PGI2, SB203580	11% MYH6+	Xu et al., 2008
Increased efficiency via Wnt inhibition	hESC	MEF	DMEM/F12, 20% KSR, FGF2	Trypsin	EB	BMP4, Activin A, FGF2, DKK1, VEGFA	50% TNNT2+	Yang et al., 2008
Concentration optimization leads to increased efficiency	hESC	MEF	DMEM, 20%, KSR, FGF2	ReproCELL dissociation solution	EB	Activin A, Wnt3A, BMP4, DKK1, FBS	98% EBs	Takei et al., 2009
Stage specific effects of Wnt/β- catenin modulation	hESC	Matrigel	MEF-CM, FGF2	Collagenase IV	Monolayer	BMP4, Activin A, FGF2, DKK1, VEGFA	49% MYH1+	Paige et al., 2010
Identification of developmentally- relevant stage- specific signaling requirements	hESC, hiPSC	MEF, then Matrigel	DMEM/F12, 20% KSR, FGF2	Collagenase B, trypsin	EB	BMP4, FGF2,	50-70% TNNT2+	Kattman et al., 2011
Optimization of chemically defined media leads to higher efficiency	hiPSC	Geltrex	MEF-CM	TrypLE	EB	BMP4, IWR-1, FBS or HSA	94% of EBs	Burridge et al., 2011
Wnt inhibition via small molecule	hiPSC	MEF	DMEM/F12, 20% KSR, FGF2	Collagenase IV	EB	BMP4, Activin A, FGF2, IWR- 1, VEGFA, FBS	16% TNNT2+	Ren et al., 2011
Serum-free, small molecule- based Wnt inhibition	hESC	MEF	KO-DMEM, 20% KSR, FGF2	Collagenase IV	EB	BMP4, Activin A, FGF2, IWR- 1, VEGFA, FBS	30% MYH6+	Willems et al., 2011
Differentiation in both EB and monolayer formats	hESC	Low density MEF	DMEM/F12, 20% KOSR, FGF2	Collagenase IV	EB	BMP4, WNT3A, Activin A, VEGFA, SCF	~25% GFP+	Elliott et al., 2011
first protocol utilizing solely small molecules	hESC	Matrigel	MEF-CM	Accutase	Monolayer	CHIR99021, IWR-1, SC431542, pumorphamine	70-70% NKX2-5+	Gonzalez et al. 2011
Use of dual GSK3β inhibitors and a novel Wnt- inhibitor	hESC, hiPSC	Gelatin or laminin-211	DMEM/F12, 20% KOSR	CTK dissociation solution	EB	CHIR99021, BIO, KY02111, XAV939	98% TNNT2+	Minami et al., 2012
Defined pluripotent media, small molecule-based differentiation	hESC, hiPSC	Matrigel or Synthemax	mTesR1	TrypLE	Monolayer	CHIR99021, IWP-4	85% TNNT2+	Lian et al., 2012 Lian et al., 2013
Protein transduction- based differentiation	hESC	Suspension	MEF-CM	Trypsin	EB	Activin A, ISL1, BMP4,	75% TNNT2+	Fonoudi et al., 2013
First chemically- defined, small molecule-based differentiation in multiple lines and various matrices	hESC, hiPSC	Synthemax or laminin- 521	E8	EDTA	Monolayer	CHIR99021, Wnt-C59	95% TNNT2+	Burridge et al.,2014

# **Table 1.8** Summary of protocols for the generation of cardiomyocytes from iPSCs.

BMP inhibition- based differentiation	hESC, hiPSC	Matrigel	mTeSR1	ReLeSR	Monolayer	CHIR99021, DMH!	75% TNNT2+	Aguilar et al., 2015
Albumin-free	hESC, hiPSC	Matrigel	FTDA	Accutase	EB or monolayer	BMP4, FGF2, CHIR, IWP-2 or Wnt-C59	80-95% TNNT2+	Zhang et al., 2015
Albumin-free, small molecule based	hESC, hiPSC	Synthemax	E8 or mTeSR1	Accutase	Monolayer	CHIR99021, IWP-2	88-98% TNNT2+	Lian et al., 2015
Use of heparin in lieu of albumin, E8-based	hESC, hiPSC	Matrigel	E8	EDTA	Monolayer	CHIR99021, IWP-2	94% TNNT2+	Lin et al., 2017 Lin et Zou, 2020
								<b>•••</b> • • • • • • • •

Table was modified to improve legibility and reproduced with permission from [623] <u>https://star-protocols.cell.com/protocols/1914</u>.

The cardiac differentiation protocol used to generate cardiac myocytes in this thesis modulates Wnt signaling [624]. In the first step of the protocol, Wnt signaling is activated using a GSK3 $\beta$  inhibitor, termed CHIR99021. Since GSK $\beta$ 3 is inhibited, the destruction complex cannot function,  $\beta$ -catenin cannot be phosphorylated allowing for its nuclear translocation. Then, Wnt signaling is sequentially inhibited using a porcupine Wnt inhibitor, IWP2. Importantly, Wnt ligands are post-translationally modified requiring a palmitoylation step for secretion and function [625, 626]. Palmitoylation is essentially performed by porcupine and IWP2 functions by inhibiting the membrane-bound O-actelytransferase porcupine, and this, in term, inhibits Wnt ligand palmitoylation, secretion and function. Several cardiomyocyte differentiation protocols also modulate the concentration of insulin as some lines of evidence has suggested a negative role of insulin in cardiac fate decisions [627]. Due to this, insulin can be omitted from culture media during the first 7 days of the differentiation protocol [627, 628]. However, in protocols that modulate Wnt signaling, the role of insulin is less important and can be used in early differentiation stages [628].

Besides Wnt signaling, other protocols support a cardiogenic role of BMP-4 and Activin A in cardiomyocyte specification and differentiation [629-631] (reviewed in [632]). Briefly, BMPs or bone morphogenic proteins belong to the family of transforming growth factor  $\beta$  and secreted BMPs bind to type I and type II serine/threonine kinase receptors forming a complex. This drives a transphosphorylation event where the type II receptor phosphorylates the type I receptor. The type I receptor then phosphorylates SMAD1, 5 and 8 which then bind with SMAD4, a co-SMAD, and this complex can translocate to the nucleus to modulate gene expression. For instance, NKX2-5, GATA4 and MYH7 are BMP-4 target genes and are upregulated during cardiac differentiation [633]. Equally, the

use of BMP inhibitors has been observed to reduce the cardiogenic fate [634]. Like Wnt signaling, timing is important as BMP-4 can exert opposing effects on the development of the cardiac mesoderm [629, 635]. All in all, several cardiomyocyte differentiation protocols have been described yielding different cTnT percentage levels. Regardless of the small molecules or growth factors used during differentiation, a metabolic selection technique is most often used to further purify the cardiomyocyte population, post-differentiation. Glucose starvation and lactate selection permits to select for cardiomyocytes as these cells can metabolize lactate whereas other non-cardiomyocyte cell types depend on glucose [636, 637]. Thus, glucose-free medium is supplemented with 4-5 mM lactate for cardiomyocyte purification. Similarly, magnetic activated cell sorting (MACS) can be used to select for iPSC-CMs and has previously been done with success [638-640].

Lactate, however, selects only for cardiomyocytes, and has no preference for ventricular versus atrial cell fates. The Lian et al (GiWi) protocol has been reported to yield a mixed population of cardiomyocytes, including ventricular, atrial and nodal cells [624]. At times, this heterogenous cardiomyocyte population may not be ideal especially if the experimental study being conducted is interested in studying atrial fibrillation for instance. Further, the generation of cardiac-specific cells is important considering that atrial and ventricular cells have distinct properties and may respond to drugs differently [641, 642]. To address this, differentiation protocols have demonstrated that the timespecific addition of retinoic acid allows for the differentiation of atrial-specific cardiomyocytes [643]. Likewise, it has been suggested that atrial and ventricular cardiomyocytes are derived from two distinct mesodermal populations. Essentially, atrial cardiomyocytes are derived from a subpopulation of RALDH2<sup>+</sup> while ventricular cardiomyocytes originate from a subpopulation of the mesoderm that is CD235a<sup>+</sup> [643]. Each of these populations can be attained though the differential activation of activin A and BMP-4 signaling where ventricular cells require enhanced levels of Activin A and BMP-4 in comparison to atrial cardiomyocytes. [643]. A protocol yielding a higher proportion of compact ventricular cells has also been described [644]. Similarly, sinoatrial pacemaker cells can also be generated using defined protocols [645].

As no cell model is inherently *perfect*, there are certain limitations that should be considered when using cardiomyocytes derived from pluripotent stem cell sources. One of the most striking limitations is their maturation status, discussed below.

# 1.3.4.2.1 Methods to drive maturation of iPSC-derived cardiomyocytes

Cardiomyocytes derived from hiPSCs have been demonstrated, in multiple cases, to resemble neonatal as opposed to adult myocytes. For instance, iPSC-CMs are rounded with mis-aligned sarcomeres instead of being elongated with organized sarcomeres as found in their adult counterparts (see Table 1.9 & Figure 1.14). To address these shortcomings, several maturation strategies have been attempted, reviewed here ([646-649]). Comparisons between neonatal and adult cardiomyocytes have identified that their metabolic signatures differ as neonatal myocytes utilize glucose as their energy source, whereas adult CMs rely on fatty acids. Thus, several strategies have modulated the availability of fatty acids in cell culture media as means to push iPSC-CMs to a more 'adult-like' phenotype. Maturation medias have also been supplemented with hormones, glucocorticoids, or various agonists [644, 650]. These media supplements have all had some degree of success in maturing cardiomyocytes. Low glucose media formulations combining fatty acids, T3 hormone, PPARa agonists have also been developed. Peroxisome-proliferator-associated receptor (PPAR) signaling has been shown to be involved in regulating cardiomyocyte maturation. An important role for PPARδ has been established promoting the metabolic switch from glycolysis to fatty acid oxidation (FAO) [651]. Relatedly, high-glucose media has been demonstrated to negatively influence cardiac maturation status [652]. As there are still several gaps in the literature, the metabolic states that govern metabolic switch involved in the maturation of iPSC-CMs are still being investigated. For example, a role for cAMP/PKA and the proteasome has been suggested to modulate the metabolic stages of cardiomyocytes [653]. This study highlighted a potential new target for driving the maturation of iPSC-CMs.

	Parameters	Immature CMs	Mature CMs
	Shape	Round or polygonal	Rod and elongated
	Size	20–30 pF	150 pF
	Nuclei per cell	Mononucleated	~25% Multinucleated
	Multicellular organization	Disorganized	Polarized
	Sarcomere appearance	Disorganized	Organized
	Sarcomere length	Shorter (~1.6 µm)	Longer (~2.2 µm)
	Sarcomeric proteins		
Morphology	MHC	β > α	β >> α
	Titin	N2BA	N2B
	Troponin I	ssTnl	cTnl
	Sarcomere units		
	Z-discs and I-bands	Formed	Formed
	H-zones and A-bands	Formed	Formed
		(prolonged differentiation)	
	M-bands and T-tubules	Absent	Present
	Action		
	potential properties		
	Resting membrane potential	~-60 mV	−90 mV
Electrophysiology	Upstroke velocity	~50 V/s	~250 V/s
Place 1 Adult CAs	Amplitude	Small	Large
F Main 2	Spontaneous automaticity	Exhibited	Absent
Phase 0 Phase 3	Ion currents		
Prose 4	Hyperpolarization-	Present	Absent
· _	activated pacemaker (1f)		
Au	Sodium ( / <sub>Na</sub> )	Low	High
	Inward rectifier	Low or absent	High
•	potassium ( <i>I</i> <sub>κ1</sub> )		
60	Iransient	Inactivated	Activated
	potassium current ( L <sub>in</sub> )	mactivated	Activated
	ATP-sensitive	P-sensitive	
	K <sup>+</sup> current ( <i>I</i> <sub>KATP</sub> )	Not reported	Present
	L- and T-type calcium		
	( $I_{Ca,L}$ and $I_{Ca,T}$ ), rapid and slow rectifier		
	potassium currents ( $I_{\rm Kr} and$ $I_{\rm Ks}$ ), Na $^{*}-$	Similar to adult CMs	
	Ca $+$ exchange current ( $I_{NCX}$ ) and		
	acetylcholine-activated K * ( I K,ACh)		
Conduction velocity	Propagation of signal	Slower (~0.1 m/s)	Faster (0.3–1.0 m/s)
Gap junctions	Distribution	Circumferential	Polarized to
			intercalated disks
	Ca <sup>2+</sup> transient	Inefficient	Efficient
	Amplitudes	Small and decrease	Increase with pacing
	of Ca <sup>2+</sup> transient	with pacing	
	Excitation-	Slow	Fast
Calcium handing		nNI rango/agil	, uN ronge/
		~nin range/Cell	~µiv range/cell
		l europe et const	Nie wee - 1
	CASQ2, RyR2, and PLN	Low or absent	Normal
	Force-	Positive	Negative

**Table 1.7** Comparison of maturation status of iPSC-CMs with mature cardiomyocytes.

	frequency relationship			
	Mitochondrial number	Low	High	
	Mitochondrial volume	Low	High	
	Mitochondrial structure	Irregular	Regular	
Mitochondrial bioenergetics		distribution, perinuclear	distribution, aligned	
	Mitochondrial proteins			
	DRP-1 and OPA1	Low	High	
	Metabolic substrate	Glycolysis (glucose)	Oxidative (fatty acid)	
	Response	Lack of inotropic reaction	Inotropic reaction	
Responses to β-adrenergic	Cardiac alpha-			
stimulation	Adrenergic	Absent	Present	
	receptor ADRA1A			

Table source Sayed N, Liu C, Wu JC. Translation of Human-Induced Pluripotent Stem Cells: From Clinical Trial in a Dish to Precision Medicine. J Am Coll Cardiol. 2016 May 10;67(18):2161-2176. doi: 10.1016/j.jacc.2016.01.083. PMID: 27151349; PMCID: PMC5086255.[654].



#### Figure 1.14 Addressing maturity concerns when working with iPSC-CM models.

(A) iPSC-CMs represent an immature model of the human adult cardiomyocyte. iPSC-CMs display sarcomeric disarray, underdeveloped mitochondria, T-tubules and other ultrastructural deficits. (B) Methods to mature iPSC-CMs. Several strategies have been developed as means to drive the maturation of iPSC-CMs. Prolonged culture, the addition of hormones or fatty addition to the culture medium as well as 3D culturing techniques. See text for more details. Panel A is reproduced from Kane et al. 2015 [655]. Panel (B) sourced from Ahmed et al. 2020 [656], with permission.

Long-term culture has also been tested and demonstrated as a viable option for iPSC-CM maturation [657, 658]. iPSC-CM cultures up to one year have shown

remarkable maturity-related signatures such as mature M-bands and other ultrastructural sarcomere changes [659]. Long-term culture of DMD organoids exhibited similar features of the disease [660]. The downside of this technique is the low throughput, having to wait extended periods of time prior to performing experiments. The extracellular matrix on which iPSC-CMs are seeded on has also been a topic of research interest [661]. In contrast to using Matrigel, one of the most common substrates used in the stem cell field, iPSC-CMs seeded on Matrix Plus, a human perinatal stem cell derived extracellular matrix (ECM) were shown to display morphological and electrophysiological signs of maturation [662]. To force iPSC-CMs to obtain an elongated shape as observed in adult CMs, patterned nano-grooved culture surfaces have been developed [663-665]. Since the heart is often referred to as a mechanical pump as it pumps blood while the muscle undergoes rhythmic contractions, mechanical stimulation has been used as a method to promote maturation, reviewed in [666]. This broadly entails modulating substrate stiffness or the topography of the culture surface as well as incorporating cyclic strain or perfusion onto the cells. Electrical pacing has also been demonstrated as a viable technique for maturation [667]. Besides, an estrogen-related receptor gamma (ERRγ) agonist and an S-phase kinase-associated protein 2 inhibitor have also been linked with cardiomyocyte maturation [668]. Silent mating type information regulation 2 homolog 1 (SIRT1) is a NAD<sup>+</sup>-dependent protein deacetylase has been demonstrated to be an important regulatory factor in cardiomyocyte alignment and maturation [669].

Since the field is still in its infancy, it is still unknown which "maturation method" is optimal and leads to the finest quality iPSC-CMs with adult-*like* signatures. However, when reflecting on 2-dimensional, monolayer culture formats, a combination of several approaches will most likely be the best option when attempting to mature iPSC-CMs *in vitro*. The myocardium is a complex tissue composed of numerous cell types communicating via an endocrine, paracrine and autocrine manner thus realistically, several cues are absent when working with unicellular cardiomyocyte cultures. The *winning* option may sensibly also be that co-cultures or 3-dimensional models will be the greatest at achieving the required maturation state. Culturing iPSC-CMs with mouse sympathetic ganglion neurons has revealed mature phenotypes such as enhanced gene

expression of ion channels and myofibril gene sets [670]. 3D cardiac microtissues composed of cardiac fibroblasts and endothelial cells also contribute to iPSC-CM maturation [671, 672]. In this study, a link between cAMP and iPSC-CM maturity was proposed where persistent cAMP activity was mentioned to be suggestive of a more mature phenotype [671]. Endothelial cells may be more efficient at maturing cardiomyocytes when incorporated with cardiac progenitor cells as a stage-specific improvement has been reported [673]. Gut tissue has also been demonstrated to support cardiomyocyte maturation through cooperative paracrine signaling and interactions [674]. 3D cultures have also shown to have increased FAO levels suggesting that a mature metabolic phenotype may be attainable using 3D cultures in contrast to common 2D monolayers [675]. Cardiac organoids have also been generated using templated substrate for geometrical control of their development [676]. The three-dimensional culture of iPSC-CMs or the generation of 3D engineered heart tissues (EHTs) have also demonstrated promising results [677].

All things considered; the field is searching for innovative methods to mature iPSC-CMs as most cardiac diseases are of adult onset. Thus, to truly be able to model disease, there is a great need for the development of protocols that yield mature and adult-*like* cardiomyocytes. Below, we examine numerous accounts of how iPSC-CMs have been used to model cardiomyopathies, in a dish.

#### 1.3.5 iPSC-CM models of cardiomyopathy

Over the years, several groups have attempted to recapitulate human cardiomyopathies, in a dish, using patient derived iPSC-CMs, in attempt to better understand disease progression and other causative disease mechanisms. As such familial DCM has been successfully recapitulated using patient-derived iPSC-CMs with a R173W mutation in the gene encoding cardiac troponin T. In comparison to healthy cardiomyocytes, these patient cells exhibited abnormal Ca<sup>2+</sup> handling, reduced contractility, and irregular sarcomeric  $\alpha$ -actinin organization which worsened by  $\beta$ -adrenergic activation [580]. These observations were in line with symptoms reported in

the clinic demonstrating the power of hiPSC-CMs for disease modelling applications. Moreover, hiPSC-CMs may have also helped uncover potential therapeutic interventions. For instance, in a dilated cardiomyopathy model carrying a mutation in troponin T (R173W), small molecule-based activation of AMP-activated protein kinase (AMPK) demonstrated partial rescue of troponin microdomain interactions as well as contractility and sarcomere alignment deficits [678]. Similarly, using familial DCM iPSC-CMs, TNNT2 R173W, impaired β-adrenergic signaling was observed in patient versus control cells [679]. Using a FRET-sensor which measures PKA activity, DCM cardiomyocytes displayed smaller PKA responses [679]. This was later attributed to higher levels of phosphodiesterase, PDE2A and PDE3A, which act to hydrolyse cAMP, in DCM cell lines [679]. The subtype-specific inhibition of phosphodiesterase may thus represent a new therapeutic avenue for dilated cardiomyopathy.

When deriving hiPSC-CMs from breast cancer patients who experienced cardiotoxic effects, the vulnerability of these patients to doxorubicin was well recapitulated in a dish [680]. Meanwhile, patients that were spared from this cardiotoxicity displayed normal iPSC-CM cultures that were not susceptible to doxorubicin's negative effects [680]. The cardiotoxic effects of trastuzumab have also been investigated in iPSC-CM cultures revealing contractile dysfunctions [681]. Reduced contractile function has also been demonstrated in a B-cell lymphoma model post treatment with doxorubicin [682]. In a rat model of doxorubicin induced cardiotoxicity, LCZ696, an angiotensin receptor–neprilysin inhibitor displayed positive outcomes by decreasing oxidative stress and may be a promising treatment option [683]. It would be interesting to test this compound in hiPSC-CMs to verify its translatability as a human therapy. Using iPSC-CMs, a role for Hippo signaling has also been demonstrated to be involved in doxorubicin mediated cardiotoxicity suggesting a protective role for the effector yes-associated protein (YAP) [684].

iPSC-CMs have also been used to test the translatability of data collected in animal models. For instance, BRCA1/2 mutations in mice models suggest a vulnerability to doxorubicin treatment. However, iPSC-CMs have demonstrated that *BRCA1/2* mutations

do not render *tested* patient susceptible to doxorubicin toxicity while assessing cell viability, contradicting animal studies [685]. Even if the iPSC-CM study was small, it demonstrates the importance of using human models when attempting to understand human disease and causative mechanisms.

As heart disease patients also present with co-morbidities, the cardiomyocyte becomes an important cell model to understand how these other diseases influence cardiac health. For this reason, iPSC-CMs have been used to study how type II diabetes impacts heart cells and has recapitulated important phenotypes [686]. iPSC-CMs can also be used to study rarer forms of heart failure such as peripartum cardiomyopathy and have identified a role of lipid metabolism in disease progression [687, 688]. Exposure to toxins can also result in a dilated cardiomyopathy phenotype and has been modelled using iPS technology. Alcohol cardiomyopathy develops due to chronic alcohol abuse and manifests with dilated left ventricle and myocardial dysfunction. To understand the negative consequences of alcohol on myocardial health, iPSC-CMs were treated acutely with 100 mM alcohol [689]. This treatment revealed negative impacts on viability, contractility and beat rate in addition to a prolonged field potential. Treated cardiomyocytes also displayed increased oxidative stress and was associated with increased AT<sub>1</sub>R expression and Ang II levels in the supernatant [689]. These negative phenotypes were rescued by treatment with an AT1 antagonist, losartan, underlining a potential treatment option to remedy alcohol toxicity [689].

When searching to identify a disease mechanism, iPSCs can be combined with genome engineering to understand the role of certain genes in disease. For instance, using CRISPR genome editing, iPSCs were engineered to carry a point mutation in troponin T,  $\Delta$ K210. These cells exhibited sarcomere disarray; an observation that was later shown to be dependent on the stiffness of the substrate on which the cells were seeded on [690].  $\Delta$ K210 iPSC-CMs had reduced sarcomere organization on rigid substrate, an environment similar to the heart failure phenotype while the disorganization was normal on 'physiologically' stiff substrates [690]. The  $\Delta$ K210 were also larger in size, an observation that was hypothesized to result from compensatory hypertrophy [690].

These observations demonstrate the value of incorporating the mechanical environment when attempting to discern underlying disease mechanisms.

As the surrounding environment provides important extracellular cues, iPSC-CMs can be organized in a 3-dimensional architecture to better mimic the human heart [691]. These organoid models provide several benefits for disease modelling applications. For instance, iPSC based engineered heart tissue have recapitulated contractile abnormalities when modelling mutations in the cardiac splicing factor RBM20 that cause dilated cardiomyopathy [692]. Further, cardiac microtissues composed of healthy iPSC-CMs but diseased cardiac fibroblasts that carried a mutation in the desmosomal protein PKP2 that causes arrhythmogenic cardiomyopathy demonstrated arrhythmic behavior [671]. This study essentially demonstrated that healthy cardiomyocytes displayed disease features through inter-cellular communication, highlighting the importance of multi-cellular and 3D culture models. Engineered heart tissues have also recapitulated features of long QT syndrome and catecholaminergic polymorphic ventricular tachycardia [693]. Cardiac spheroids have also been used to model doxorubicin toxicity [694], hypertrophic cardiomyopathy [695] and myocardial infarctions [696].

Considering that iPSC-derived cardiomyocytes have been successful, thus far, at modelling various forms of cardiomyopathies, they may realistically be able to help the scientific community uncover novel therapeutic targets to help with cardiac disease management. To this end, unbiased, high-throughput as well as automated methods may help systematically study and phenotype patient-derived hiPSC-CMs. Below, we introduce biosensors as tools that may help accomplish this, as they represent an ideal method to study protein conformation as well as cellular signaling in control versus diseased cells.

1.4 Tools for studying GPCR conformational and signaling dynamics

To gain insight into the molecular mechanisms of GPCR signal transduction, several elegant tools have been developed. Of central importance to this thesis, several generations of biosensors have been built and allowed to "sense" GPCR conformation as well as the activation of their downstream signaling transduction pathways. When expressed in cell-based systems, these biosensors are designed to track cellular signaling in real-time and are further discussed below.

#### 1.4.1 Resonance energy transfer versus intensiometric-based biosensors

Over the years, a number of interesting tools have been developed and refined to measure biological outcomes downstream of GPCR activation. In this thesis, biosensors built on the principle of resonance energy transfer (RET) or fluorophore intensity are of particular relevance. Both biosensor configurations rely on optical processes that result in the emission of light that can be captured using luminometers, fluorometers or microscopes. Biosensors based on resonance energy transfer rely on an optical method where non-radiative excitation energy of a donor molecule is transferred to an acceptor through dipole-dipole interactions (reviewed in [697]). This optical principle requires proximity between donor and acceptor molecules, within 10 nm or 100 Angstroms, and the donor's emission wavelength must overlap with the absorption spectrum of the acceptor (see Figure 1.15). The efficiency of the energy transfer is distance dependent and is proportional to the inverse sixth power of the distance between the donor and acceptor. Since this technique is highly sensitive to distance, it has often been referred to as a "molecular ruler" as it can measure distances within and between GPCRs, and other proteins tagged with compatible donors and acceptor molecules [698]. When acceptor and donor molecules tag the same protein, RET is a ratiometric approach and measures changes in donor and acceptor intensity when the cellular system in which the biosensor is expressed is introduced to drug treatments or other external stimuli (see Figure 1.16). In contrast, intensiometric biosensors typically report on a cellular event through the quantification of intensity of a single fluorophore. When assessing cellular signaling with intensiometric sensors, an increase or decrease in biosensor intensity output can reflect pathway activation. The evident disadvantage of these biosensor types is that the

intensity of the fluorophore in response to a given stimulation may also be influenced by transfection efficiency and cell confluency. To circumvent this, excitation ratiometricbased biosensors have been developed which are single colour, and pathway activation is computed using a ratio of GFP excitation wavelengths [699]. These biosensors have been shown to report on small rapid changes in cellular behavior and have the benefit of being amenable to multiplexing. By co-transfecting blue, green and red versions of different biosensors, it allows the simultaneous monitoring of multiple signaling pathways in a single cell.



Figure 1.15 Jablonski diagram depicting FRET mechanism.

(A) Fluorophore absorption of light brings an electron to an excited energy state. As this is transiently lived, the electron drops back to the ground state and the fluorophore emits light at a longer wavelength. In the context of FRET, the relaxation energy is transferred to the acceptor moiety, proximity permitting, and the acceptor emits light at a characteristic wavelength. (B) FRET is distance-dependent, when acceptor and donor are nearby, within 10 nm, resonance energy can be transferred from donor to acceptor. If the distance is surpassed, the donor emits light and no FRET is measured. (C) Spectral overlap is required for FRET to occur, and donor (D) emission must overlap with acceptor (A) excitation. Figure from Skruzny et al 2019 [700], with permission.

RET biosensors exist in two different *flavours*. The donor can be a bioluminescent protein such as Renilla luciferase or a fluorophore such as CFP. When the donor is bioluminescent, the technique is referred to as BRET where FRET uses fluorophores for both donor and acceptor molecules. BRET systems use bioluminescent enzymes as donors and when exposed to the coelenterazine substrate, catalysis of the substrate results in the emission of light, explained in [701-703]. If the wavelength of the light emitted falls within the spectrum of the acceptor, it can transfer non-radiative resonance energy to the acceptor molecule. Once the acceptor has captured this energy, the acceptor itself can then emit light at another characteristic wavelength. The light emitted can be quantified using a luminometer. Due to the low light intensity signals, BRET imaging is not compatible with most current microscopy setups as *most* cameras are not sensitive enough. BRET data is thus frequently acquired using microplate readers that enable population-level measurements. To circumvent this limitation, FRET can be used as it takes advantage of a fluorescent donor and acceptor molecules. FRET microscopy also allows to study pathway activation at the single cell level, providing greater granularity [704-706]. In the FRET set-up, a laser line is required to excite the donor as opposed to an enzymatic reaction occurring in BRET. Besides these key differences, BRET and FRET configurations follow the same "molecular rules" however there are certain advantages and disadvantages associated with each system [707]. For instance, since a laser excites the donor in FRET, fluorophore photobleaching may arise as a potential issue as well as undesired direct excitation of the acceptor. Thus, the main advantage of BRET over FRET is the fact that BRET does not require an external excitation source. Cell autofluorescence and high background is also not an issue when performing BRET experiments. Orientation of both BRET and FRET pairs is also an important parameter to consider when performing experiments since if the donor/acceptor molecules are in opposing orientations, the system may interpret this as a lack of signal
even if the two molecules are within 10 nm. Certain RET pairs suffer less of this orientation-related concern.



Figure 1.16 Two common types of biosensor readouts.

**Biosensors are generally built based on two types of readouts.** Biosensors can be intensiometric (left), referring to their ability to report on analyte concentration or pathway activation based on fluctuations in single fluorophore's emission intensity. Biosensors can also be ratiometric (right) based on changes in emission of a donor and acceptor chromophore. Figure adapted from Kaczmarski et al. 2019 [708], with permission.

# 1.4.2 Biosensors that report on GPCR conformational dynamics

Several BRET- and FRET- based biosensors have been designed to monitor the conformational dynamics within GPCRs. Conformation-sensitive biosensors use intramolecular designs and commonly insert the donor moiety at the end of the carboxyl terminus of the receptor. Within the same coding sequence, an acceptor moiety is introduced within the intracellular loops (ICLs). These intracellular locations are conformationally sensitive as GPCR activation has been demonstrated to result in a 14Å outward movement of transmembrane 6 which propagates within the intracellular loops to activate effectors initiating cellular signaling [709]. Since the distance between the third intracellular loop and the C-terminus has been shown to be within the RET distance, these

biosensors can be used to monitor receptor activation and conformational outputs when stimulated with different ligands (*see* **Figure 1.17**) [710, 711]. Since GFP is a large protein, 27 kDa, its insertion within intracellular loops can be quite disruptive to protein function. Thus, to avoid this issue, smaller tags have been developed. One example is the tetracysteine tag (CCXXCC) [712]. If the tetracysteine tag is used as an acceptor, an extra labeling step is required with a green-fluorescent FIAsH-EDT<sub>2</sub> dye. Once labeled, the tetracysteine tag can fluoresce. Similarly, Nanoluc luciferase, 19.1 kDa vs. 36 kDa of Rluc, has also been combined with the Halotag to monitor conformational signatures [713, 714]. Using the BRET or FRET approach, GPCR conformation has been assessed in numerous receptor systems such as the AT<sub>1</sub>R [715], F prostanoid receptor [716],  $\beta_2$ AR [713, 717, 718], 5-HT<sub>2A</sub> [719],  $\alpha_{2A}$  adrenergic receptor [720], parathyroid hormone receptor 1 PTH1 [713] and adenosine A(2A) receptors [720].

#### A) Receptor-based Conformational Biosensors





(A)Schematic depiction of a GPCR-based conformation-sensitive biosensor. Conformational biosensors can either be BRET- or FRET-based and report on changes within the structure of a GPCR. Donor and acceptor chromophores are introduced within the coding sequence of the receptor. Agonist binding incites a conformational change within the GPCR, a change that is measurable through variations in resonance energy transfer between donor and acceptor. (B) The FIAsH dye can be used to label conformation-sensitive biosensors that use the FIAsH-tag as an acceptor (CCPGCC). (C) The FIAsH dye only fluoresces when it binds to the FIAsH-binding tetracysteine tag (+TC). Figure (A) is modified from Olson et. al 2022 [721], while Figure (B) and (C) are modified from Pomorski et al. 2020 [722], with permission.

The development of conformation-sensitive biosensors engineered within GPCR structures was and still is driven by the expectation that they will be useful tools to help identify novel compounds for drug development purposes. Conformation-sensitive biosensors have illuminated various aspects of GPCR biology [721]. For instance, conformational outputs from biosensors engineered within the human  $\beta_2$ -adrenergic receptor have shown to correlate well with the efficacy and potency of ligands tested [718]. Sensors built within the human AT<sub>1</sub>R sequence were able to distinguish between balanced and biased agonists such as SI and SII [715]. Moreover, AT<sub>1</sub>R sensors revealed that the cellular context including the expression of G protein and β-arrestins influenced the conformational outputs measured [715]. In addition, sensors engineered within the AT<sub>1</sub>R, and the F Prostanoid receptor have demonstrated the transmission of allosteric information from the AT<sub>1</sub>R to FP suggestive of heterodimer interactions [723]. This allosteric communication was also shown to be dependent on G protein partner  $G\alpha_{q}$  [723]. Besides monitoring GPCR activation and ensuing conformations, biosensors have been extremely useful tools to resolve activation patterns of key GPCR effectors and is discussed below.

#### 1.4.3 Biosensors that report on GPCR signaling and effector activation

RET and intensity-based biosensors have also been built to measure numerous signaling endpoints downstream GPCR activation. Several biosensor designs have measured cAMP, PKA, Rho, ERK<sub>1/2</sub> and  $\beta$ -arrestin activity among other signaling modalities. These biosensors can be intramolecular or intermolecular in design. Intramolecular versions are classically designed with donor and acceptor chromophores fused to a "sensing domain" which can *sense* the activity of the effector being probed (*see* **Figure 1.18**). Effector activation results in a conformational change within the biosensor which either increases or decreases the proximity between the donor and acceptor.

Correspondingly, more or less resonance energy is transferred which correlates with the degree of activity of the effector being assessed. In contrast, intermolecular types have donor and acceptor molecules on distinct effector proteins and typically involve a recruitment or dissociation event as means to report on effector activation or activity. These biosensors are portable as they can be expressed in different *in vitro* cellular vehicles ranging from HEK 293 cells to primary and stem cell-based model systems. These biosensors have also been used in an *in vivo* context to measure activity of kinases in awake, living animals [724, 725].



Figure 1.18 Example of a FRET-based biosensor that reports on ERK<sub>1/2</sub> signaling in live-cells.

(A)Cartoon depiction of a typical FRET-based biosensor. The EKAREV biosensor, used as an example, is built with a substrate binding domain as well as with a WW-phosphopeptide binding domain linked to two reporting fluorophores. Upon the activation of effector molecules such as  $ERK_{1/2}$  kinase, the kinase itself, for example can phosphorylate the substrate domain of the biosensor inciting a conformational change altering the distance between the acceptor (YFP) and donor (CFP) moieties. Changes in the FRET ratio, computed as acceptor emission divided by donor emission, can then be correlated to the level of effector activation/activity. Lower panel reflects  $ERK_{1/2}$  activation patterns, where the 'folded' biosensor configuration is indicative of higher  $ERK_{1/2}$  activity within cells. Figure modified from Hirashima et al. 2022 [726], with permission.

Furthermore, RET-based biosensors can report on compartmentalized signaling within cells. Biosensors have been tagged with nuclear localization (NLS) as well as nuclear export signals (NES) to report on nuclear and cytosolic signaling events respectively. Tags enabling the measurement of Golgi-, mitochondria- [727-729], as well

as myofilament-localized [730, 731] signaling have also been designed. One of the main advantages of the FRET configuration is the notion that it is amenable to microscopy imaging facilitating the assessment of compartmentalized signaling. Using automated high-content microscopes, single-cell resolution is also feasible and provides the ability to parse out distinct cellular populations that response differently to given agonist stimulations [706]. However, one limitation of RET-based sensors, is their limited ability for multiplexing, a strength of intensiometric biosensors discussed hereunder.

#### 1.4.4 Intensiometric-based biosensors

Intensiometric biosensors are circular permutated fluorophores (cpF) that are sensitive to their cellular and subcellular environments (reviewed in [732]). They are usually fused to a sensing domain which acts to sense intracellular changes or protein behaviours within a cell, such as calcium levels, kinase or phosphatase activities which affect the fluorophore's conformation and corresponding optical properties (see Figure **1.19**). The sensing domain can be a single protein with high affinity for the molecule being probed or two proteins which upon binding to the molecule being surveyed leads to a conformational change that affects the spectral properties of cpFP. The most widespread example refers to the fusion of a calmodulin and a M13 binding peptide; a calmodulin binding domain of myosin light chain kinase [733]. In this exemplar, an increase in intracellular calcium causes calmodulin to bind the M13 peptide which translates in changes in the optical properties in the cpFP, increasing its fluorescent output. Changes in optical properties in the cpFP have been associated with the changes in the protonation state, fluorescence quantum yield and the extinction coefficient (explored in [734]). Initially, cpFPs were designed using a circularly permutated GFP. With time, blue, yellow, and red variants have been developed allowing the generation of a toolbox of intensiometric sensors that simultaneously monitor multiple signaling behaviours in a single cell.

The GCaMP variety of cpFPs are calcium-sensitive where an increase in intracellular calcium levels leads to an increase in fluorescence being measured

(reviewed in [735]). Initial intensiometric biosensors were based on a single fluorophore intensity quantified by the amount of light emitted at a characteristic wavelength. However, as mentioned above, this can result in the misrepresentation of the biological event under investigation as fluorescent intensity can also fluctuate due to transfection efficiency, cell density or tissue thickness. To bypass this issue, excitation ratiometric biosensors have been developed. The suite of ExRai biosensors measure biological events through a cpEGFP excitation ratio (see Figure 1.19) [736-738]. This suite of biosensors can monitor PKA, Akt as well as PKC activity in various cellular compartments. These biosensors have been shown to exhibit high signal to noise and high expression in various experimental systems.



#### Figure 1.19 Example of an excitation-ratiometric intensity-based biosensor.

Cartoon depiction of the ExRai-AKAR2 biosensor that reports on PKA activity in living cells. The cpEGFP-based biosensor contains a PKA substrate domain as well as a sensing unit in the form of a phosphor-amino acid-binding domain. Upon PKA activation, the biosensor undergoes a conformational rearrangement and activation is measured through an excitation ratio of 400 nm to 480 nm. Figure from Zhang et al 2022, [738] with permission.

Through the years, numerous types of biosensors have been used to track cellular signaling events in health and disease. Below, we briefly summarise some key accounts that demonstrate the power of biosensors for disease modelling applications.

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#### 1.4.5 Biosensors as tools to track physiological and pathophysiological signaling

There have been some superb reports that have exemplified how FRET or intensiometric biosensors can help illuminate underlying disease mechanisms in the myocardium. For instance, a FRET-based PKA biosensor helped reveal an attenuated PKA response to adrenergic stimulation in DCM iPSC-CMs compared to control [679]. This reduced PKA response was recognized as being a result of higher phosphodiesterase levels since inhibitors helped rescue the PKA response, as discussed earlier [679]. In another study, a sarcomeric FRET-based biosensor, TPNI-CUTie helped demonstrate that TnT-R173W DCM iPSC-CMs exhibited increased cAMP signaling [678]. This enhancement was reasoned to be compensatory in nature due to decreased PKA responsiveness and contractility in DCM [678]. Both accounts demonstrate the ability of biosensors to help elucidate molecular underpinnings of disease.

A FRET-based cAMP sensor has also been used to track signaling over time examining day 30-, 60- and 90-day old iPSC-CMs [739]. In mature, day 90 iPSC-CMs, cytosolic cAMP activity was smaller compared to day 30 cells, an observation identified because of higher  $\beta_2AR$  compartmentalization in caveolar microdomains at later stages [739]. The authors also urged caution, as even their 90-day old iPSC-CMs resembled more neonatal counterparts. Thus, besides disease modelling, biosensors can also help track how maturation impacts signaling transduction mechanisms within iPSC-CMs. Relatedly, it has also been shown that day 30 cardiomyocytes exhibit more  $\beta_2AR$ signaling while a larger portion of older cultures signal via  $\beta_1$ -adrenergic receptor pools [740].

In adult rat cardiomyocytes, EKAR, a biosensor that monitors ERK<sub>1/2</sub> signaling was able to illuminate signaling at the single-cell level. Stimulation of adult myocytes with phenylephrine that targets  $\alpha_1$ -adrenergic receptors revealed that 60% of the cells responded to the agonist [704]. This population was sensitive to a MEK-inhibitor demonstrating specificity. In comparison, stimulation of myocytes with Ang II did not result

in significant ERK<sub>1/2</sub> activation [704]. Likewise, in adult ventricular myocytes, AKAR3, a FRET-based PKA sensor revealed that  $\beta_1$ AR and  $\beta_2$ AR receptor pools distinctively control nuclear PKA signaling [741]. It will be interesting to compare how signal transduction events occurring in iPSC-CMs relate to neonatal versus adult primary cardiomyocytes derived from rodent models. Adult rodent cardiomyocytes may be useful as means to compare how various maturation strategies influence pathway activation.

Besides 2-dimensional models, biosensors have also been used in 3D contexts of the heart. For example, in an organoid model of myocardial infarction, GCaMP6, a calcium biosensor was used to examine whether control versus infarct organoids displayed different calcium transients [696]. In infarct organoids, reduced calcium transients were observed and further attributed to the fibrotic response [696]. Similarly, in engineered heart tissue (EHT), genetically-encoded calcium and voltage indicators have been used. Under the control of cTnT, GCaMP5, a calcium sensor, revealed characteristic calcium transients that fluctuated during contractions and were sensitive to drug stimulations [693]. In the same vein, GEVIs such as ArcLight, which are genetically-encoded voltage indicators have also been used in EHT [693]. These indicators allow to monitor cardiac action potentials and may have great utility for drug screening or disease modelling of arrhythmogenic conditions.

#### **1.5 Rationale and objectives**

When I began in the lab several years ago, the incubators were filled with HEK 293 cell cultures. Apart from one graduate student who cultured primary cells, HEK 293 cells were used to study various aspects of GPCR biosynthesis, internalization, and signaling. In the first chapter of my dissertation, I also used HEK 293 cells as vehicles to study the conformational dynamics of the  $\beta_2$ -adrenergic receptor. Following this study, my supervisor presented a lab meeting discussing the need to transition to more physiologically relevant cellular models. It was essentially argued that the current attrition rates in drug discovery spoke to the much-needed modernization of how drug discovery was performed. Better disease models and better tools were required to address this. From then on, my goal was to develop tools and more physiologically relevant models for research, disease modelling and drug discovery purposes.

Of particular note, I was interested in developing an *in vitro* cellular model of the human cardiomyocyte for our laboratory. With advances in the field of stem cell research, a routine blood draw offered enough patient material for somatic cell reprogramming into iPSCs followed by the generation of cardiomyocytes for disease modelling or drug discovery. Stem cell and affiliated technologies thus opened a new chapter in cardiac disease modelling as we can study disease mechanisms in patient derived cells - ranging from cardiomyocytes to cardiac fibroblasts including 2- and 3-dimensional models. This is the basis of this thesis, leveraging state-of-the-art experimental systems including 2dimensional stem cell models of the human cardiomyocyte to understand how signaling, or other cellular perturbations result in disease. Being able to model human disease progression in iPSC-derived cardiomyocyte cultures in a patient-specific manner is unique and allows for the development of a "bedside to bench back to bedside" pipeline for cardiovascular disease modelling. By characterizing the pathobiology of the disease in a patient-specific manner, we aim to understand personalized mechanisms for the occurrence and development of dilated cardiomyopathy. Uniting basic and clinical research platforms is thus essential to understand and discover novel therapeutic strategies to treat cardiomyopathies.

#### 1.5.1 Statement of purpose

The overall objectives of this thesis were, first, to design and develop biosensors that can be used in the context of drug discovery to track GPCR activation and conformation. Secondly, I aimed to develop innovative physiological and pathophysiological human models of the cardiomyocyte that can be used in the context of disease modelling as means to progressively replace heterologous cell systems and primary rodent cultures. Lastly, I aimed to show that induced pluripotent stem cell-derived cardiomyocytes can be combined with biosensor-based approaches to assess cellular signaling in relevant human contexts and to understand how dysregulated signaling contributes to disease progression.

The individual objectives of the three chapters presented in this thesis are as follows:

- To design and develop conformation-sensitive biosensors to report on the conformational dynamics of the human β<sub>2</sub>-adrenergic receptor.
- 2) To study cellular signaling patterns at single-cell resolution in primary rat neonatal cardiomyocytes and human iPSC-derived cardiomyocytes. We used FRET-based and intensiometric biosensors and performed a head-to-head comparison evaluating how rodent and human iPSC-derived cardiomyocytes behaved in response to numerous ligands that target cardiac-relevant GPCRs. These studies were complemented with bulk transcriptomics.
- 3) To develop an iterative bedside-to-bench-bedside platform that can be used to inform rational and personalized treatment options for patients diagnosed with idiopathic dilated cardiomyopathy. Here, we demonstrate proof of concept, where patient blood cells can be reprogrammed into induced pluripotent stem cells and further differentiated into cardiomyocytes as vehicles to study the underlying disease-causing mechanisms and test potential therapies. We established a pipeline that uses genetically-encoded biosensors to evaluate signaling signatures

in control versus diseased iPSC-derived cardiomyocytes as means to identify dysregulated pathways that may be targeted therapeutically to improve patient outcomes.

# **Chapter 2 :** Distinct Conformational Dynamics of Three G Protein-Coupled Receptors Measured Using FIAsH-BRET Biosensors

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Running Title: GPCRs show distinct conformational dynamics

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#### 2.1 Preface

At the time, receptor conformation was a hot topic as several GPCR crystal structures were being captured using x-ray crystallography. In a sense, conformation was coined "king" considering the well accepted notion that receptor conformation dictates signaling transduction and overall receptor function. However, trials of GPCR structure determination required immense time and efforts and the structures provided "snap-shots' of receptor states that were representative of the most stable receptor conformations. There was a need to develop assays that would assess GPCR conformation on a *larger* scale. A scale amenable for drug discovery platforms, for instance. In this vein, our lab aimed at developing assays with higher throughput with the ability of capturing the dynamic conformational landscapes of GPCRs. This brought about the FIAsH-BRET approach that allowed the measurement of GPCR conformation in real-time and in live cells. Here, the FIAsH tag was essentially "walked" throughout the coding sequence of various GPCRs for the development and validation of biosensors for drug screening applications. The goal was to develop sensors that could be used in high-throughput drug screens yielding the identification of novel drugs based on the conformation they stabilized. In theory, the conformations observed could be correlated with various types of agonists such that they would be able to distinguish full, partial, inverse, biased and neutral agonists based on the conformational signature observed. In addition, the portability of this approach was of value and these sensors can be expressed in various cell types and contexts and report on receptor conformation. Conformation-sensitive biosensors designed in the human angiotensin II type I as well as in the F Prostanoid receptor had previously been designed and validated in our lab. This chapter intended to expand on this earlier work, aiming for the design of conformation-sensitive sensors within the cardiac relevant  $\beta_2$ -adrenergic receptor.

#### 2.2 Abstract

A number of studies have profiled G protein-coupled receptor (GPCR) conformation using fluorescent biarsenical hairpin binders (FIAsH) as acceptors for BRET or FRET. These conformation-sensitive biosensors allow reporting of movements occurring on the intracellular surface of a receptor to investigate mechanisms of receptor activation and function. Here, we generated eight FIAsH-BRET-based biosensors within the sequence of the  $\beta_2$ -adrenergic receptor ( $\beta_2AR$ ) and compared agonist-induced responses to the angiotensin II receptor type I (AT<sub>1</sub>R) and the prostaglandin F2a receptor (FP). Although all three receptors had FIAsH-binding sequences engineered into the third intracellular loops and carboxyl-terminal domain, both the magnitude and kinetics of the BRET responses to ligand were receptor-specific. Biosensors in ICL3 of both the AT<sub>1</sub>R and FP responded robustly when stimulated with their respective full agonists as opposed to the  $\beta_2$ AR where responses in the third intracellular loop were weak and transient when engaged by isoproterenol. C-tail sensors responses were more robust in the  $\beta_2AR$  and AT<sub>1</sub>R but not in FP. Even though GPCRs share the heptahelical topology and are expressed in the same cellular background, different receptors have unique conformational fingerprints.

#### **2.3 Introduction**

Understanding the dynamic nature of G protein-coupled receptors (GPCRs) is critical given their capacity to modulate numerous biological responses in health and disease. Largely localized to the plasma membrane, GPCRs respond to an array of extracellular stimuli including photons, odors, hormones, peptides, lipids, and sugars [1]. With over 800 genes expressed in the human genome, they are found in nearly every organ of the body [2, 3]. The  $\beta_2$ -adrenergic receptor ( $\beta_2AR$ ) is one of the most studied GPCRs and is tightly regulated as part of elaborate multicomponent signaling networks. Upon ligand binding, the receptor undergoes a conformational change that stimulates the exchange of guanine diphosphate for guanine triphosphate in the G $\alpha_s$  subunit leading to the functional dissociation of the G $\beta\gamma$  dimer from G $\alpha$  [1]. These G proteins then independently act on downstream effector molecules in a number of signaling cascades. This simplified notion of receptor activation provides only a glimpse into the complex processes of signal transduction, of which we have much to learn.

Understanding GPCR function involves determining how agonist binding translates into receptor activation. The traditional view of receptor activation has evolved from where it was initially thought of as a switch from a single inactive state to a single active state. Now it is widely accepted that the receptor pool in any given cell can occupy a number of different inactive and active conformations [4–6]. At equilibrium, there are numerous conformations within the receptor population and different orthosteric and allosteric ligands can stabilize diverse receptor states. The fundamental mechanisms of GPCR activation have been investigated by several groups using diverse techniques, including but not limited to nuclear magnetic resonance, double electron–electron resonance, and fluorescence spectroscopy [4, 7–9]. Both fluorescence and bioluminescence resonance energy transfer (FRET and BRET) approaches have also been used to explore the conformational dynamics of GPCRs [9–14]. The site-specific introduction of the short tetracysteine motif CCPGCC within the coding frame of a receptor when labeled with a fluorescence derivative can be used in resonance energy

transfer (RET) applications to report on conformations adopted by the receptor upon ligand binding in living cells [15, 16].

We have explored the use of FIAsH BRET in the conformational profiling of the prostaglandin F2α receptor [FP; [11]], and the angiotensin II type I receptor [AT<sub>1</sub>R; [17]]. Here, we introduced this tetracysteine tag at various locations within the coding sequence of the  $\beta_2AR$  in order to report on conformational changes upon agonist stimulation. Eight such biosensors were constructed; two within the second intracellular loop, three in the third intracellular loop, and three in the carboxyl terminus of the receptor. In a previous study, the  $\beta_2$ AR was tagged using FIAsH FRET [18]. In that work, the third intracellular loop was tagged with the FIAsH motif and the carboxyl terminus with CFP after having truncated the C-tail at amino acid 343 [18]. Upon agonist stimulation, an increase in the FRET ratio was observed suggesting that the third intracellular loop approaches the Cterminus [18]. Other groups have also attempted to understand the conformational dynamics of the  $\beta_2AR$  while using fluorescence-based probes as indicators of conformational changes occurring in real-time. Lohse and colleagues have generated FRET-based biosensors incorporating YFP in the third loop and CFP in the C-terminus of the  $\beta_2$ AR [19]. Again, the receptor was truncated at amino acid 369. To our knowledge, our study is the first report of using the full-length  $\beta_2AR$  tagged with reporter proteins to monitor conformations adopted by the receptor upon agonist stimulation. Further, we compare and contrast three distinct GPCRs and show that even though they share a similar seven transmembrane architecture, they behave very differently in regards to the magnitude and kinetics of their BRET responses.

#### 2.4 Materials and Methods

#### 2.4.1 Materials

#### 2.4.1.1 Primers

All primers were synthesized and purchased by Integrated DNA Technologies (Coralville, IA, USA, see Table 2.1).

Table 2.1 List of primers used for the generation of the  $\beta_2$ -adrenergic receptor

Position	Sequence $(5' \rightarrow 3')$
ICL2 p1	F: TGCTGCCCCGGCTGCTGCAGCCTGCTGA R: GCAGCAGCCGGGGCAGCACTGGTACTTG
ICL2 p2	F: TGCTGCCCCGGCTGCTGCCCTTTCAAGTACCAGAGC R: GCAGCAGCCGGGGCAGCATGAAGTAATGGCAAAGTAGC
ICL3 p1	F: TGCTGCCCCGGCTGCTGCCATGTCCAGA R:GCAGCAGCCGGGGCAGCAGAAGCGGCC
ICL3 p2	F: TGCTGCCCCGGCTGCTGCGAGCAGGATG R: GCAGCAGCCGGGGCAGCACCTGGCT
ICL3 p3	F: TGCTGCCCCGGCTGCTGCGGACTCCGCA R: GCAGCAGCCGGGGCAGCAATGCCCCGT
C-tail p1	F: TGCTGCCCCGGCTGCTGCGCCTATGGGA R: GCAGCAGCCGGGGCAGCACTTCAAAGA
C-tail p2	F: TGCTGCCCCGGCTGCTGCAATAAACTGC R: GCAGCAGCCGGGGCAGCATTCTTTCTCC
C-tail p3	F: TGCTGCCCCGGCTGCTGCCATCAAGGTA R: GCAGCAGCCGGGGCAGCAGCCCACAAA
Name	Sequence $(5' \rightarrow 3')$
BamHI β₂AF	F: CAGTGGATCCATGGGGCAACCCGGGAAC
β₂AR EcoRI Ba	mHI R: CTCCGGATCCGAATTCCAGCAGTGAGTC
Nhel Xhol Koz SP	zak F: CCTAGCTAGCTCGAGGCCACCATGAA

 $(\beta_2AR)$  FIAsH-BRET-based recombinant biosensors.

#### 2.4.1.2 Constructs

The recombinant receptors used in this paper are as follows: SP-FLAG-hAT<sub>1</sub>R-CCPGCC-ICL3-p3-RlucII or SP-FLAG-hAT<sub>1</sub>R-CCPGCC-C-tail-p1-RlucII in a pIRESH plasmid backbone [17] along with SP-HA-hFP-CCPGCC-ICL3-p4-RlucII in a pcDNA3.1(–) backbone [11], in addition to the panel of eight  $\beta_2$ AR biosensors expressed in a pIRESpuro3 plasmid backbone.

# 2.4.2 Generation of FIAsH-BRET-Based Biosensors

The intramolecular biosensors were designed to harbor the tetracysteine tag positioned at various locations within the intracellular surface of the receptor in addition to a C-terminally fused Renilla luciferase. More precisely, the CCPGCC tag was inserted in two positions within the second intracellular loop, three within the third, and three within the carboxyl terminus domain of the receptor. For ease of cloning, compatible restriction sites were introduced by polymerase chain reaction (PCR) at the 5' and 3' ends of the receptor to facilitate its insertion into its corresponding mammalian expression vector. Briefly, HA-tagged h $\beta_2$ AR [20] in a pcDNA3.1(–) backbone vector was used as a template and amplified by PCR using the BamHI- $\beta_2$ AR forward and the  $\beta_2$ AR-EcoRI-BamHI reverse primers. The resulting PCR product was cloned into an accepting vector; pIRESpuro3-signal peptide-HA-RlucII using *Bam*HI. We screened for correct orientation using *Pst*. The introduction of the CCPGCC motif was accomplished by overlapping PCR where the wild-type receptor was flanked by the appropriate primers (Table 2.1) in order to introduce the desired TC tag within the coding sequence [11]. In the first round, fragment one was generated using Nhel-Xhol-forward primer and the appropriate FIAsH internal reverse primer. Fragment 2 was generated using the appropriate FIAsH internal forward primer and  $\beta_2 AR$ -*Eco*RI-*Bam*HI reverse primer. Both fragments were then combined in equal portions and used as templates for the second round of PCR using *Nhel-Xhol-Kozak-* $\beta_2$ AR forward and  $\beta_2$ AR-*Eco*RI-*Bam*HI reverse primer. This product was cloned into pIRESpuro3-SP-HA-RlucII backbone using Nhel and EcoRI. All constructs were confirmed by bidirectional sequencing (Génome Québec).

#### 2.4.3 Cell Culture

HEK 293 SL cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 5% vol/vol fetal bovine serum and 1% w/v penicillin–streptomycin from Wisent. The cells were maintained in a controlled environment, 37°C in a humidified atmosphere at 95% air and 5% CO<sub>2</sub>.

# 2.4.4 Transient Transfection

HEK 293 SL cells were plated at a density of  $2.0 \times 10^5$  cells per well in clear 6-well plates (Thermo Scientific, 140675) prior to transfection. On the following day, cells were

transfected with 1 µg of each of the eight  $\beta_2$ AR FIAsH biosensors along with pcDNA3.1(–) for a total of 1.5 µg per well using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. Alternatively, 1 µg of AT<sub>1</sub>R-ICL3-p3-RlucII or AT<sub>1</sub>R-C-tail-p1-RlucII and 500 ng of the FP-ICL3-p4-RlucII biosensor was also used.

#### 2.4.5 Immunofluorescence

The day following transfection, cells were detached with 0.25% Trypsin–EDTA (Wisent) and 2.0 × 10<sup>4</sup> cells were re-plated onto a poly-l-ornithine (Sigma-Aldrich) treated clear bottom black 96-well plate (Thermo Scientific, 165305). The next day, the cells were washed once with phosphate-buffered saline (PBS) and fixed with 2% paraformaldehyde (Sigma-Aldrich) for 10 min at room temperature. Successively, the cells were blocked with a 1% bovine serum albumin (Fisher Scientific) PBS solution for 1 h at room temperature to prevent non-specific interactions of the antibodies. Cells were then incubated with a monoclonal mouse anti-HA primary antibody for 1 h (BioLegend, 1:200, previously Covance). Afterward, the cells were washed three times with PBS and an Alexa fluor-488 goat anti-mouse IgG secondary antibody (Life Technologies, 1:1,000) was used to label cells. To confirm the ability of recombinant receptors to localize to the cell surface, the Operetta High Content Imaging system (Perkin Elmer) with a 20× WD objective was used. The excitation filter was set at 475/15 nm and its corresponding emission filter at 525/25 which permitted to capture the signal produced by Alexa fluor-488.

# 2.4.6 Gas Coupling and Downstream cAMP Production

HEK 293 SL cells were transfected with 1  $\mu$ g of each of the eight  $\beta_2$ AR FIAsH biosensors and the  $\beta_2$ AR-WT-RlucII aconstruct supplemented with 0.5  $\mu$ g of the previously described H188 EPAC FRET sensor [21] using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. The day following transfection, cells were detached with 0.25% Trypsin–EDTA (Wisent) and 4.0 × 10<sup>4</sup> cells were re-plated onto a poly-I-ornithine (Sigma-Aldrich) treated black flat bottom 96-well plate (Costar, 3916). The day of the experiment, cells were washed once in 150  $\mu$ L of Krebs buffer and the cells then sat in 90  $\mu$ L of Krebs at 37°C prior to the start of the assay. A Synergy 2

plate reader (Biotek) was used to assay coupling of the  $\beta_2$ AR FIAsH biosensors to G $\alpha_s$  by investigating accumulation of cAMP. The temperature of the instrument was set at 37°C and kinetic measurements were taken. The 420/50 excitation filter was used to excite the donor molecule, mTurquoise2, and light was captured by the emission filters 485/20 (mTurquoise2) and 528/20 (Venus). Basal FRET was measured continuously every 5 s for a total of 20 s. Cells were then treated with either the vehicle (ascorbic acid) or the full agonist, 10 µM isoproterenol (in ascorbic acid) using the injector module. Stimulated FRET readings were then captured every 5 s for a total time of 2 min. FRET ratios were computed by dividing the Venus emission channel by the mTurquoise2 emission channel.  $\Delta$ FRET ratios were calculated by subtracting the averaged isoproterenol stimulated FRET ratio by the averaged basal FRET ratio, as shown;  $\Delta$ FRET = (avgFRET<sub>stimulated</sub> – avgFRET<sub>basal</sub>).

#### 2.4.7 ERK<sub>1/2</sub> MAP Kinase Activation

Twenty-four hours post-transfection, cells were detached with 0.25% Trypsin– EDTA (Wisent) and 400  $\mu$ L of cell suspension was re-plated onto a clear 12-well plate (Costar, 3513). On the day of the experiment, the cells were starved in DMEM without serum supplementation for 5 h. Afterward, cells were stimulated with either vehicle or 10  $\mu$ M isoproterenol for 5 min at 37°C. The plate was then placed on ice, where the cells were washed once with an ice-cold PBS solution. The cells were lysed in 200  $\mu$ L Laemmli buffer (2% SDS, 10% glycerol, 60 mM Tris pH 6.8, 0.02% bromophenol blue, 5%  $\beta$ mercaptoethanol). In order to shear the genomic DNA, lysates were sonicated three times, each repetition for 5 s at 3 W using a Sonicatior 3000 (Misonix). Lysates were then heated at 65°C for 15 min.

MAP kinase activation was measured by western blot. Correspondingly, 30 µL of cell lysate was loaded and proteins were, respectively, separated by SDS-PAGE and then transferred onto a PVDF membrane *via* a wet transfer technique. To prevent non-specific binding of the primary antibody, the membrane was blocked in a 5% non-fat milk solution in Tris-buffered saline and 0.0005% Tween20 solution. An anti-phospho-ERK<sub>1/2</sub> rabbit primary antibody was used (Cell Signalling Technologies, 1:1,000) followed by an anti-

rabbit polyclonal IgG peroxidase secondary antibody (Santa Cruz Biotechnology, 1:20,000). Immuno-detection was accomplished *via* chemiluminescence using Western Lightning plus-ECL (Perkin Elmer) or ECL-Select western blotting detection reagent (GE Healthcare) given that the secondary antibody was conjugated to the horseradish peroxidase enzyme.

# 2.4.8 FIAsH Labeling

Twenty-four hours post-transfection, cells were detached with 0.25% Trypsin-EDTA (Wisent) and  $4.0 \times 10^4$  cells were re-plated onto a poly-I-ornithine (Sigma-Aldrich) treated white 96-well plate (Thermo Scientific, 236105). The next morning, a 25 mM solution of 1,2-ethanedithiol (EDT) was prepared by diluting it in dimethyl sulfoxide. Then, one volume of FIAsH reagent (2 mM) was added to two volumes of EDT to make a 667 µM FIAsH (Invitrogen) solution, which was incubated for 10 min at room temperature. Following the incubation, 100 µL of Hank's balanced salt solution (HBSS) without phenol red, with sodium bicarbonate, calcium, and magnesium was added to the 667 µM FIAsH solution and further incubated for 5 min at room temperature (Wisent). Then, HBSS was added to make a solution with final concentration of 750 nM FIAsH-EDT<sub>2</sub>. In parallel, cells were washed in 150 µL of HBSS prior to the FIAsH labeling. Subsequently, 60 µL of the 750 nM FIAsH-EDT<sub>2</sub> solution was added to the cells and incubated for 1 h at 37°C, protected from any source of direct light. Following the incubation, cells were washed once with 100 µL of 100 µM 2,3-dimercapto-1-propanol (BAL, Invitrogen) diluted in HBSS buffer and then incubated for 10 min at 37°C. The cells were washed once again with BAL without incubation. Afterward, cells were washed once with 150 µL of the assay buffer: Krebs (146 mM NaCl, 4.2 mM KCl, 0.5 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 10 mM HEPES pH 7.4, 0.1% glucose). The cells then sat in 80 µL of Krebs for 2 h at room temperature, in an environment protected from light, prior to the BRET assay. The FIAsH labeling procedure has been previously described elsewhere [11].

# 2.4.9 BRET Measurements

A TriStar2 LB 942 multimode plate reader from Berthold Technologies was used to measure BRET using the pre-determined BRET1 filter pair F485 and F530. Light was produced via enzymatic catalysis of the luciferase substrate coelenterazine h by the donor Rlucll. Accordingly, 10  $\mu$ L of a 2  $\mu$ M coelenterazine h solution (NanoLight Technologies) was added to the cells and incubated for 5 min whereafter the luminescence was measured. Basal BRET corrected from spectral overlap of the donor and acceptor channels were calculated by subtracting the BRET value obtained from unlabeled cells expressing solely the donor from the corresponding BRET value obtained from the labeled FIAsH recombinant receptors. Additionally, ligand-induced changes were investigated and kinetic readings were reported. Correspondingly, the counting time of the two filters was analyzed continuously every 0.2 s for a total of 50 repeats. Subsequently, either vehicle or a saturating concentration of the agonist, 10 µM isoproterenol, was injected using the injector module. For the AT1R, 1 µM angiotensin II was used and 1  $\mu$ M PGF2 $\alpha$  for FP. Thereafter, the luminescence was again captured every 0.2 s and a total of 100 repeats. The change in BRET, as a response to the addition of agonist or the  $\Delta BRET$ , as referred to in this paper, was computed by subtracting the average BRET across all reads pre-injection from the average BRET across all reads post-injection:  $\Delta BRET = (avgBRET_{post-injection}) - (avgBRET_{pre-injection})$ .

#### 2.4.10 Statistical Analysis

All statistical analysis was performed using GraphPad Prism 7.0 software. Data are reported as mean  $\pm$  SE. The Prism software performed a Brown–Forsythe test to determine if parametric or non-parametric statistics should be performed. The degree of G $\alpha_s$  coupling was evaluated using a one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test comparing the various FIAsH positions to the wild type (Figures 2.3A, B). When determining the basal BRET exhibited by each of the recombinant  $\beta_2$ AR biosensors, a one-way ANOVA was performed. A Dunnett's post hoc test was successively completed with the purpose of comparing the basal BRET of the eight recombinant constructs to the wild-type receptor (Figure 2.4A). When evaluating the agonist-induced BRET response, a two-way ANOVA was carried out followed by a Bonferroni corrected Student's t-test aimed at comparing the response of the vehicle to the response of the agonist for each individual sensor position (Figure 2.4B).

#### 2.5 Results

#### 2.5.1 Biosensor Validation

We constructed a number of FIAsH-BRET biosensors in the  $\beta_2$ AR with a FIAsHbinding site engineered into various intracellular sites and Renilla luciferase placed on the carboxy terminus (Figure 2.1). If the biosensor components are positioned at appropriate sites within the receptor then this would allow profiling of conformational changes in the receptor upon ligand stimulation. In order for our intramolecular BRET constructs to be meaningful tools for the study of receptor conformational dynamics, recombinant receptors must maintain their native function. If they do not function in a manner similar to the wildtype receptor, then conformational analysis will be meaningless. Immunofluorescence was first used to verify the surface localization of the recombinant receptors generated. An anti-HA antibody was used to label the recombinant receptors, followed by an Alexa fluor-488 conjugated secondary antibody. As illustrated in Figure **2.2**, almost all the FIAsH-tagged  $\beta_2$ AR constructs trafficked to the cell surface. Receptors tagged within the second intracellular loop were less robustly expressed compared to the wild type. However, the fluorescence intensity for all other positions was similar to the wild type providing us with at least six positions to carry forward.



в

β<sub>2</sub>AR ICL2 p1 (Q142-S143) DRYFAITSPFKYQ CCPGCC SLLTKNK

BAR ICL2 p1 (S137-P138) DRYFAITS CCPECC PFKYQSLITKNK BAR ICL3 p1 (F240-H241) RVFQEAKRQLQKIDKSEGRF CCPGCC HVQNLSQVEQDGRTGHGLRRSSKFCLKEHKALKT

5-AR ICL3 p2 (V248-E249) RVFQEAKRQLQKIDKSEGRFHVQNLSQV CCPGCC EQDGRTGHGLRRSSKFCLKEHKALKT

β<sub>2</sub>AR ICL3 p3 (H256-G257) RVFQEAKRQLQKIDKSEGRFHVQNLSQVEQDGRTGH CCPGCC GLRRSSKFCLKEHKALKT B-AR C-tail p1 (K348-A349) CRSPDFRIAFQELLCLRRSSLK CCPGCC AYGNGYSSNGNTGEQSGYHVEQEKENKLLCEDLPGTEDFVGHQGTVPSDNIDSQGRNCSTNDSLL β<sub>2</sub>AR C-tail p2 (E373-N374) CRSPDFRIAFQELLCLRRSSLKAYGNGYSSNGNTGEQSGYHVEQEKE CCFGCC NKLLCEDLPGTEDFVGHQGTVPSDNIDSQGRNCSTNDSLL β<sub>2</sub>AR C-tail p3 (G389-H390) CRSPDFRIAFQELLCLRRSSLKAYGNGYSSNGNTGEQSGYHVEQEKENKLLCEDLPGTEDFVG CCPGCC HQGTVPSDNIDSQGRNCSTNDSLL

# Figure 2.1 Positions of $\beta_2$ -adrenergic receptor ( $\beta_2 AR$ ) conformation sensitive biosensors.

(A) Schematic of the  $\beta_2AR$  FIAsH BRET-based intramolecular biosensors. The Nterminus and C-terminus are fused with an HA tag and RlucII respectively. The FIAsH motif was introduced at specific sites within the second, third loop and carboxyl terminus of the receptor. (B) Positioning of the FIAsH tags within the sequence of the  $\beta_2AR$ . The sequence of the receptor is shown in black where the position of the FIAsH tag is highlighted in red.

Next, to further validate the functionality of each construct, we measured isoproterenol-mediated cAMP accumulation as well as ERK<sub>1/2</sub> MAPK activation. We studied the relative accumulation of cAMP as an indication of Ga<sub>s</sub> activation using the H188 FRET-based EPAC sensor. FRET was used as BRET-based EPAC biosensors could not be used with the BRET-based conformational biosensors (as both would be activated). As shown in **Figures 2.3A**, **B**, the majority of constructs displayed similar levels of cAMP accumulation (measured as a decrease in FRET) as compared to the untagged wild-type receptor. As the  $\beta_2$ AR shows agonist-independent basal activity, we examined both basal (**Figure 2.3A**) and agonist-stimulated FRET (**Figure 2.3B**). We observed that certain sensor positions exhibited high basal activity as shown as the reduced FRET ratio at baseline. This may have been as a result of higher expression levels of these biosensors. For example, the C-tail P3 position may seem as though there is less cAMP production than the wild type in response to agonist; however, the lack of a robust decrease in FRET as a response to agonist is probably due to having attained the threshold of detection at basal levels.



Figure 2.2 Cell surface localization of the  $\beta_2$ -adrenergic receptor ( $\beta_2 AR$ ) FIAsH BRET-based biosensors.

(A) Fluorescence microscopy validating cell surface localization of the FIAsH tagged intramolecular  $\beta_2AR$  biosensors. Immunofluorescence images of nonpermeabilized HEK 293 SL cells transiently transfected with the recombinant  $\beta_2AR$  constructs demonstrating their membrane localization. The cells were incubated with an anti-HA primary antibody and then stained with an Alexa-fluor-488 conjugated secondary antibody. Images were taken using the Operetta high content microscope (Perkin Elmer). We then examined a more distal readout of receptor functionality; the  $\beta_2$ ARmediated MAPK (Raf/Ras/MEK/ERK) signaling pathway that has been previously characterized by various groups [22, 23]. As demonstrated by **Figure 2.3C**, all the recombinant  $\beta_2$ AR constructs exhibited MAPK activation at similar intensities as the wildtype receptor. As a result, the third intracellular loop sensors as well as the C-tail sensors passed the validation stage although some caution again must be taken when interpreting results using the sensors engineered into the second intracellular loop. It must be noted here that all our transfections were transient and no attempt was made to normalize levels of expression *per se*.



# Figure 2.3 Functional characterization of the $\beta_2$ -adrenergic receptor ( $\beta_2 AR$ ) FIAsH BRET-based biosensors.

(A) Basal FRET demonstrating agonist-independent activity of  $\beta_2AR$  FIAsH-BRETbased biosensors. Data represent results of three independent experiments. Error bars represent mean  $\pm$  SE. One-way analysis of variance (ANOVA) was performed followed by Dunnett's post hoc test. (B) Assessment of cAMP accumulation using the H188 FRET-based EPAC sensor in response to 10 µM isoproterenol. The  $\Delta$ FRET ratio was obtained by subtracting the averaged basal FRET from the averaged isoproterenolstimulated FRET ratio (post – pre-injection FRET). One-way ANOVA was performed on results from three independent experiments followed by Dunnett's post hoc test; asterisk represents \*p ≤ 0.05. (C) Representative western blot probing activation of the ERK<sub>1/2</sub> mitogen-activated protein kinase signaling pathway. HEK 293 SL cells were transiently transfected with the wild-type or recombinant  $\beta_2AR$  constructs. Cells were stimulated with vehicle (–) or 10  $\mu$ M isoproterenol (+) for 5 min. Cell lysates were collected and a western blot was performed using an ERK<sub>1/2</sub> monoclonal rabbit primary antibody followed by an anti-rabbit secondary antibody conjugated to the horseradish peroxidase substrate. Phospho-ERK<sub>1/2</sub> is shown in the upper panel while total ERK<sub>1/2</sub> is shown in the lower panel. Data are representative of three independent experiments.

# 2.5.2 BRET Measurements

Next, we measured basal BRET between the FIAsH-labeled receptors and the Ctail luciferase. Basal BRET or the BRET ratio after it has been corrected for spectral overlap of the donor and acceptor channels was determined by subtracting BRET where cells were not labeled with FIAsH. All receptor biosensors showed basal BRET to varying degrees (**Figure 2.4A**). The larger the basal BRET, the closer the donor–acceptor pair was at the outset. As a result, there is a greater dynamic range to capture relative changes in receptor conformation. The  $\beta_2AR$  biosensor with the greatest basal BRET was the third position within the C-tail. There was a position-dependent increase in the basal BRET, as one moves farther down the tail of the receptor, as acceptor and donor moieties get closer together. As for the third loop, the second position showed the largest basal BRET which is in accordance to its position in the middle of the loop (**Figure 2.4A**).



# Figure 2.4 Assessment of the agonist induced conformational change in the intracellular surface of the $\beta_2$ -adrenergic receptor ( $\beta_2AR$ ).

(A) Basal BRET for the panel of eight  $\beta_2$ AR FIAsH BRET-based biosensors. For each recombinant receptor, basal BRET readings were calculated after having corrected them for spectral overlap by subtracting the BRET ratio obtained from unlabeled receptors expressing solely the donor from basal BRET readings of the recombinant receptors. Six technical replicates were performed and subsequently averaged; error bars represent mean  $\pm$  s.e.m. One-way ANOVA was performed followed by Dunnett's post-hoc test. (B)

Conformational changes within the cytoplasmic region of the  $\beta_2AR$  in response to isoproterenol were measured.  $\Delta BRET$  was calculated by subtracting averaged preinjection BRET from post-injection readings. Population-based measurements were recorded on 40,000 cells except for the  $AT_1R$  C-tail p1 where 30,000 cells were used. All readings were taken using the Tristar multimode plate reader (Berthold Technologies) except for the  $AT_1R$  C-tail p1 which was assayed on the Victor X Light (Perkin Elmer). Data represent means of three or more independent experiments; error bars represent mean  $\pm$  S.E. One-way ANOVA was performed followed by Bonferroni corrected t-tests. Asterisks represent \* p ≤ 0.05, \*\* p ≤ 0.01, \*\*\* p ≤ 0.001, \*\*\*\* p ≤ 0.0001.

ΔBRET in response to ligand was measured by subtracting the averaged postinjection BRET from the averaged pre-injection BRET readings. BRET ratios could potentially increase or decrease depending on the ligand used and the subsequent conformation adopted by the receptor. It was hoped that our biosensors would differentially respond to ligands and provide a conformational fingerprint to better understand the dynamic nature of the receptor which could be exploited for validating new drugs in early phases of development. Of all the biosensors tested, only the C-tail positions P2 and P3 showed a robust conformational change upon isoproterenol stimulation (**Figure 2.4B**). The lack of response in ICL3 was somewhat of a surprise but the functional data (**Figures 2.2 and 2.3**) suggested that ICL2 sensors may not be correctly folded.

In order to make a comprehensive assessment of the isoproterenol-induced responses of the β<sub>2</sub>AR biosensors, we also examined the underlying kinetics. As mentioned, neither the second or third loop positions captured a sustained conformational change in response to isoproterenol (**Figures 2.4B and 2.5**). Oddly, a small spike was a consistent feature of the ligand-induced response in these sensors with the exception of ICL3 P1 (**Figures 2.5D–F**). The presence of this spike was not an artifact originating from the sampling instrument as no such spikes were seen when vehicle was similarly injected and it was also absent from kinetic traces of the wild-type receptor expressing RlucII with no FIAsH-binding sequences (**Figure 2.5A**). The C-tail P1 sensor displayed similar features as the second and third loop positions (**Figures 2.5 and 2.6A**). However, the responses in the other C-tail sensors were much more robust and sustained (**Figures 2.6B, C**). We have previously analyzed responses to ligand in both FP [11] and in the

AT<sub>1</sub>R [17]. Responses in ICL3 in AT<sub>1</sub>R and FP were both robust and sustained (**Figures 2.6D**, **E**) compared to the  $\beta_2$ AR. Further, robust sustained responses have also been detected in both ICL2 and the C-terminus of AT<sub>1</sub>R [[17]; **Figure 2.6F**]. Interestingly, no responses were detected in similar constructs built into either ICL2 or the C-tail of FP (**data not shown**). Taken together, our data paint a picture which highlights the conformational heterogeneity of different GPCRs in response to ligand stimulation.



Figure 2.5 BRET kinetics in the second and third intracellular loops of the  $\beta_2$ -adrenergic receptor ( $\beta_2 AR$ ).

HEK 293 SL cells transiently expressing the ICL2 and ICL3  $\beta_2AR$  biosensors were labeled with the FIAsH reagent. (A) WT untagged receptor, (B) ICL2 p1, (C) ICL2 p2, (D) ICL3 p1, (E) ICL3 p2, and (F) ICL3 p3. Open boxes refer to vehicle and solid boxes refer to isoproterenol treatment. Basal BRET was captured prior to the injection of the full agonist, 10  $\mu$ M isoproterenol. After ligand stimulation, data were continuously captured to observe the corresponding change in the BRET signal. The BRET ratio was calculated by dividing the fluorescence by the luminescence and plotted as a function of time. The dotted line represents the time at which the injection took place. The inset at the top right corner of each graph zooms in at the time points close to the injection. The data are representative of three or more independent experiments.



Figure 2.6 BRET kinetics in the C-terminal  $\beta_2$ -adrenergic receptor ( $\beta_2 AR$ ) FIAsH constructs as well as in AT<sub>1</sub>R and FP biosensors.

HEK 293 SL cells transiently transfected with the three C-tail  $\beta_2AR$  recombinant biosensors or with FP ICL3 p4 or AT<sub>1</sub>R ICL3 p3 and C-tail p1 and then labeled with the FIAsH reagent. (A) C-tail p1, (B) C-tail p2, (C) C-tail p3, (D) FP ICL3 p4, (E) AT<sub>1</sub>R ICL3 p3, and (F) AT<sub>1</sub>R C-tail p1. Open boxes refer to vehicle and solid boxes refer to agonist treatment. Basal BRET was captured prior to the injection of each receptor's respective full agonist, 10 µM isoproterenol, 1 µM PGF2 $\alpha$ , or 1 µM angiotensin II. After ligand stimulation, data were continuously captured to observe the corresponding change in the BRET signal. The BRET ratio was calculated by dividing the fluorescence by the luminescence and plotted as a function of time. The dotted line represents the time at which the injection took place. The inset at the top right corner of each graph zooms in at the time points close to the injection. Measurements were recorded on 40,000 cells except for the AT<sub>1</sub>R C-tail p1 where 30,000 cells were used. All readings were taken using the Tristar multimode plate reader (Berthold Technologies) except the AT<sub>1</sub>R C-tail p1 which was assayed on the Victor X Light (Perkin Elmer). The data are representative of three or more independent experiments.

#### 2.6 Discussion

Crystal structures offer snapshot images of receptor structure that can be complemented using more dynamic measures such as RET approaches. Kobilka and coworkers reported that transmembrane domain VI experiences a 14 Å outward movement when comparing the inactive carazolol bound  $\beta_2AR$  versus the active-G $\alpha_s$ bound crystal structure [24]. We show here that three different GPCRs show distinct patterns of BRET in response to ligand even when biosensors are placed in similar positions (**Figure 2.7**).



Figure 2.7 Homology-based representation of the positioning of the FIAsH tag in three Class A G protein-coupled receptors.

(A) Homology model of the  $h\beta_2AR$  (P07550-1) based on PDB identifier: 2rh1A with truncated C-tail. Positions highlighted in orange correspond to the first position, in blue the second position, and in green the third position within each respective loop structure. (B) Homology model of the human angiotensin II type 1 receptor (P30556-1) modeled upon the existing crystal structure with PBD accession 4yayA, the C-tail was then truncated (25). The ICL3 p3 biosensor is shown in green. (C) Homology model of the human FP (P43088-1) based on the PBD ID: 3emIA. Insertion of the TC tag in ICL3 position 4 is shown in red. (D) Superimposing the models of the  $h\beta_2AR$ ,  $AT_1R$ , and FP. Overlay of three receptors reveals the relative similarities in the transmembrane domains and differences in the cytoplasmic regions. Approximately 20 residues were removed from the N-terminus and the C-terminus was truncated to facilitate the visualization of the overall structure. Inset shows expanded versions of ICL2 (left) or ICL3 (right). The I-TASSER models (26, 27) were exported into PyMOL where the CCPGCC motifs were inserted at their respective positions and color coded to facilitate the visualization of the positioning of the FIAsH tags.

For the  $\beta_2AR$ , our data showed that ICL2 and ICL3 did not respond to the full agonist isoproterenol, whereas two of our C-tail biosensors exhibited sustained BRET responses. As the acceptor was progressively walked down the C-terminus, resonance energy was more efficiently transferred from donor to acceptor under basal conditions and this may explain why BRET was not detected in biosensors with acceptor and donor farther apart. This pattern was distinct in the AT<sub>1</sub>R and in FP receptor. The AT<sub>1</sub>R exhibits the most conformational heterogeneity in that sensors engineered into ICL2, ICL3, or the C-tail all reported robustly on conformational changes in response to either canonical (Ang II or Ang III) or biased (SI) ligands [17]. Further, only ICL3 biosensors reported responses upon stimulation with PGF2α in FP ([11], data not shown for ICL2 or the Ctail). This may suggest that the movement of the intracellular loops in the  $\beta_2$ AR or FP is constrained by a protein within the vicinity of the fifth, sixth or seventh transmembrane domain. Even if this constrained conformation does not allow us to use these biosensors in this cellular background, it does highlight the advantage of using a six amino acid tag since this reduced size allows us to probe receptor conformation. For instance, if GFP or one of its variants were used instead of the FIAsH tag, perhaps the 238 amino acid (27 kDa) insertion would have significantly distorted receptor structure.

G protein-coupled receptors have many associated interacting partners that may pose conformational constraints on the receptor which translates into distinct conformational profiles. One of the major differences between the three receptors is that both the AT<sub>1</sub>R and FP couple to  $G\alpha_q$  whereas the  $\beta_2AR$  couples to  $G\alpha_s$ . The  $\beta_2AR$  has also been reported to differentially couple to  $G\alpha_i$  [28–30]. It may be interesting to explore the propensity of the receptor to couple to different G proteins in a particular biological context. Such differential coupling may lead to distinct conformations adopted by the receptor. Alternatively, it is well known that all three GPCRs form oligomers [31–35]. Homo- and heterodimers or larger oligomers are not fully characterized and their physiological roles are not fully understood. Perhaps the formation of such larger arrays imposes additional conformational constraints on the receptor. These effects must be considered as early events occurring in receptor biosynthesis [36, 37]. Further exploring the lifecycle of a receptor is merited since oligomerization can alter several aspects of receptor function [37]. Likewise, the  $\beta_2$ AR experiences a high level of basal activity which some believe is due to the higher availability of G proteins and other effectors; proteins that might restrict receptor movement [38].

The length of intracellular loops in each receptor may also be related to measured conformational flexibility. The  $\beta_2AR$  has a much longer third loop than the other two receptors. Taking this into account, we could imagine that the  $\beta_2AR$  might be more free to adopt a larger range of conformations compared to the AT<sub>1</sub>R and FP (**Figure 2.7**). This may be a contributing factor explaining the different conformational patterns exhibited by all three receptors. These receptors are all classed into the same family of class A GPCRs, yet, they show different conformational behaviors. It must also be noted that this method is limited by the orientation of the reporter proteins. If the receptor folds in such a way where the enzymatic pocket of *Rlucll* orients itself facing away from the FIAsH tag, the transfer of resonance energy will be less efficient with respect to RET.

In conclusion, we have demonstrated that the  $\beta_2AR$ ,  $AT_1R$ , and FP display distinct conformational signatures when assayed in HEK 293 cells. Certainly cell context will matter in such experiments. The introduction of these BRET-based biosensors into diverse cell types may result in the detection of multiple different conformations adopted by the receptor depending on the cellular and subcellular contexts. Such receptor-based biosensors will be portable in this regard. Combined with genome editing approaches, these sensors are simple tools that could be used to uncover the complex mechanisms of GPCR activation and function.

#### **2.7 Author Contributions**

K.B, D.D, R.S, and T.E.H designed the study, and wrote and edited the paper. K.B, D.D, R.S, D.P, and A.Z performed experiments. K.B, D.D, and R.S analyzed the data. K.B, D.D, R.S, and D.P generated figures.

#### 2.8 Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# **Chapter 3 :** Comparing the signaling and transcriptome profiling landscapes of human iPSC-derived and primary rat neonatal cardiomyocytes

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## 3.1 Preface

In chapter one, HEK 293 cells were used as vehicle to study GPCR conformational dynamics. However, during these studies, we began questioning the relevance of using heterologous cell systems due to their low physiological resemblance to human cells, especially human heart cells. Akin, a then PhD candidate, Dr. Ryan Martin was using neonatal rat cardiomyocytes to model and study the transcriptional regulation governing the hypertrophic phenotype. In the past, rat neonatal cardiomyocytes (RNCMs) represented the ideal cell choice for such studies; inherently being cardiomyocytes albeit from another species. RNCMs are also considerably easier to isolate compared to adult rat cardiomyocytes, facilitating experiments using this cell type. Of course, we were aware that using neonatal rat cardiomyocytes to model an adult-onset disease that essentially takes years to develop was less than favourable however, this model system was the *best-* and accessible, at the time. Since primary neonatal rat cardiomyocytes have a short shelf life and essentially begin to de-differentiate after 5-7 days in culture, this imposed another limitation to studying chronic disease such as the human heart failure. At this point, we had reached a juncture and a decision had to be made. This prompted the choice to transition towards using human derived cell-based systems to model the cardiomyocyte. With advancements in the field of stem cell technology, I began searching PubMed for robust protocols that could yield high quantities of cardiomyocytes derived from human stem cells. Therefore, pioneering the use of hiPSC-based technology, as described in this chapter, resulted from my efforts to introduce physiologically relevant models to the lab. However, since rat neonatal cardiomyocyte did represent the traditional model system for decades prior, we decided that before universally adopting stem cell derived cardiomyocytes in the Hébert lab, we would conduct a 'head-on' comparison of primary neonatal and hiPSC-derived cardiomyocytes. To this end, we explored both signaling outcomes downstream the activation of select cardiac-relevant GPCRs as well as bulk transcriptomic profiling of both cell types.

#### 3.2 Abstract

The inaccessibility of human cardiomyocytes significantly hindered years of cardiovascular research efforts. Post-mortem tissue or biopsies from diseased patients, which remain scarcely available, rendered it possible to study end-stage heart disease yet the inclusion of healthy human cardiac materials for basic science research was beyond reach. To overcome these limitations, non-human cell sources were used as proxies to study heart function and associated diseases. Rodent models became increasingly acceptable surrogates to model the human heart either in vivo or through in vitro cultures. More recently, due to concerns regarding animal to human translation, including cross-species differences, the use of human inducible stem cell derived cardiomyocytes presented a renewed opportunity. We thought it necessary to conduct a comparative study, assessing cellular signaling through cardiac G protein-coupled receptors and bulk transcriptomics of traditional rat neonatal cardiomyocytes and human iPSC-CMs. Genetically-encoded biosensors were used to interrogate nuclear protein kinase A (PKA) and extracellular signal-regulated kinase 1/2 (ERK<sub>1/2</sub>) in rat and humanderived cardiomyocyte populations. To increase data granularity, a single-cell analytical approach was conducted for an in-depth examination of existing differences between both in vitro cardiomyocyte models. Using automated high content microscopy, our analyses of nuclear PKA and ERK<sub>1/2</sub> signaling revealed distinct response clusters in rat and human CMs. In line with this, bulk RNA-seq demonstrated key differences regarding the expression patterns of GPCRs, G proteins and effectors. Overall, our study demonstrates that human stem cell derived models of the cardiomyocyte do provide significant advantages and should be taken advantage of.

#### **3.3 Introduction**

Cardiac physiology is regulated, to some extent, by G protein-coupled receptors (GPCRs) including adrenergic, angiotensin and endothelin receptor systems. These receptors and their associated signaling effectors modulate cardiac contractility, force generation, vascular tone, as well as cardiomyocyte growth and survival [1, 2]. The activation of prototypical cardiac  $G\alpha_q$ -coupled and  $G\alpha_s$ -coupled GPCRs,  $\alpha$ - and  $\beta$ adrenergic receptors for instance, result in calcium mobilization or protein kinase A (PKA) activity, correspondingly influencing cardiomyocyte contractile responses [3]. Moreover, the activation of the extracellular signal-regulated kinase 1/2 (ERK<sub>1/2</sub>) downstream adrenergic and angiotensin input has relatedly been linked with cardiomyocyte viability and survival [4]. Yet, signals propagated by these kinases have also been associated with maladaptive cardiac remodelling and hypertrophic growth. The same receptors and effectors can thus drive adaptive and maladaptive cardiac functions. These complex signaling circuits regulating cardiomyocyte function and pathway activation have been extensively studied, in numerous model systems, as dysregulated activity downstream GPCR activation has been implicated in the progression of several heart muscle diseases.

Generally, the study of human cardiac physiology and pathophysiology has been challenging. The most notable barrier has been the inaccessibility of human cardiac tissue and cell sources for *in vitro* experimental studies. Human heart tissue obtained from diseased non-transplantable hearts only provide insight on end-stage heart disease and is not informative of disease evolution through time. The few adult primary human cardiomyocytes that may be rarely obtained, only last one or two days in culture, thereby limiting the types of experiments that can be conducted and reducing the chance to explore disease progression. Recently, a new isolation protocol has been described that was shown to maintain human primary cardiomyocytes in culture for 7 days as well as a procedure for successful cryopreservation and thawing [5]. Needless to say, the small quantities obtained still represent a substantial bottleneck and beyond the reach of most laboratories. To model the human heart, other representative cell sources were exploited, most prominently being cardiomyocyte cultures derived from rodents. With large litters

and relatively low-cost for maintenance compared to large-animal models, the isolation of cardiomyocytes from these species provides millions of functional cells for study. Frequently, neonatal rat cardiomyocyte cultures are used as they can typically be kept in culture for 5-7 days compared to adult rodent cultures that tend to de-differentiate after 2-3 days in vitro. Numerous reports have demonstrated that cardiomyocytes isolated from mice and rat hearts are signaling competent and hypertrophy in response to known inducers [6, 7]. With certain genetic and phenotypic similarities to humans, rodents have helped researchers study numerous aspects of cardiac physiology. Nonetheless, rodent hearts do not completely recapitulate heart conduction and electrophysiological properties of the adult human heart. Most remarkably, while at rest, the human heart beats on average 60-70 beats per minute, while mice and rat hearts beat at 500-600 and 260-450 bpm, respectively [8]. Further, differences at the level of action potentials, myofilaments, isoforms of certain proteins and their corresponding phosphorylation status have also been reported [9]. Besides the species barrier, 5-7 days in culture represents a serious limitation when attempting to model long-term and slow onset diseases leading to heart failure. In addition, neonatal cultures may also limit the translatability of experimental results when modeling adult-onset diseases. This raises the ever-present underlying question, how well does the experimental data collected in neonatal rat cardiomyocytes translate to the adult human heart or at the level of the individual cardiomyocyte?

With the advent of human induced pluripotent stem cells (hiPSCs), the influence of cross-species differences has been a topic of interest due to the establishment of robust protocols that permit the differentiation of hiPSCs towards mesodermal cell types like cardiomyocytes (CMs) (reviewed in [10, 11]). Human derived iPSC-CMs represent a clinically relevant cell population of high purity, quality and quantity [12]. These differentiated cells express the relevant cell- and tissue-specific receptors and ion channels compared to their *in vivo* counterparts. They can even be cryopreserved providing access to large numbers of cells with less batch-to-batch variation [13]. Considering that certain lines of evidence suggest that cellular signaling networks that control myocardial function are cell- and tissue-type specific, it becomes vital to assess

experimental endpoints in a human background. As such, we sought to examine whether there were differences between primary rodent and human derived cardiomyocytes through dual profiling of cellular signaling pathways and the transcriptome. Briefly, we measured pathway activation downstream of four cardiac relevant GPCR families, namely  $\alpha_1$ -,  $\beta_1$ - and  $\beta_2$ -adrenergic receptors ( $\alpha_1AR$ ,  $\beta_1$ - and  $\beta_2AR$ ), AT<sub>1</sub>R angiotensin, and ET<sub>A</sub> endothelin receptors. We assessed signaling transduction pathways using genetically-encoded biosensors that measure nuclear PKA and ERK<sub>1/2</sub> activity. Pathway activation was analyzed on a single-cell basis in order to parse out subtle differences within the overall rat neonatal or human iPSC-derived cardiomyocyte populations. To complement these assays, we also investigated gene expression via bulk transcriptomics using RNA-seq. Further, gene expression profiles of rat and hiPSC-CMs were compared to HEK 293 cells as these have been a valuable resource to study the biological function of cardiac relevant GPCRs.

# **3.4 Materials and Methods**

# 3.4.1 Reagents

Unless specified, all common laboratory reagents were purchased from Sigma-Aldrich. Drugs were purchased from various vendors as follows: forskolin (Bioshop, FRS393.5), phorbol-12-myristate-13-acetate (PMA-, Cedarlane 10008014-1), angiotensin II (Sigma, A9525), phenylephrine (Sigma Aldrich, P6126), endothelin-1 (Bachem, H6995.0001), isoproterenol (Sigma Aldrich, I6504), norepinephrine (Sigma Aldrich, A9512), epinephrine (Sigma Aldrich, E4375), ascorbic acid (Sigma Aldrich, A4544), acetic acid (Fisherbrand, 351270-212).

# 3.4.2 Rat neonatal cardiomyocyte isolation and culture

All procedures involving animals were approved by the McGill University Animal Care Committee, in accordance with Canadian Council on Animal Care Guidelines. Sprague-Dawley dams with postnatal day 1-3 pups were purchased from Charles River, Saint-Constant QC, Canada. Complete litters, including both female and male neonatal pups were sacrificed by decapitation, as previously described [14-16]. Using forceps,

whole hearts were isolated from the chest cavity and placed in cold HBSS (Wisent, 311-511-CL). Using surgical scissors, hearts were kept whole and cut 3-5 times to increase the surface area for overnight enzymatic digestion at 4 °C with 0.1% trypsin in HBSS. The next morning, the trypsin reaction was inhibited with 7% FBS-supplemented DMEM low glucose + penicillin/streptomycin (P/S). Five serial collagenase digestions were then performed. Cell suspensions containing cardiomyocytes and cardiac fibroblasts from whole heart extracts were then seeded onto tissue-culture treated plastic 10-cm dishes. Non-cardiomyocyte cells such as fibroblasts can attach to plastic while cardiomyocytes do not adhere to plastic surfaces. The cell suspension after this incubation period is thus enriched for cardiomyocytes. After two 75-minute incubations, the cell suspension typically contains >90% cardiomyocytes and were seeded in black optical bottom 96-well plates (Thermo Scientific, 165305) coated with human plasma fibronectin + 0.1% gelatin in DMEM low glucose + 7% (vol/vol) FBS + P/S + 10  $\mu$ M cytosine- $\beta$ -d-arabinoside (AraC, Sigma Aldrich, C1768-500MG). The mitotic inhibitor, AraC, was added to prevent proliferation of remaining dividing fibroblasts. Cardiomyocytes were then maintained in a humidified atmosphere at 37 °C with 5% CO<sub>2</sub>. The next day, cardiomyocytes were washed three times with DMEM low glucose and exchanged with maintenance medium containing DMEM low glucose and universal ITS supplement composed of insulin, transferrin, and selenium (Wisent, 315-081-QL) prior to being transduced with adenoassociated viruses carrying the genetically-encoded biosensor of interest. For the RNAseq experiment, RNCMs were seeded in a 6-well dish coated with human plasma fibronectin + 0.1% gelatin. After vehicle or agonist stimulation, RNCMs were incubated for 1 hour at physiological conditions whereafter RNCMs were harvested, and RNA was isolated.

# 3.4.3 Differentiation of human induced pluripotent stem cells into cardiomyocytes

The use of human derived hiPSCs in this research was approved by the McGill University Health Centre Research Ethics Board. In this paper, the hiPSC line derived from a healthy male donor (AIW002-2) was provided from the Montreal Neurological Institute through the Open Biorepository, C-BIGR [17]. This iPSC line has previsouly been validated by investigating gene/protein expression of pluripotency genes, tri-lineage differentiation as well as other genomic integrity assays, described in [17]. Human iPSCs, between passages 2 to 8 post-thawing, were differentiated into cardiomyocytes following the established GiWi protocol with slight modifications [18]. hiPSC-CMs were routinely screened for mycoplasma contamination and both iPSCs and differentiated iPSC-CMs used in this study were mycoplasma-free. For cardiomyocyte lineage commitment, a monolayer of 500,000 iPSCs, dissociated with Accutase, were seeded onto Matrigel (Corning, 354277) coated 24-well dishes in mTeSRPlus media supplemented with Rho kinase inhibitor, Y-27632-HCI (Selleckchem, S1049). The next day, the media was exchanged for fresh mTeSRPlus media. On day 0 of the protocol, iPS cells were stimulated with Wnt activator, CHIR99021 (Cayman Chemical, 13122) for 24 hours in RPMI 1640 media supplemented with B27 minus insulin (ThermoFisher, A1895602). At the same time the next day, the media was exchanged for fresh RPMI 1640 supplemented with B27 minus insulin. On day 3, Wnt was inhibited using the IWP2 compound (Selleckchem, S7085). The media was exchanged 48 hours later with RPMI 1640 supplemented with B27 minus insulin. On day 7 and day 10 of the protocol, the media was exchanged for RPMI 1640 supplemented with regular B27 (ThermoFisher, 17504001). Starting at day 12, metabolic selection was conducted where the cells were starved from glucose for 5-6 days. To promote cardiomyocyte survival and deplete undifferentiated cells, RPMI 1640 without glucose (Wisent, 350-060-CL), supplemented with B27 as well as with 4 mM lactate was used [19]. Wells that contained spontaneously beating cardiomyocytes were reseeded into 6-well dishes coated with fibronectin in PBS and maintained in RPMI 1640 media supplemented with B27 until day 28 where hiPSC-CMs were collected for RNA extraction. Prior to signaling experiments, hiPSC-CMs were seeded into optical bottom, black 96-well plates for cellular signaling experiments (Thermo Scientific, 165305).

# 3.4.4 Cloning biosensors in AAV plasmids

The FRET-based ERK<sub>1/2</sub> protein kinase biosensors EKAR-EV were generously provided by Dr. Michiyuki Matsuda and carried a nuclear localization (NLS) signal sequence [20]. This FRET-based biosensor was introduced into an AAV compatible backbone, pENN-AAV-CAG-tdTomato (Addgene catalog #105554) using a 'cut-and-

paste' method using BamHI and BstBI restriction enzymes and ligase. The ExRai-AKAR2-NLS biosensor was obtained from Dr. Jin Zhang's lab [21]. For cloning ExRai-AKAR2 biosensor into an AAV backbone, a multiple cloning site (MCS) was first generated in pAAV-CAG-hChR2-H134R-tdTomato (Addgene catalog #28017). The MCS was generated annealing the following two primers 5'by gatccgctagcgtttaaacttaaggtaccgagctcactagtgaattctgcagatatccagcacagtggcggccgctcgagg 3'gcccttcga-3' and gcgatcgcaaatttgaattccatggctcgagtgatcacttaagacgtctataggtcgtgtcaccgccggcgagctccc gggaagcttcga-5'. To introduce the ExRai-AKAR2 biosensors within the pAAV-CAG-MCS backbone, biosensors were amplified by PCR and inserted using 5'-Nhel and 3'-HindIII. Universal forward primer sequence was 5'-gctagctagcgccaccatgctgcgtcgcgccaccctg-3'. Reverse primer was used 5'-catagaagcttttatgcgtcttccacctttc-3' for ExRai-AKAR2-NLS.

# 3.4.5 Transduction of primary neonatal rat and iPSC-derived cardiomyocytes

Adeno-associated viruses (AAVs) used in this study were produced by the Neurophotonics Platform Viral Vector Core at Laval University, Québec, Canada. Upon arrival, AAVs were aliquoted in low-retention Eppendorf tubes to minimize freeze-thaw cycles. AAVs were stored for long-term in the -80°C and once thawed, were stored at 4°C for a maximum of 7-10 days. Cardiomyocyte cultures were transduced with biosensors packaged within AAV serotype 6 which is aligned with observations made by other groups demonstrating this serotypes effectiveness at infecting cardiomyocytes [22, 23]. During the transduction protocol, AAVs were kept on ice and diluted in maintenance media for each type of cardiomyocyte. Prior to transduction, cardiomyocyte cultures were washed three times to remove excess cell debris carried over during primary cell isolation or the hiPSC-CM re-seeding step. For effective infection, a multiplicity of infection of 5000, indicative of 5000 virons per cell,  $1.5 \times 10^8$  virons per well, was used for all experiments. Both RNCMs as well as hiPSC-CMs, were transduced for 72 hours to allow for sufficient biosensor expression. This time frame was chosen as RNCMs have limited time in culture.

# 3.4.6 High-content imaging of primary neonatal rat and hiPSC-derived cardiomyocytes

Prior to conducting the signaling experiments, cardiomyocyte cultures were inspected under a phase-contrast microscope to ensure RNCMs and hiPSC-CMs were healthy. Visual inspection of the cardiomyocytes permitted us to confirm that the hiPSC-CMs were spontaneously contracting and that both hiPSC-CMs and RNCMs appeared to have cardiomyocyte-like morphologies. In this study, the assay buffer was clear HBSS with calcium, magnesium, and sodium bicarbonate (Wisent, 311-513-CL). Sterile assay buffer was warmed to physiological temperature, 37°C, and cardiomyocytes were washed 2-3 times prior to imaging. Cells were left bathing in 90 µL of HBSS for imaging. Postwashing, cardiomyocyte cultures were re-incubated in a humidified atmosphere of 37°C with 5% CO<sub>2</sub> for appropriately 1 hour before imaging to allow cells to re-equilibrate in the assay buffer. During this time, the temperature control settings (TCO) of Perkin Elmer's Opera PHENIX high-content screening system were set allowing sufficient time for to warm up to 37°C with 3% CO<sub>2</sub> for live-cell imaging. All drugs were prepared 10-fold more concentrated as 10 µL would be added in each well representative of a 10-fold dilution, 100 µL final volume. For nuclear ERK<sub>1/2</sub> assays, images were acquired using a 20X air objective using a 425 nm laser for excitation of CFP. Emissions were detected with filters at 435-515 nm (CFP-donor) and 500-550 nm (YFP-acceptor). For the ExRai-AKAR2-NLS biosensor, images were acquired using a 20X air objective using a 375 nm and 480 nm laser for excitation and 500-550 nm emission filter. Within Perkin Elmer's Harmony software, the experiment was set up in such a way where the imager would first acquire a ligand-independent measurement before the microwell plate would be ejected allowing time to perform drug stimulations. The plate itself was not displaced during drug treatment. Post-treatment measurements were automated and continued for another 70 minutes for a total of 8 readings, acquired at 10-min intervals.

# 3.4.7 Image analysis and data processing in R

After live-cell imaging, images were imported into Perkin Elmer's Columbus analysis software. Raw image files were also transferred onto external hard drives for long-term safe keeping. In Columbus, images were processed by first identifying the nuclei of individual cardiomyocytes followed by the calculation of morphology features. This output provided information regarding individual nuclei size and shape including roundness. To remove cell debris from the analysis, a size threshold was set as well as a roundness criterion. Intensity properties were calculated for both CFP and YFP fluorophores for EKAR-NLS and the same was done for the single fluorophore ExRai-AKAR2-NLS biosensor. Next, FRET- and excitation-ratios were computed by dividing acceptor/donor (YFP/CFP) for EKAR-NLS and 488 nm / 375 nm for the ExRai-AKAR2-NLS sensors, respectively. Columbus was then queried to output these data for each cardiomyocyte that fit the criteria listed and data was exported as text files for further analysis.

Data were then processed in R based on a previously published in-house singlecell analytical approach with slight modifications, formerly applied to neuronal cultures [24]. Briefly, cardiomyocyte nuclei that appeared in all 8 timepoints were carried forward. As nuclei tend to drift slightly overtime, occasionally due to minor microplate positional changes occurring during ligand addition, we set a parameter where the fluorescence intensity of each nucleus could only deviate by ≤20% intensity to be considered as the same object/nuclei. To calculate the delta FRET or change in FRET in response to drug stimulations, we subtracted the ligand induced FRET from basal, ligand independent FRET. This expression was then converted into a percentage change in FRET ( $\Delta F/F$ ) where the denominator (F) represented basal FRET. For consistency, (F) was computed by averaging the basal FRET across all nuclei in the same microwell. To detect whether cardiomyocyte sub-populations exhibited differential agonist-induced responses, when comparing RNCMs and hiPSC-CMs, we applied a clustering algorithm using the TSclust package [25] in R. Nuclei responses were clustered based on their magnitude of response over time, using the 'pam' function. For the single cell clustering, hiPSC-CMs and RNCMs nuclei were merged into a single dataset. Merging both sets of data allowed for output clusters to be reliable across RNCMs and hiPSC-CMs as means to obtain matched patterns and clusters within the two populations being compared. The algorithm could yield distinct clusters when applied to independent data sets. Following clustering, the merged hiPSC-CM and RNCM datasets were split and further plotted as heatmaps for

visualization using pheatmap package. Stacked bar charts were also generated to better summarize the data using the ggplot2 package.

# 3.4.8 RNAseq sample preparation and analysis

RNCM and hiPSC-CM cultures were seeded on fibronectin-coated 6-well dishes at a density of 1 million cells per well. After vehicle and drug stimulations, followed by 1 hour incubation, RNA was collected and isolated with the QiAshredder kit (Qiagen, 79656) followed by RNeasy<sup>™</sup> Mini Kit (Qiagen, 74106) according to manufacturer's instructions. RNA guality was later assessed by using a NanoDrop to ensure for RNA purity. Libraries were prepared using the NEBNext<sup>™</sup> rRNA-depleted (HMR) stranded library kit and paired-end 100 bp sequencing to a depth of 25 million reads per sample. Sequencing was performed on the Illumina NovaSeq<sup>™</sup> 6000 at the McGill University and Génome Québec Innovation Centre. RNAseq analysis was performed as we have previously described [15, 26]. Briefly, quality of reads was determined using FastQC, and trimmed with TrimGalore. Trimmed reads were then aligned to the Ensembl rat reference genome (Rattus\_norvegicus.Rnor\_6.0.100) or the reference human genome (Homo\_sapiens.GRCh38.104) with STAR. Transcripts were then assembled with StringTie. Normalized transcript abundance, measured as TPM, transcript per million, was plotted using pheatmap package in R. Curated gene sets were downloaded from https://www.gsea-msigdb.org/gsea/index.jsp.

# 3.4.9 Statistical analysis

All statistical analyses were performed using GraphPad Prism software where summary data processed and computed in R was imported. For cellular signaling datasets, Figures 2, 3 and 4 and supplementary Figure 4, non-parametric Welch's t-test were conducted as test statistic to determine whether the RNCM and hiPSC-CM populations and clusters had equal means. In supplemental Figure 3, we performed Welch's ANOVA followed by Dunnett's multiple comparisons test, to test whether the response means were equal across different fields measured. Welch's ANOVA was chosen as Bartlett's test statistics indicated that there was a significant difference between the variances.

# 3.5 Results and Discussion

# 3.5.1 Distinct protein kinase activation patterns noted in hiPSC-CM and RNCM cultures

#### 3.5.1.1 Nuclear PKA signaling

To compare the cellular contexts of hiPSC-CMs against primary rat neonatal cardiomyocytes, we began probing for differences in cellular signaling. Here, we set out to test the null hypothesis that RNCMs and hiPSC-CMs are equivalent cellular models. With this in mind, we measured kinase activation in RNCMs and hiPSC-CMs, at the single cell level, every 10 minutes for 70-minutes, as in the experimental pipeline depicted in Fig 3.1A. To probe agonist-induced PKA activation profiles, cardiomyocyte cultures were transduced with AAV2/6-ExRai-AKAR2-NLS (Fig 3.1B, C). A few days later, cardiomyocyte cultures were imaged using an automated high content imaging system. Baseline measurements were recorded prior to stimulating cells with a panel of saturating doses of GPCR agonists targeting either endothelin, adrenergic or angiotensin systems. (Fig. 3.1D). As seen in Fig. 3.1E, the expression level of the nuclear-localized PKA biosensor, as represented on the x-axis, was considerably higher in hiPSC-CMs compared to RNCMs, despite being transduced for the same length of time. When testing the relationship between biosensor expression (RFU) and biosensor output ( $\Delta F/F$ ), we did not observe a significant linear correlation in the RNCM cultures as shown with small R correlation coefficient and nonsignificant p-value (p =0.23). In the hiPSC-CMs, the R coefficient was small, yet the p-value was significant, an observation that is suggesting greater variability around the regression line and a larger prediction interval when correlating both variables. Thus, to have a more uniform population of RNCMs and hiPSC-CMs, removing plausible confounds caused by biosensor expression, we set a threshold and analysed CMs on the lower end of the spectrum. Cardiomyocytes that expressed the PKA sensor between 0 and 5000 RFU were carried forward, and no cells exhibited zero intensity as the lowest unit measured was 462 RFU (Fig. 3.1E, inset).



Figure 3.1 Measuring protein kinase activity in RNCMs and hiPSC-CMs.

(A) Depiction of the experimental pipeline used to compare signaling signatures in two cardiomyocyte model systems. The experimental pipeline begins with the differentiation of hiPSCs into cardiomyocytes or the isolation of rat neonatal cardiomyocytes. Cardiomyocyte cultures are then transduced with adeno-associated virus serotype 6 (AAV6) to introduce the biosensor of interest. Next, RNCM and hiPSC-CM cultures are imaged using high content microscopy and further analyzed using a single cell analytical approach. Representative fluorescent microscopy images illustrating the expression of ExRai-AKAR2-NLS biosensor in (B) hiPSC-CMs and (C) RNCMs. (D) Diagram depicting the drugs used in this study. After a baseline reading was taken, RNCMs and hiPSC-CMs were stimulated with saturating doses of a panel of ligands that targeted cardiac relevant GPCRs. Norepinephrine, epinephrine, isoproterenol, phenylephrine, SI and SII were used at 10 µM. Ang II and PMA were used at 1 µM. Forskolin was prepared as 5 µM and ET-1 at 100 nM. (E) Basal, ligand independent single fluorophore intensities of hiPSC-CM and RNCM ExRai-AKAR2-NLS datasets. Biosensors were expressed at higher levels in hiPSC-CMs compared to RNCMs despite being transduced for the same length of time. Panel (A) was created with BioRender.com

Next, we averaged the nuclear PKA responses of all CMs, across our biological replicates, and plotted the responses as a function of time (**Suppl. Fig. 3.1**). RNCMs and hiPSC-CMs displayed distinct averaged PKA signatures when stimulated by various agonists, when evaluating both kinetics and magnitude of the responses. In both CM types, forskolin, a direct activator of adenylyl cyclase, was the most potent activator of the PKA pathway followed by epinephrine. Markedly, epinephrine and norepinephrine led to

larger nuclear PKA responses in RNCMs. Phenylephrine and ET-1 also resulted in PKA activation; an observation examined later. To move beyond averaged responses, and as means to fully appreciate subtle differences or granularity between RNCMs and hiPSC-CMs, we applied a single cell analytical approach to our biosensor datasets [24]. To identify cardiomyocyte populations or clusters that responded differently within the overall population, we merged RNCM and hiPSC-CM datasets and ran them through a clustering algorithm [25]. The clustering technique revealed that the overall population of cardiomyocytes could be sub-divided into 4 main clusters (**Suppl. Fig. 3.2**). We arbitrarily named them, 'sustained-responders', 'transient-responders', 'non-responders' and 'negative-responders'. To test whether the expression of the biosensor influenced the clustering method, cardiomyocytes at different fluorescent intensities thresholds were independently ran through the algorithm (Suppl. Fig. 3.2A). The inclusion of cells at varying fluorophore intensities did not appreciably impact how the cells were clustered. We also noticed that while requesting the clustering algorithm to devise four clusters instead of three (Suppl. Fig. 3.2B), the data was partitioned into more informative patterns. By requesting four clusters, we saw that the population of 'responders' could be further sub-divided based on kinetics, with some nuclei exhibiting 'transient' while other displayed 'sustained' effects. Four clusters were carried forward throughout further analyses. As another internal control, we investigated whether all cardiomyocytes seeded in the microwell were exposed to a uniform concentration of drug. When stimulating the cells, we were vigilant to break the surface tension barrier with the microtip to ensure effective drug diffusion, as we were working with small, 10 µL volumes. The 21 fields imaged were plotted against the percentage change in response compared to baseline, %ΔF/F, post-drug stimulation (Suppl. Fig. 3.3). No significant differences were observed in response to stimulation with norepinephrine while responses to forskolin and isoproterenol showed minor positional effects by field. Still, these observations may just as likely be correlated with the proportion of nuclei that fall within a response cluster, irrespective of drug diffusion. Thus, the fraction of nuclei exhibiting distinct behaviors, categorized as non-responders or responders may be skewing the average response per field.

To visualize the overall spread of single nuclei PKA responses, we built heatmaps with single nuclei responses plotted on the y-axis as a function of time (x-axis). In hiPSC-CMs, adrenergic targeting ligands, isoproterenol, norepinephrine and epinephrine, resulted in modest activation of nuclear PKA (Fig. 3.2A). More specifically, the distribution revealed that 18.72 ± 5.73 % of hiPSC-CMs exhibited a sustained response to isoproterenol compared to 2.66 ± 1.5 % of RNCMs (Fig. 3.2B). Further, in response to epinephrine, 57.83 ± 2.3 % of hiPSC-CMs led to a transient response compared to 35.51 ± 13.5 % of RNCMs. Forskolin steered a similar response profile in both species with slightly more sustained responders in the overall RNCM population. Most remarkably, phenylephrine resulted in nuclear PKA activity in RNCMs but not in hiPSC-CMs (Fig. **3.2B**). A greater density of  $\alpha_1 AR$  in RNCMs could explain the prominent PKA response upon stimulation with phenylephrine. Similarly, ET-1 drove a transient PKA activation profile in RNCMs and hiPSC-CMs, albeit to a smaller degree in iPSC-CMs. As the ETA receptor is canonically coupled to  $G\alpha_q$ , this observation was unpredicted. However, this finding has been previously observed in both HeLa cells overexpressing the ETA as well as in rat aortic smooth muscle cells [27]. In these models, PKA activation was reported to be independent of cAMP production. Further, in rat aortic vascular smooth muscle cells,  $ET_A$  coupling to  $G\alpha_i$  was shown to result in transient PKA activation, with a return to baseline after 20 minutes [28]. An observation analogous to what we observed in RNCM cultures with approximately  $45 \pm 15$  % of cells exhibiting this transient behaviour (Fig. **3.2B**). G proteins may be expressed at varying stoichiometries in both cell types, examined in a later section. However, this observation contrasts with one of our previous reports which demonstrated phenylephrine's ability to induce nuclear PKA responses in RNCMs but not ET-1 [15]. However, as ExRai-AKAR2-NLS is more sensitive than the AKAR4-NLS sensors used in our previous study, this discrepancy may simply reflect differential affinity and sensitivities of the biosensors themselves. Nonetheless, this result is worth follow-up studies as it may be implicated in shaping transcriptional programs involved in the development of cardiac hypertrophy [15]. With a greater proportion of hiPSC-CMs exhibiting a decrease in PKA activity compared to baseline, this behaviour may suggest that adrenergic receptors exhibit a greater degree of constitutive activity in this context. Lastly and as expected, compounds that targeted the AT<sub>1</sub>R did not result in

PKA activation (**Suppl. Fig. 3.4**). Interestingly, Ang II and related compounds led to a measurable negative response in hiPSC-CMs. PMA, a direct activator of PKC, also failed to trigger PKA activation, except for a small population of hiPSC-CM. This may be reflect signaling cross-talk [29]. Altogether, there are trends that indicate distinct signaling landscapes in both species.



Figure 3.2 Single nuclei PKA activation patterns as observed in hiPSC-CM and RNCM cultures in response to adrenergic agonists and vasoactive ET-1 peptide.

Heatmaps displaying nuclear PKA activity as measured in (A) hiPSC-CMs and (B) RNCMs. Single nuclear PKA data summarized as  $\%\Delta F/F$  (y-axis) as a function of time (x-axis). The data was partitioned into four clusters representing distinct nuclear behaviors, either exhibited sustained or transient responses to agonists while other nuclei failed to respond or experienced a decrease in activity compared to baseline. Graphical representation of the four response clusters was plotted as a bar chart with percentages of nuclei belonging to each response cluster as observed in (C) hiPSC-CMs and (D) RNCMs. Isoproterenol, epinephrine, and norepinephrine were dissolved in ascorbic acid. ET-1 and phenylephrine were dissolved in acetic acid. Experiments were performed using RNCMs isolated from 4 different neonatal rat pup litters and 3 independent cardiac differentiations.

# 3.5.1.2 Nuclear ERK<sub>1/2</sub> signaling

We were also interested in probing for activation of extracellular signal-regulated kinase, ERK<sub>1/2</sub>. Correspondingly, RNCMs and hiPSC-CMs were transduced with AAV2/6-EKAREV-NLS to measure nuclear ERK<sub>1/2</sub> activity (Suppl. Fig. 3.5A, B). As observed in our PKA experiments, hiPSC-CMs expressed the EKAREV-NLS biosensor at higher donor, CFP intensities compared to RNCMs (Suppl. Fig. 3.5C). With our available computer memory (RAM), it was not possible to include all CMs as too many objects within the R clustering exhausted the memory limit. Therefore, for consistency in our data analysis, we filtered our CM populations and carried forward CMs that expressed the biosensor between 2000 and 5000 RFU. The response clusters did not appear to change greatly with higher CFP intensities except at higher CFP intensities as less RNCMs fit that criterion (data not shown). However, before dissecting single cell response profiles, we plotted averaged ERK<sub>1/2</sub> responses over time. The summarized data demonstrated that PMA and ET-1 were the most robust responders in RNCMs and hiPSC-CMs, displaying the greatest response magnitudes, while epinephrine, norepinephrine and phenylephrine exhibited larger ERK<sub>1/2</sub> activation in RNCMs compared to hiPSC-CMs (Suppl. Fig. 3.5D).

Next, we plotted the single cell ERK<sub>1/2</sub> responses using heatmaps (**Fig. 3.3, 3.4**). In hiPSC-CM profiles, adrenergic ligands displayed considerable variability, ~30% of nuclei exhibited a decrease in ERK<sub>1/2</sub> activation compared to baseline. Adrenergic receptors may be exhibiting a greater degree of constitutive activity in this context, or this observation may expose a sub-population of cells signaling through a  $\beta_2$ AR-G $\alpha_i$  pathway. In hiPSC-CMs, ET-1 treatment led to the most robust and sustained response over time in 97.8 ± 0.5% of the nuclei population. However, even vehicle showed ERK<sub>1/2</sub> activity albeit to a considerably smaller magnitude. To further dissect this response range, low and high responses, we applied the clustering algorithm on a select population, those previously categorized as 'responders'. This yielded two more sub-clusters, arbitrarily named 'low-responders' and 'high-responders' (**Suppl. Fig. 3.6C**). This extra step allowed us to summarize the data in a way that better reflected the spread as observed with the heatmaps (**Fig. 3.3 C, D inset**).



Figure 3.3 Single nuclei ERK<sub>1/2</sub> activation patterns as observed in hiPSC-CM and RNCM cultures in response to adrenergic agonists and vasoactive ET-1 peptide.

Heatmaps displaying nuclear ERK<sub>1/2</sub> activity as measured in (A) hiPSC-CMs and (B) RNCMs. Single nuclear ERK<sub>1/2</sub> data summarized as  $\%\Delta F/F$  (y-axis) as a function of time (x-axis). The data was partitioned into three clusters representing distinct nuclear behaviors. CM nuclei either responded to the drug stimulations, resulting in ERK<sub>1/2</sub> activation, while others did not respond and the last cluster of nuclei represented those that exhibited a decrease in ERK<sub>1/2</sub> activity compared to baseline. The three response clusters were plotted as stacked bar charts with percentages of nuclei belonging to each cluster as observed in (C) hiPSC-CMs and (D) RNCMs. Insets reflect subclustering applied to the 'responding' nuclei cluster. It is apparent that within the 'responding' sub-population, two other clusters can be identified based on their responding magnitudes- low or high responders. Isoproterenol, epinephrine and norepinephrine were dissolved in ascorbic acid. ET-1 and phenylephrine were dissolved in acetic acid. Experiments were performed using RNCMs isolated from 3 different neonatal rat pup litters and 3 independent cardiac differentiations.

In hiPSC-CMs, phenylephrine effects were less pronounced but not significantly stronger than vehicle (**Fig. 3.3A**). In strike contrast, adrenergic ligands resulted in significant nuclear ERK<sub>1/2</sub> activity, specifically in response to epinephrine (p=0.033) and

norepinephrine (p=0.038) in RNCMs compared to hiPSC-CMs (**Fig. 3.3B**, **D inset**). In RNCMs, transient activation patterns were observed in response to isoproterenol (p= 0.047), an observation that was not mirrored in hiPSC-CMs. Phenylephrine drove ERK<sub>1/2</sub> activation to a greater extent in RNCMs compared to hiPSC-CMs, once again suggesting that hiPSC-CM and RNCMs may express  $\alpha_1$ ARs at different levels. In adult mouse cardiomyocyte cultures, phenylephrine has been shown to result in ERK activation in 60% of CMs, as  $\alpha_1$ AR receptors have been demonstrated to be expressed in 60% of ventricular cardiomyocytes [30, 31]. This observation is similar to our ERK<sub>1/2</sub> assays conducted in hiPSC-CMs, at 58% (**Fig. 3.3C**). This finding is aligned with reports suggesting that mouse CM cultures are better mimics of the human CM, when studying  $\alpha_1$ AR-mediated myocardial effects, as  $\alpha_1$ AR expression levels are more comparable. In contrast, rats express 5-10-fold more  $\alpha_1$ AR, a finding that is aligned with the strong phenylephrine responses observed in our RNCM experiments (**Fig. 3.3 B, D**) [32, 33].



Figure 3.4 Single nuclei ERK<sub>1/2</sub> activation patterns as observed in hiPSC-CM and RNCM cultures in response to Ang II and  $\beta$ -arrestin biased peptides.

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Heatmaps displaying nuclear ERK<sub>1/2</sub> activity as measured in (A) hiPSC-CMs and (B) RNCMs. Single nuclear ERK<sub>1/2</sub> data summarized as  $\%\Delta F/F$  (y-axis) as a function of time (x-axis). The data was partitioned into three clusters representing distinct nuclear behaviors. CM nuclei either responded to the drug stimulations, resulting in ERK<sub>1/2</sub> activation, while others did not respond and the last cluster of nuclei represented those that exhibited a decrease in ERK<sub>1/2</sub> activity compared to baseline. The three response clusters were plotted as stacked bar charts with percentages of nuclei belonging to each cluster as observed in (C) hiPSC-CMs and (D) RNCMs. Insets reflect the subclustering applied on the 'responding' nuclei cluster. It is apparent that within the 'responding' sub-population, two other clusters can be identified based on their responding magnitudes- low or high responders. Ang II and  $\beta$ -arrestin biased peptides were dissolved in H<sub>2</sub>O. Experiments were performed using RNCMs isolated from 3 different neonatal rat pup litters and 3 independent cardiac differentiations.

Unsurprisingly, forskolin failed to produce significant  $ERK_{1/2}$  signals in either CM models while PMA drove activation in 99% of CMs, irrespective of the species. Similarly, the Ang II peptide and  $\beta$ -arrestin biased drugs, SI and SII did not drive significant  $ERK_{1/2}$  activity in comparison to the vehicle (**Fig. 3.4C, D inset**). It is possible that by the time our cells were imaged, at the 10-minute time point, the early  $ERK_{1/2}$  activation phase had passed as we have western blot data that supports  $ERK_{1/2}$  activity as early as 5 minutes in RNCMs (**Suppl. Fig. 3.7**). However, in the above-mentioned single cell FRET study, the authors also reported the lack of an Ang II-mediated ERK response.

3.5.2 Bulk RNA-seq reveals transcriptome-level differences in three cells types; hiPSC-CMs, RNCMs and HEK 293 cells.

# 3.5.2.1 GPCR-mediated signal transduction-related gene sets

As distinct signaling signatures were observed at the level of protein kinase activation, we next sought to test whether these differences could be explained via mRNA expression analyses. Thus, we set out to determine if RNCMs and hiPSC-CMs were wired differently, at the endogenous level. To characterize the transcriptional landscapes in hiPSC-CMs and RNCMs, we surveyed genes linked with GPCR signaling at baseline, independent of ligand. Since HEK 293 cells have been a valuable model system for dissecting GPCR functions *in vitro*, we included these cells in our analysis.

Basal abundance of select cardiac-relevant Class A GPCRs, reported as transcript per kilobase million, TPM, revealed striking differences between the three in vitro cell models assessed (Fig. 3.5). As suggested earlier, indeed, the α1-adrenergic receptor was more highly expressed in RNCMs compared to hiPSC-CMs and HEK 293 cells (Fig. 3.5A, **Suppl. Fig. 3.8A**). The  $\beta_1$ -adrenergic receptor was also more abundant in RNCMs. The endothelin A (ET<sub>A</sub>) receptor was expressed at higher levels in hiPSC-CMs compared to RNCMs. In contrast, angiotensin II type I (AT<sub>1</sub>) receptors were expressed at low amounts in all three cell types. An observation aligned with certain reports that state features of cardiac remodelling associated with the angiotensin system are linked through fibroblast activation and paracrine mechanisms [34]. Regarding the endogenous signaling machinery, including heterotrimeric G proteins,  $G\alpha_s$  was shown to be well expressed in all three cell types (Fig. 3.5B, Suppl. Fig. 3.8C). Gα<sub>q</sub> was demonstrated to be expressed at comparable levels in all three cell types, though at slightly higher levels in RNCMs. Gai2 expression was reported as 3-fold higher in RNCMs. Conceivably, this may be associated with the PKA response to ET-1, as observed in Fig. 3.2B. Gβ isoforms were all observed, albeit to varying degrees, with G $\beta$ 1 being most abundant followed by G $\beta$ 2 (**Fig. 3.5C**, Suppl. Fig. 3.8D). Equally, all Gy isoforms were detected with Gy5, 10 and 12 being most abundant (Fig. 3.5D, Suppl. Fig. 3.8E). Lastly, distinct expression patterns were observed for select GPCR effector molecules (Fig. 3.5E, Suppl. Fig. 3.8F). For example, adenylyl cyclase isoforms 5 and 6 were more abundant in cardiomyocytes compared to HEK 293 cells, an observation consistent with previous reports [35]. In contrast, βarrestin2 was expressed at greater levels in HEK 293 cells, perhaps affecting GPCR desensitization or post G protein signaling. PKA enzyme subunits were well represented as well as members of the MAPK cascade, albeit at different level (Fig. 3.5E, Suppl. **3.8F).** All in all, mRNA levels seem to align well with the cellular signaling signatures reported above. Further, differential DESeq2 analysis demonstrated that genes that were either significantly up- or down-regulated in RNCMs and hiPSC-CMs after 60-min agonist stimulation differed (Suppl. Fig. 3.9). A 60-min time point was selected to parallel the time course of our signaling experiments. This finding indicates that their transcriptional landscapes are also different, driving distinct cellular effects. Taken together, evidence presented implies that cell context is a critical determinant dictating receptor outcomes.

For physiologically relevant conclusions to be drawn, it is important to consider the cell context and its influence especially when translating research findings for human predictability [36].



# *Figure 3.5 Ligand-independent RNA-seq based comparison of the endogenous signaling machinery expressed in RNCMs, hiPSC-CMs and HEK 293 cells.*

Exploratory analysis of gene sets associated with GPCR signal transduction investigating (A) select cardiac relevant class A GPCRs, heterotrimeric G proteins (B) G $\alpha$ , (C) G $\beta$  and (D) G $\gamma$  as well as (E) effector expression profiles. Colored heatmaps display comparative expression relative to all three cell models being assessed normalized as transcripts per million. For comparison sake, data from HEK 293 cells was included (published originally as Lukasheva et al., 2020 Sci Rep 10, 8779. https://doi.org/10.1038/s41598-020-65636-3.

3.5.2.2 Myocyte-, maturity- and metabolism-related gene sets in hiPSC and RNCMs

We next examined gene sets associated with cardiomyocyte fate, maturity and 'adult-*like*' identity. When observing normalized transcript abundance of a curated gene

list for cardiac progenitor differentiation, RNCMs as well as iPSC-CMs expressed GATA4, Nkx2-5 and Mef2c, key transcription factors that control cardiomyocyte fate (Fig. 3.6A). Sarcomeric proteins were also abundant including TNNT2, TNNi3, Actc1 and MYH6. In our cultures, the fetal troponin isoform, TNNI1 was more abundant in hiPSC-CMs compared to the adult equivalent, TNNI3 (Fig. 3.6B). RNCMs express approximately 5fold more TTNI3 and 2.5-fold TNNI1 compared to day 28 hiPSC-CMs. This data is consistent with the current literature equating hiPSC-CMs as neonatal as opposed to adult cardiomyocytes. Both hiPSC-CMs and RNCM express MYH6, present in the developing ventricle and neither expressed the adult isoform MYH7 (Fig. 3.6C). This echoes to the neonatal nature of both of these cardiomyocyte cell models. Genes involved in glycolytic metabolism, such as ALDOA, BPGM, ENO1 and LDHA were more abundant in RNCMs compared to hiPSC-CMs (Suppl. Fig. 3.10A) [37]. These genes have been shown to be downregulated in 'matured' hiPSC-CMs that undergo a metabolic switch from glycolysis to fatty acid oxidation. Overall, transcript reads, measured as TPM, were higher in glycolytic metabolism pathways compared to gene sets of fatty acid related metabolism. This result is suggestive that both RNCMs and iPSC-CMs use glycolysis as an energy source.



Figure 3.6 Ligand-independent comparative gene expression analysis in hiPSC-CMs and RNCMs.

**RNA-seq inferred transcript abundance measured as TPM of curated gene sets associated with (A)** cardiac progenitor differentiation, **(B)** maturation-related genes and **(C)** cardiac ventricular development. Large colored heat maps represent gene expression based on transcripts per million while smaller colored heatmaps display comparative expression (hiPSC-CM vs RNCMs). Colorless heatmap depict normalized transcript abundance.

In the context of cardiomyocyte functionality and calcium regulation, both RNCM and iPSC-CMs express key genes: RyR2, Cacna1c, Camk2d, Atp2a2 (SERCA2), Casq2, Slc8a1 (NCX1) and Tmp1 (**Fig. 3.7A**). Transcript levels for Bin1, a gene that participates in T-tubule formation was low in both RNCM and iPSC-CMs, an observation consistent with the literature (**Fig. 3.7A**, **B**). In connection with genes involved in propagating the cardiac action potential, the voltage gated potassium channel, KCNH2 (hERG), was more abundant in hiPSC-CMs than RNCMs (**Fig. 3.7C**). The HCN4 subunit of the funny current,

I<sub>f</sub>, was shown to be expressed at 2-fold higher abundance in hiPSC-CMs compared to RNCMs. This observation is in line with the automacity seen in iPSC-CMs cultures and not in RNCMs [38]. The low (negligible: 0.35) transcript levels of KCNJ2 encoding for the inward rectifier potassium current IK1 has also been associated with the spontaneous contractile properties of hiPSC-CMs [39]. All in all, our hiPSC-CMs display similar gene expression patterns as hiPSC-CMs cultures previously published and are similar to RNCMs in terms of maturity.



Figure 3.7 Gene-level examination of get sets associated with cardiomyocyte behavior and function.

**RNA-seq deduced transcript abundance of genes related with (A)** cardiac muscle contraction **(B)** regulation of heart rate by cardiac conduction and **(C)** ion channels that generate cardiac action potential. Large colored heat maps represent gene expression based on TPM while smaller colored heatmaps display comparative expression (hiPSC-CM vs. RNCMs vs. HEK 293 cells). Colorless heatmap depict normalized transcript abundance. For comparison sake, data from HEK 293 cells was included (published originally as Lukasheva et al., 2020 Sci Rep 10, 8779. https://doi.org/10.1038/s41598-020-65636-3.

# 3.6 Concluding remarks

Translational research programs take advantage of numerous immortalized, primary cell and now iPSC-derived cellular models to mimic the human condition. Several

reports have attested to the usefulness of hiPSCs and their differentiated derivatives as human model systems especially when attempting to recapitulate disease in a dish. Still, we deemed it necessary to perform a comparative study with primary rat cardiomyocytes as hiPSC-CMs are associated with certain limitations, ie. their inherent immaturity. As such, we combined single cell signaling with bulk transcriptomics to compare the traditional rat neonatal cardiomyocyte with human iPSC-CMs. Overall, our data serves to suggest that RNCMs and hiPSC-CMs exhibit unique signaling signatures that are, in part, rationalized by mRNA levels and cross-species differences. As these differences were captured in non-matured hiPSC-CMs, these observations point towards the notion that even if our hiPSC-CMs are not adult-*like*, they are still superior cellular human proxies compared to traditional rat primary cardiomyocytes.

Selecting the correct model system becomes even more relevant in the context of cardiovascular disease modelling as receptor and effector expression levels have been reported to be altered. In the human failing heart, for example, the ratios of β-adrenergic receptors, G protein, GRKs, are different compared to non-failing hearts [40]. These changes undoubtedly affect cellular signaling profiles, rewriting gene transcription. Therefore, as working with hiPSC-CM enables patient access, these model systems allow us to gain a glimpse at how cellular context affects signaling in disease. As hiPSC-CMs endogenously express the main therapeutic targets in heart failure, there is no need for the overexpression of recombinant proteins thereby generating undesired artificial systems, as commonly done in HEK 293 cells. Even if RNCMs express the relevant players and effectors, their stoichiometries are different. Since their limited time in culture poses an extra experimental constraint, hiPSC-CMs represent an excellent proxy for modelling the human healthy or diseased heart in an *in vitro* setting. Lastly, hiPSC-CMs also express the hERG channel making them ideal models for cardiotoxicity studies as all drugs need to demonstrate the lack of pro-arrhythmic risk prior to approval.

This study does have potential limitations. It is important to consider certain confounding variables when analysing our single cell datasets. The rat neonatal cultures were essentially in an *in vivo* context 5 days prior to the assay and data collection while iPSC-CMs were in an *in vitro* context for 40-46 before being assayed. We attempted to

control for this by obtaining rat iPSCs however the cells did not survive post-thawing. Secondly, even if the RNCM maintenance media was supplemented with a mitotic inhibitor, it is noteworthy to be aware that a small population of cardiac fibroblasts renders the overall population somewhat impure. However, our culturing method has been shown to yield a population composed of ≥90% cardiomyocytes. Besides, the differentiation protocol used in this study does yield a mixed population of ventricular, atrial and pacemaker cardiomyocytes. Thus, some of the clusters observed may reflect these different cell types as they do exhibit distinct behaviours. Thirdly, iPSC-CMs were not matured in this study, and it would be interesting to follow up and assess how hiPSC-CMs would compare to those used here as well as to RNCMs or adult primary cultures. Nevertheless, the main message of our study remains, that the cellular vehicle needs to express the correct stoichiometry of receptors and effectors for meaningful conclusions to be drawn from the data particularly for translation efforts. All in all, our study demonstrates that human stem cell derived models of the cardiomyocyte do provide significant advantages, even if immature, and should be taken advantage of if their use is appropriate and aligned with the research objective.

# 3.7 Supplemental Figures and Legends



Supplemental Figure 3.1 Mean nuclear PKA activity over a 70-minute timeframe. Baseline normalized averaged nuclear PKA responses measured in (A) human iPSC-derived cardiomyocytes (hiPSC-CMs) and (B) rat neonatal cardiomyocytes (RNCMs). Ang II and  $\beta$ -arrestin biased peptides were dissolved in H<sub>2</sub>O. Isoproterenol, epinephrine and norepinephrine were dissolved in ascorbic acid. ET-1 and phenylephrine were dissolved in acetic acid. Grey shadow represents SEM.



Supplemental Figure 3.2 Graphical representations of PKA response clusters in hiPSC-CMs and RNCMs. Response clusters were plotted as a function of ExRai-AKAR2-NLS biosensor expression (RFU), ranging from (A) 0 - 5000, 1000 - 5000 and  $1000 - 10\ 000$ . Biosensor expression did not appreciably influence the response clusters generated. Requesting the clustering algorithm to unearth four clusters instead of (B) three provided a better fit for the data. Ang II and  $\beta$ -arrestin biased peptides were dissolved in H2O. Isoproterenol, epinephrine and norepinephrine were dissolved in acetic acid.



Supplemental Figure 3.3 Bar plot representing drug diffusion patterns within different fields of imaged hiPSC-cardiomyocytes expressing ExRai-AKAR2-NLS. Charts display drug diffusion at the first timepoint post-stimulation with (A) norepinephrine (Welch's ANOVA p = 0.3260), (B) forskolin (Welch's ANOVA p = 0.0214), (C) isoproterenol (Welch's ANOVA = 0.0043, Dunnett's multiple comparisons test field 8 vs. 20 p = 0.0404), Error bars represent SEM.



Supplemental Figure 3.4 Single nuclei PKA activation patterns as observed in hiPSC-CM and RNCM cultures in response to angiotensinergic drugs. Heatmaps displaying nuclear PKA activity as measured in (A) hiPSC-CMs and (B) RNCMs. Single nuclear PKA data summarized as  $\%\Delta$ F/F (y-axis) as a function of time (x-axis). The data was partitioned into four clusters representing distinct nuclear behaviors, either exhibited sustained or transient responses to agonists while other nuclei failed to respond or experienced a decrease in activity compared to baseline. Representation of the four response clusters was plotted as a bar chart with percentages of nuclei belonging

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to each response cluster as observed in (C) hiPSC-CMs and (D) RNCMs. Ang II and  $\beta$ -arrestin biased peptides were dissolved in H<sub>2</sub>O. Experiments were performed using RNCMs isolated from 4 different neonatal rat pup litters and 3 independent cardiac differentiations.



Supplemental Figure 3.5 Nuclear ERK<sub>1/2</sub> investigations in hiPSC-CM and RNCMs. Fluorescent microscopy image depicting expression of EKAREV-NLS donor in (A) hiPSC-CMs and (B) RNCMs. Agonist induced FRET responses plotted as a function of donor intensity in (C) hiPSC-CMs and RNCMs. Donor is expressed at higher intensities in hiPSC-CMs. (D) Averaged ERK<sub>1/2</sub> response profiles in both cardiomyocytes cell types. Ang II and  $\beta$ -arrestin biased peptides were dissolved in H<sub>2</sub>O.

Isoproterenol, epinephrine and norepinephrine were dissolved in ascorbic acid. ET-1 and phenylephrine were dissolved in acetic acid. Grey shadow represents SEM.



Supplemental Figure 3.6 Graphical depiction of nuclear ERK<sub>1/2</sub> response clusters. The clustering algorithm was requested to partition data in either (A) three or (B) four clusters. Based on the clustering output, nuclei appeared to experience three response profiles as opposed to four. This is exemplified in panel (B) as two clusters, displayed with orange and plum colours, appear to overlap with one another. (C) **Depiction of the sub-clustering applied on the 'Responder' population.** It is apparent that the 'responding' nuclei can be further divided into two clusters, referred as low- and high-responders respectively. Ang II and  $\beta$ -arrestin biased peptides were dissolved in H<sub>2</sub>O. Isoproterenol, epinephrine and norepinephrine were dissolved in ascorbic acid. ET-1 and phenylephrine were dissolved in acetic acid.



**Supplemental Figure 3.7 Ang II results in ERK**<sub>1/2</sub> action in RNCMs. (A) Representative western blot demonstrating that Ang II stimulation results in ERK<sub>1/2</sub> activity, depicted by pERK antibody at early timepoints, 5 minutes. (B) Western blot experiments summarized as bar plot, n=3.



Supplemental Figure 3.8 Ligand independent RNA-seq based comparison of the endogenous signaling machinery expressed in RNCMs, hiPSC-CMs and HEK 293 cells. Exploratory analysis of gene sets associated with GPCR signal transduction investigating (A) class A GPCRs, heterotrimeric G proteins (B) Gα, (C) Gβ and (D) Gγ as well as (E) effector expression profiles. Colorless heatmaps show transcript abundance measured in TPM, related to Figure 3.5. For comparison sake, data from HEK
### 293 cells was included (published originally as Lukasheva et al., 2020 Sci Rep 10, 8779. <u>https://doi.org/10.1038/s41598-020-65636-3</u>.



Supplemental Figure 3.9 Volcano plots demonstrating up- and downregulated genes as determined by bulk RNAseq, 60 minutes after stimulation with various agonists.



**Supplemental Figure 3.10 Transcript profiles of select energy metabolism-related gene sets. Transcript abundance (TPM) of gene involved in (A)** glycolysis and **(B)** fatty acid metabolism. Large colored heat maps represent gene expression based on TPM while smaller colored heatmaps display comparative expression (hiPSC-CM vs RNCMs). Colorless heatmap depict normalized transcript abundance.

#### **3.7 Author Contributions**

K.B. contributed to conception of the project, conducted experiments in all figures, analyzed data, produced figures, and wrote the original manuscript. J.J.T and R.D.M provided original computer code for analysis of single cell FRET data. D.P. performed several RNCM isolations. T.E.H. contributed to conception of the project, provided supervisory support, contributed to experimental design and interpretation of results and edited the manuscript.

#### **3.8 Conflict of Interest Statement**

The authors declare that they have no known competing financial interest or personal relationships that could have influenced the work reported here.

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#### 3.10 Chapter 3 – References

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# **Chapter 4 :** Effective use of genetically-encoded biosensors for profiling signaling signatures in iPSC-CMs derived from idiopathic dilated cardiomyopathy patients.

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**Keywords:** iPSC-CM, idiopathic cardiomyopathy, disease modelling, biosensors, signaling, calcium imaging, biobank.

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#### 4.1 Preface

After working with stem cells as discussed in Chapter 3, we quickly recognized their potential as state-of-the-art models for basic research but also for disease modelling applications. Then, on a late afternoon in the lab, I recall receiving a phone call where a cardiologist at the Glen site, Dr. Nadia Giannetti, requested to speak with me. Effectively, Dr. Giannetti was given my name from a member of the McGill Regenerative Medicine Network, as she was interested in generating a 'Heart-in-a-Dish' model of dilated cardiomyopathy. Dr. Giannetti mentioned that several of her patients were diagnosed with idiopathic forms of dilated cardiomyopathy and the general idea was to create stem cell derived cardiomyocytes from these patients to be able to better understand the disease at a molecular level as means to inform better treatment options. During the phone call, Dr. Giannetti essentially informed me that she was in the process of acquiring funding, from a private donor, and was simply looking for the expertise to generate cardiomyocytes and launch this project forward. As pleased as I was, I informed Dr. Giannetti that I was a PhD student and all collaborations needed to be approved by my supervisor even if I was evidently, extremely interested in the proposal. About one year later, we pitched our ideas and skillsets to Mr. Jacques Courtois who graciously offered to donate funding to begin what is now officially called the "Heart-in-a-Dish" project. Unfortunately, these exciting events unfolded during March 2020, also known as the beginning of the COVID-19 pandemic. Even if the project was off to a slow start, in the past two years, we have been able to demonstrate the development of a functioning pipeline between cardiologists and colleagues at the Glen hospital center and basic science researchers at the McIntyre Medical building. Essentially, as part of the project, our collaborators have successfully reprogrammed peripheral blood mononuclear cells into iPSCs, and we further differentiated them into spontaneously beating cardiomyocytes. These cells have also successfully entered our phenotyping platform where we are actively investigating how cellular signaling, morphology and viability is altered in dilated cardiomyopathy. We deem this essential for the development of personalized targeted therapies and to inform rational drug design for cardiovascular disease. Ultimately, with a better understanding of DCM, it may help reduce the number of patients requiring heart transplantation.

#### 4.2 Abstract

Dilated cardiomyopathy (DCM) is a cardiovascular condition that develops when the left ventricle of the heart enlarges, compromising its function and diminishing its capacity to pump oxygenated blood throughout the body. After patients are diagnosed with DCM, disease progression can lead to heart failure and the need for a heart transplantation. DCM is a complex disease where underlying causes can be idiopathic, genetic, or environmental. An incomplete molecular understanding of disease progression poses challenges for drug discovery efforts as effective therapeutic strategies remain elusive. Decades of research using primary cells or animal models have increased our understanding of DCM, but has been hampered due to the inaccessibility of human cardiomyocytes, to model cardiac disease, *in vitro*, in a dish. Here, our goal is to leverage patient-derived hiPSC-CMs and to combine them with biosensors to understand how cellular signaling is altered in DCM. With high sensitivity and versatility, optical biosensors represent the ideal tools to dissect the molecular determinants of cardiovascular disease, in an unbiased manner and in real-time at the level of single cells. By characterizing the pathobiology of dilated cardiomyopathy in a patient-specific manner using high content biosensor-based assays, we aim to uncover personalized mechanisms for the occurrence and development of DCM and as a pathway towards the development of personalized therapeutics.

#### **4.3 Introduction**

Cardiomyopathy is a heart muscle disease characterized by increased myocardial stress caused by the diminished capacity of the heart to adequately pump oxygenated blood throughout the body. There are five types of cardiomyopathies: dilated (DCM), hypertrophic (HCM), restrictive (RCM), arrhythmogenic right ventricular dysplasia (ARVD) and unclassified which includes Takotsubo cardiomyopathy. Each disease impacts patient prognosis differently. Irrespective of the etiological form: genetic or non-genetic, cardiomyopathy patients progress towards heart failure and many eventually require heart transplantation. Unfortunately, 50% of individuals diagnosed with heart failure (HF) succumb to this disease within 5 years of diagnosis. Of interest, dilated cardiomyopathies are characterized by left- or bi-ventricular dilation/enlargement and systolic dysfunction. A large fraction of DCM patients have a genetic etiology with 25% of these patients harbouring mutations in the titin gene. Besides the giant sarcomeric filament titin, mutations in several other sarcomere-related genes result in DCM (reviewed in [1,2]). DCM pathology can also be caused due to infection, autoimmunity as well as chemical and toxin exposure [3], such as alcohol or heavy metals [4,5]. Further, some prescription [6] or illicit drugs [7] have been associated with DCM. Of significance, certain chemotherapeutic agents have important side effects that progress towards a DCM phenotype either acutely or years after administration [8-11]. In addition, endocrine dysfunction and certain metabolic syndromes have been linked with the disease [12,13]. Dilated cardiomyopathy can also occur secondarily to neuromuscular causes [14]. However, many patients are diagnosed with idiopathic forms, in part, due to inadequate genetic testing [15]. Thus, with such heterologous underlying etiologies, challenges arise in developing novel therapies to treat DCM or cardiomyopathies in general-necessitating a more personalized attention.

A better understanding of the underlying molecular determinants that drive disease progression or therapeutic response is needed. The advent of induced pluripotent stem cells (iPSCs) allows the conversion of somatic cells, including patient-derived samples, into stem cells that can later be differentiated into functional cardiomyocytes (CMs) or other relevant cell types for phenotypic studies [16,17]. Numerous reports have demonstrated the power of human-derived iPSC-CMs to model various aspects of DCM, particularly genetic and chemotherapy-induced cases (Table 4.1). Several reviews have also been published showing how iPSC-CMs have been used to model genetic or sarcomeric cardiomyopathies [18-22].

Model	Disease	Cause	Key experimental observations	Speculative or Identified Therapeutic interventions	Reference
iPSC-CMs	DCM	R173W in cardiac troponin T	Abnormal Ca <sup>2+</sup> handling, reduced contractile force, disorganized sarcomeres.	β-adrenergic blocker treatment and over- expression of SERCA2a.	Sun et al. 2012 [31]
iPSC-CMs	DCM	TTN Ser14450fsX4	Defective myofibril assembly and stability.	Antisense oligonucleotide mediated skipping of <i>TTN</i> exon 326	Gramlich et al. 2015 [91]
iPSC-CMs, Engineered iPS- CM microtissues	DCM	I- and A-band TTNtvs	Sarcomere deficits, contractile deficits.	TTN gene expression, diminish miRNAs that inhibit sarcomerogenesis, or stimulate cardiomyocyte signals that improve function.	Hinson et al. 2015 [92]
iPSC-CMs	DCM	TNNT2 R173W	Reduced β-adrenergic signaling, increased (PDEs) 2A and PDE3A expression.	Pharmacological inhibition of PDE2A and PDE3A	Wu et al. 2015 [74]
iPSC-CMs	DCM	(cTnT-R173W)	Myofibrillar disruption, reduced sarcomeric assembly.	Hypocontractile phenotype reversed by the myosin activator omecamtiv mecarbil.	Broughton et al. 2016 [93]
iPSC-CMs	DCM	RBM20 R636S missense mutation	Defective Ca <sup>2+</sup> homeostasis, apoptotic changes, and sarcomeric disarray.	Protective effects seen with β-blocker, carvedilol, or Ca <sup>2+</sup> -channel blocker, verapamil.	Wyles et al. 2016 [94]
iPSC-CMs +	DCM	TNNT2 p.R173W	Sarcomere disarray and altered Ca <sup>2+</sup> cycling	Allelic-specific knockout could ameliorate the DCM	<u>Karakikes</u> et al. 2017

**Table 4.1** Non-exhaustive literature search of ESC- or iPSC-CM based *in vitro* models of dilated cardiomyopathy.

iPSC-CMs	DCM	TNNT2 R173W	signaling, increased (PDEs) 2A and PDE3A expression.	inhibition of PDE2A and PDE3A	Wu et al. 2015 [74]
iPSC-CMs	DCM	(cTnT-R173W)	Myofibrillar disruption, reduced sarcomeric assembly.	Hypocontractile phenotype reversed by the myosin activator omecamtiv mecarbil.	Broughton et al. 2016 [93]
iPSC-CMs	DCM	RBM20 R636S missense mutation	Defective Ca <sup>2+</sup> homeostasis, apoptotic changes, and sarcomeric disarray.	Protective effects seen with β-blocker, carvedilol, or Ca <sup>2+</sup> -channel blocker, verapamil.	Wyles et al. 2016 [94]
iPSC-CMs + TALEN	DCM	TNNT2 p.R173W	Sarcomere disarray and altered Ca <sup>2+</sup> cycling parameters.	Allelic-specific knockout could ameliorate the DCM phenotype.	<u>Karakikes</u> et al. 2017 [95]
iPSC-CMs + EHT	DCM	RBM20 mutation S635A	Visco-elasticity and reduced contractile force, dysregulated sarcomeric organization and defective Ca <sup>2+</sup> handling.	N/A	Streckfuss- Bömeke et al. 2017 [96]
iPSC-CMs	DCM	Genetic	Telomere shortening as a hallmark of genetic cardiomyopathies.	N/A	Chang et al. 2018 [97]
iPSC-CMs + CRISPR-Cas9	DCM	Heterozygous A-band TTNtv	Impaired sarcomerogenesis, decrease in diastolic traction stresses.	N/A	Chopra et al. 2018 [98]
iPSC-CMs	DCM	TTN mutation c.86076dupA, c.70690dupAT	Disorganized sarcomeres, attenuated inotropic response.	N/A	Schick et al. 2018 [99]
iPSC-CMs	DCM	BAG3 mutation (R477H), BAG3 (KO)	Myofibrillar disarray, proteosome inhibition.	Overexpression of the stress response protein heat shock factor 1.	McDermott- Roe et al. 2019 [100]
iPSC-CMs + CRISPR-Cas9	DCM	RBM20 deficient	mRNA splicing defects, altered calcium and impaired contractility.	All-trans retinoic acid.	Briganti et al. 2020 [101]
iPSC-CMs + CRISPR/Cas9- engineered isogenic controls	DCM	Troponin T (TnT)-R173W	Disorganized sarcomere and impaired contractility.	Small molecule-based activation of AMPK.	Dai et al. 2020 [57]

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iPSC-CMs+ CRISPR-Cas9	DCM	Mutation (1n1), isogenic 1n1- KO	and reduced contractile force.	N/A	Malkovskiy et al. 2021 [102]
iPSC-CMs	DCM	p.C335R sodium voltage-gated channel alpha subunit 5 (SCN5a), TTN variant (p.Ser24998LysfsTer28)	Na+ channel currents were also reduced in iPSC-CMs dysregulation of sarcomere structures	N/A	Sedaghat- Hamedani et al. 2021 [103]
iPSC-CMs, iPSC- cFbs	DCM	LMNA mutations	Hyperactivated ERK, elevated levels of apoptosis markers, sarcomeric and nuclear abnormalities.	N/A	Yang et al. 2021 [104]
iPSC-CMs	DCM	Cardiac troponin T (R173W)	[Ca2+]-alternans, increased myofilament sensitivity to Ca <sup>2+</sup> .	Ca <sup>2+</sup> Desensitiser, blebbistatin, normalized calcium abnormalities.	Jung et al. 2022 [65]
iPSC-CMs	DCM	TTNtv c.70051C > T, p.Arg23351Ter	N/A	N/A	Lee et al. 2022 [105]
iPSC-CMs	Cardiotoxicity	RARG variant rs2229774	Suppression of topoisomerase 2b expression and activation of the ERK pathway.	RARG agonists, CD1530	Magdy et al. 2021 [106]
hESC-CMs	Cardiotoxicity	Doxorubicin-induced	Cytotoxicity, altered morphology, reduced contractility.	cTnT release can be used as a measurement of acute cardiotoxicity	Holmgren et al 2015 [107]
hES-CM	Cardiotoxicity	Doxorubicin-induced	Unsynchronized beating, lower protein concentrations	MicroRNAs as potential biomarkers for drug- induced cardiotoxicity.	Holmgren et al. 2016 [108]
iPSC-CM	DCM	Doxorubicin-induced	Increased apoptotic and necrotic cell death, reactive oxygen species production, mitochondrial dysfunction and increased intracellular Ca <sup>2+</sup> concentration.	CRISPR-Cas9-mediated disruption of TOP2B.	<u>Maillet</u> et al. 2016 [109]
hiPSC-CMs	Cardiotoxicity	Doxorubicin-induced	Arrhythmic beating	Identified gene panel that can be applied in the safety assessment of novel drug candidates	Chaudhari et al. 2016 [110]
iPSC-CMs	Cardiotoxicity	Doxorubicin-induced	Cytotoxicity, altered morphology, reduced contractility.	N/A	Holmgren et al. 2018 [111]
mESC-CMs (line E14TG2A )	Cardiotoxicity	Doxorubicin-induced	N/A	CM-mESC transplantation	Silva dos Santos et al. 2018 [112]
iPSC-CMs	Cardiotoxicity	Trastuzumab-induced	Impaired contractile and calcium-handling.	AMP-activated protein kinase activators.	Kitani et al. 2019 [113]
iPSC-CMs	Cardiotoxicity	Doxorubicin-induced	Cationic transporters upregulated in patients experiencing toxicity.	Identification of OCT3 as uptake transporter of doxorubicin in cardiomyocytes.	Huang et al. 2021 [114]
iPSC-CM of aggressive B cell lymphoma cancer patients	Cardiotoxicity	Doxorubicin-induced	Arrhythmogenic events and contractile dysfunction	CamKIIδ inhibition.	Haupt et al. 2022 [115]

To begin to understand the specific molecular events that drive idiopathic DCM disease progression, we have established an hiPSC-based program for personalized cardiovascular medicine enabled by access to patient-derived blood samples followed by iPSC generation. Our biobank of hiPSCs when differentiated into cardiomyocytes are combined with an optical biosensor-based screening platform to identify patient specific signalosomes as means to better track idiopathic disease mechanisms. Genetically-

encoded optical biosensors (GEBs) are chromophore-based and can be used to probe cellular responses to specific stimulations [23,24].

To obtain a mechanistic understanding of DCM at the molecular level, we have established proof of concept data that use biosensors to model idiopathic DCM, in vitro, in a dish. The biosensors used here target pathways downstream G protein-coupled receptors (GPCRs) as the activation of these signaling cascades is interconnected with the pathobiology and pharmacotherapy of dilated cardiomyopathy. We explored protein kinase A (PKA) [25,26] and extracellular signal-regulated protein kinase (ERK<sub>1/2</sub>) activity [27] as well as calcium handling in patient and control iPSC-derived cardiomyocytes (iPSC-CMs) as an approach to uncover the unknown molecular anonymities causing idiopathic DCM. PKA and calcium handling were selected as important regulators of cardiac contractility [28] while ERK<sub>1/2</sub> signaling is an important cell survival signal in the myocardium [29,30]. We are specifically interested in assembling detailed patient-specific cellular signaling profiles to obtain a first glance at the diversity or similarities across the entire patient and control cohort. In our study, control subjects are healthy individuals with no known cardiac conditions. Ultimately, we would like to uncover whether features of the disease process are shared amongst male and female subjects or whether age, etiology, etc are determining factors of the molecular profiles being gathered. At the outset, we want to know whether the idiopathic nature of the disease will seep into the iPSC-CM model as well as genetic DCM which has been relatively well recapitulated in a dish [31]. As idiopathic disease could also be genetic, due to insufficient testing in the clinic, exome sequencing may be required in follow-up studies. With the diverse etiologies of our patient cohort, described below, we will have the inherent capacity to examine the molecular fingerprint of idiopathic, genetic, familial and chemotherapy induced DCM. Our novel approach can also assess whether iPSC-CM maturity influences in vitro modeling of idiopathic disease. Based on the current logistical model, maturation does not seem to influence DCM disease modeling when assessing genetic etiologies [31]. However, some conflicting evidence has been reported such as when performing clinical trials in a dish [32,33]. Yet, to what extent will this be the case for idiopathic DCM? Since biosensors allow for repeated measures, signal profiles can be assessed at distinct timepoints (day

30, 45, 60) to control for cardiomyocyte maturity while removing potential batch effects by assaying the same cells. This data will feed the development of personalized interventions through improved understanding of individual disease mechanisms and therapeutic responses. Below, we describe our patient population and show preliminary data that supports using optical biosensors for idiopathic disease modelling.

#### 4.4 Materials and Methods

#### 4.4.1 Recruitment of patients and control subjects

Male and female patients as well as control subjects were recruited at the cardiology clinic at the McGill University Health Center. Control subjects were either healthy family members of the patients or participants with no known cardio-pulmonary disease. All enrolled participants provided informed consent to be a part of the 'Heart-in-a-Dish" project with REB approval HID-B/2020-6362. Each individual completed a questionnaire that recorded information concerning their biological sex, age, ethnicity, cardiovascular family history, smoking, cholesterol, past cancer diagnosis, current medications and a genesis praxy gender questionnaire.

# 4.4.2 Generation and validation of hiPSCs from healthy control volunteers and DCM patients

#### 4.4.2.1 Reprogramming of PBMCs into induced pluripotent stem cells

Peripheral blood mononuclear cells (PBMCs) were grown in StemSpan SFEMII supplemented media (STEMCELL Technologies, CA) which are distinct since they can be observed to float in culture. Once PBMCs were grown to sufficient numbers, at least 1x10<sup>6</sup> PBMCs, the Epi5<sup>™</sup> Episomal iPSC Reprogramming Kit (Thermofisher, USA) was used along with the Neon Transfection System (Invitrogen) to introduce Oct4, Sox2, Lin28, Klf4, and L-Myc into the cells using the following parameters: 3 pulses, 10 ms, 1650 V. Once electroporated, cells were seeded onto freshly prepared Matrigel-coated plates (Corning), to help with the attachment of the iPSCs. Reprogramming, as per [34], was regarded as successful when floating cells began to attach to the cell culture dish, observed on approximately day 7 after electroporation. Once attached, iPSC colonies

slowly expanded and were mechanically split under the microscope. Briefly, a 20- or 22gauge needle was used to delineate the border of the colony, and a grid was then made for colony splitting. iPSC colonies were selected after gross morphological inspection under a brightfield microscope. High quality iPSCs that were picked displayed large nuclei, were tightly packed, and colony borders had a clear delineation, without any differentiation or fibroblast-like cells at the borders [16,35].

#### 4.4.2.2 Validation of pluripotency and quality of iPS cell lines

All iPSC lines will be subsequently validated [36-38] by 1) colony morphology; 2) alkaline phosphatase; 3) qRT-PCR and immunocytochemistry assays of pluripotency markers (e.g., Nanog, SSEA4, SOX2, Klf4, Tra 1-60). iPSC colonies at passages 6 and onward were immuno-stained to assess expression of core pluripotency genes at the protein level. For immunofluorescence studies, hiPSCs were replated onto Matrigel-coated 24-well plates, and colonies were fixed with 4% PFA once they measured approximately at 100 – 500 µm in diameter. Next, colonies were permeabilized using 0.5% Triton X-100 in PBS for 15 minutes at RT. Cells were then stained with primary antibodies overnight, at 4°C, for nuclear pluripotency markers OCT4 (Cell Signaling, #2750) and NANOG (Cell Signaling, #4903), as well as surface markers SSEA-4 (Cell Signalling, #4755) and TRA-1-60 (Cell Signaling, #4746). The following day, iPSC colonies were co-stained with secondary antibody AlexaFluor-488, anti-rabbit or antimouse, for 1 hour at RT. Representative images were captured using a fluorescent microscope. If the localization and intensity of the staining of each respective antibody was found to be appropriate across all colonies imaged, this confirmed pluripotency.

Further, to functionally validate the ability of iPSC to differentiate into the three germ layers- ectoderm, mesoderm, and endoderm, a trilineage differentiation assay was performed (R&D Systems, SC027B). The expression of Otx2 was assessed as it is a nuclear protein responsible for the primitive streak stage of embryonic development – it is closely related and expressed in the ectoderm, which gives rise to neurons, eyes, skin, etc. In addition, the expression of Brachyury was examined as it is a transcription/mesodermal factor expressed in tissues fated to become the heart, bone

marrow, etc. Brachyury is found in the nucleus. Lastly, Sox17, found in the nucleus and cytoplasm, was studied as it is an important factor during endoderm formation throughout the gastrulation process. Based on the manufacturer's instructions, iPSC colonies were seeded into 2x3 wells of a 24-well plate and grown according to the R&D Systems' protocol. Each well (in duplicate) was differentiated and stained with the respective markers above. This assay essentially allowed for the qualitative assessment of the quality and potential of our iPSC lines to differentiate.

To ensure chromosomal integrity, hiPSC lines were screened for normal karyotype by G-banding technique. Briefly, iPSC colonies were seeded into two Matrigel-coated 60mm tissue culture dishes and harvested at 50-60% confluency. The morning of the harvest, Colcemid (ThermoFisher, 15212012) was added onto the iPSCs at two different concentrations; 0.10 µg/mL and 0.15 µg/mL, and incubated at 37°C for 30 min. Cells were then washed three times with PBS prior to being incubated with Accutase solution. Dissociated iPSCs were collected and spun down at 1000 RPM for 10 min in a 15 mL conical tube. If the cell pellet was between 2 to 4 mm, the protocol was continued, otherwise pellets were discarded. Once the supernatant was removed, leaving approximately 0.5 mL, a pre-warmed, 37°C, 0.075 M KCl hypotonic solution was added onto the pellet and the tube was inverted  $\sim$ 7 times. In the fume hood, cells were prefixed by adding 8 drops of RT Carnoy's fixing solution (3 methanol: 1 glacial acetic acid) to the KCI solution. The tube was then inverted 10 times followed by centrifugation at 1000 RPM for 10 min. In the fume hood, the supernatant was aspirated until 0.5 mL and the pellet was resuspended by flicking the tube. Cold fixing solution, 1 mL, was then added and the tube was inverted 10 times. Cells were then incubated at -20°C for 1 hour. After being inverted a couple times, cells were spun down at 1000 RPM for 20 min at 4°C. The supernatant was removed until 0.5 mL and the pellet was resuspended using flicking motion before being resuspended in 14 mL cold Carnoy's fixing solution. Cells were then shipped to Cytogenomics Facility at the Hospital for Sick Kids in Toronto, Ontario, Canada for karyotyping. All iPSC lines were also screened for mycoplasma contamination using a mycoplasma detection kit (ThermoFisher, 4460623). Once the test came back negative,

iPSC lines were established within our biorepository and subsequently differentiated into cardiomyocytes.

#### 4.4.3 Differentiation of hiPSCs into functional cardiomyocytes

Control and patient-derived iPSCs were differentiated into cardiomyocytes following the previously published GiWi protocol with minor modifications [39]. To achieve a 95% confluent monolayer of iPSCs, 500,000 cells were seeded onto Matrigel (Corning, 354277) coated 24-well dishes in mTeSRPlus media supplemented with 10 µM Rho kinase inhibitor, Y-27632-HCI (Selleckchem, S1049). The following day, spent media was removed and 1 mL fresh mTeSRPlus media was added onto the iPSC and cells were returned to the incubator. On day 0 of the GiWi protocol, Wht signaling was activated by supplementing the RPMI 1640 + B27 minus insulin media (ThermoFisher A1895602) with 12 µM Wnt activator or GSK-3 inhibitor, CHIR99021 (Cayman Chemical, 13122). The time was recorded to ensure the media would be exchanged 24 hours later with fresh RPMI 1640 supplemented with B27 minus insulin. On day 3, Wnt signaling was inhibited using the IWP2 compound (Selleckchem S7085). Precisely 48 hours later, on day 5, the spent media was removed and exchanged with RPMI 1640 supplemented with B27 minus insulin. On day 7 and day 10 of the protocol, the media was changed for RPMI 1640 supplemented with regular B27 (ThermoFisher, 17504001). At this time, spontaneous beating iPSC-CMs should be observed under a brightfield microscope using 10X or 20X magnification. Starting at day 10-12, metabolic selection was performed and the iPSC-CMs were starved from glucose for 4-6 days. RPMI 1640 without glucose, supplemented with B27 as well as with 4 mM lactate was used to purify the cardiomyocyte population [40]. Wells embedded with monolayers of spontaneously beating cardiomyocytes were replated into fibronectin-coated 6-well dishes and maintained in RPMI 1640 media supplemented with B27 until day 35-40 where 30,000 iPSC-CMs were re-seeded into optical bottom, black 96-well plates for cellular signaling experiments (Nunc, 165305).

#### 4.4.3.1 Transduction of hiPSC-CMs with adeno-associated viruses

Adeno-associated viruses (AAVs) used in this study were produced by the Neurophotonics Platform Viral Vector Core at Laval University, Québec. To minimize

freeze-thaw cycles, AAVs were aliquoted in low-retention Eppendorf tubes and stored for long-term at -80°C. Post-thawing, AAVs were kept at 4°C for a maximum of 7-10 days. To determine the optimal serotype to transduce our iPSC-CM cultures, we used the serotype selection kit offered by the Neurophotonics Platform (data not shown). The kit contains AAV serotypes 1, 2, 5, 6, 8, 9, Rh10, DJ, DJ8, php.B, php.eB, php.S and retro for testing. Based on these results, iPSC-CMs were transduced with biosensors packaged within AAV serotype 6, which had previously been reported to be effective at infecting cardiomyocytes [41,42]. During transduction, AAVs were kept on ice before diluting them in RPMI 1640 + B27 media. To minimize excessive cell debris carried over during cell seeding into the microwell dish, cardiomyocyte cultures were washed three times with basal RPMI 1640 media before being transduced. A multiplicity of infection (MOI) of 5000, representative of 5000 viral genomes per cell was used for all experiments. Post virus addition, iPSC-CM cultures were returned to the 37°C incubator and kept there for a minimum of 72 hours to allow for sufficient biosensor expression.

#### 4.4.4 Single-fluorophore intensity-based and dual-colour FRET imaging

On the day of the experiment, cardiomyocyte cultures were visualized under a phase-contrast microscope to confirm that the iPSC-CMs were of good quality. Essentially, we confirmed that the iPSC-CMs were spontaneously beating and appeared to have a cardiomyocyte-like morphology. If the cells were deemed healthy, the media was exchanged for pre-warmed, 37°C, assay buffer, HBSS without phenol red, with calcium, magnesium, and sodium bicarbonate (Wisent 311-513-CL). Cells were washed three times prior to leaving the cells in 90  $\mu$ L HBSS for the assay. The microwell plate was then returned to a humidified atmosphere of 37°C with 5% CO<sub>2</sub> for 45 minutes to an hour before imaging. This permitted the iPSC-CMs sufficient time to re-equilibrate in the assay buffer post-washing. Meanwhile, the temperature control settings of Perkin Elmer's Opera PHENIX high-content screening system were turned on. The microscope would thus have sufficient time to warm up to 37°C with 3% CO<sub>2</sub> for live-cell imaging. Concurrently, drugs were prepared at the benchtop at a 10-fold more concentration as 10  $\mu$ L would be added in each well, which is equivalent to a 10-fold dilution. For EKAREV-NLS based experiments, FRET imaging was collected using a 20X air objective using a

425 nm laser for excitation of the CFP donor. Emissions were detected with filters at 435-515 nm (CFP-donor) and 500-550 nm (YFP-acceptor). For the ExRai-AKAR2-NLS biosensor, images were acquired using a 20X air objective using a 375 nm and 480 nm laser for excitation and 500-550 nm emission filter. In the experimental setup within Perkin Elmer's Harmony software, parameters were set, where the automated microscope would capture an initial basal, ligand independent, measurement. Subsequently, the assay microplate was ejected from the system, and drug stimulations were conducted manually. To minimize cellular perturbations, we were cautious not to move/displace the microplate during agonist addition. The microscope then continued collecting 7 measurements post-drug stimulation for another 70 minutes at 10-minute intervals as programmed.

#### 4.4.4.1 Single-fluorophore intensity-based and dual-colour FRET analysis

Post-imaging raw image files were imported into Perkin Elmer's Columbus image analysis software. As biosensors were localized to the nucleus, iPSC-CM nuclei were identified using the 'find nuclei' feature. Subsequently, nuclear morphology features, including nuclei roundness and area were calculated. The morphology criteria permitted the sorting of the nuclei's to then select a population of 'healthy' nuclei without including unwanted auto-fluorescent cell debris. Roundness and size thresholds were set to remove cellular debris which had rough edges and were smaller in size compared to a *typical* cell's nucleus. To calculate the YFP/CFP FRET ratio for EKAREV-NLS biosensor, the intensity properties of the acceptor and donor fluorophores were computed. The same calculations were performed by Columbus to calculate the GFP excitation ratio for the ExRai-AKAR2-NLS biosensor. Data of each individual nucleus that was categorized as 'healthy' was then exported as text files for further analysis in R.

Single nuclei datasets were treated in R based on previously published single-cell analytical approach with minor modifications [43]. Briefly, cardiomyocyte nuclei that appeared in all 8 timepoints (t=0 and post-stimulation) were carried forward. As nuclei tend to drift marginally once the microplate is loaded back into the microscope post drug

addition, a threshold of fluorescence intensity and distance was set for each nucleus. To be considered as the same object (nucleus), the fluorescence intensity could only deviate by ≤20%. Nuclei that did not conform to this parameter were excluded from the analysis. To compute the change in FRET in response to drug addition, a  $\Delta$ FRET parameter was calculated. AFRET represents an individual's nuclei FRET relative to basal, ligand independent FRET. This was then converted into a percentage change in FRET ( $\Delta \Delta F/F$ ). In this percentage change in FRET, the denominator (F) was computed by averaging the basal FRET across all nuclei in the same microwell. The TSclust package [44] in R was then used to cluster the cells by the magnitude of response over time. In this package, the 'pam' function was used. For single nuclei clustering, patient/control or male/female nuclei were merged into a single dataset. This allowed for consistency within the patterns and clusters identified within the two populations that were being assessed. Performing the clustering algorithm on separate datasets may result in distinct clusters being identified. This method would assure the clusters identified would be correlated in the two datasets. Following the clustering, the merged patient/control or male/female datasets were split and further plotted as heatmaps for visualization using pheatmap in R.

#### 4.4.5 Calcium imaging and analysis

Single cell calcium handling was measured using RGECO-TnT, a red-shifted single-colour intensiometric biosensor that localizes to troponin T in the myofilament [45]. iPSC-CMs were seeded at a density of 20,000 cells per microwell of a black, optical bottom, 96-well plate. Approximately three days later, iPSC-CMs were washed three times with basal RPMI 1640 media before being transduced with AAV2/6-RGECO-TnT using an MOI of 5000. Prior to recording calcium transients, iPSC-CM cultures were inspected under a phase-contrast microscope to ensure cells were healthy. Then, media was exchanged with RPMI 1640 without phenol red supplemented with B27 to minimize background caused by autofluorescent phenol red. Spontaneous, ligand independent calcium transients in iPSC-CMs were captured at 10.4 frames per second for 15 seconds using a Zeiss Axio Observer fully automated inverted microscope with a Zeiss 20x PLAN APOCHROMAT (NA 0.8), X-Cyte 120 LED light source, and FS-14 RFP filter set (560/26

nm excitation, 620/60 nm emission, 565 dichroic mirror). For the duration of the experiment, hiPSC-CMs were kept in a temperature- and CO<sub>2</sub>-controlled chamber, mimicking physiological conditions, 37°C and 5% CO<sub>2</sub>. The open-source image processing software, Image J, was used to trace single cells from image stacks. Analysis of recorded videos was performed using a custom Transient Analysis app in OriginPro 2021b v9.8.5 (OriginLab). This software has the built-in capacity to determine features of calcium transients including but not limited to the calcium transient frequency, time to peak, peak amplitude, time between peaks, transient duration, area under the curve, time to reach 50% baseline, time to reach 90% baseline and upstroke velocity. Analysis was performed on a single-cell basis. Statistical analysis was done using GraphPad Prism v9.3.1 with a Welch's ANOVA test and Dunnett's multiple comparisons controlling for unequal variances.

#### 4.5 Results and Discussion

#### 4.5.1 Establishment of control and DCM iPSC lines

To develop a more personalized approach to cardiac care, we created a biorepository of patient samples from those diagnosed with dilated cardiomyopathy as well as age-matched healthy control subjects (**Fig. 4.1 & Suppl. Table 4.1, 4.2**). Enrolled patients had different suspected primary etiologies of their respective dilated cardiomyopathic phenotypes ranging from idiopathic, viral, genetic, chemotherapy-induced etc. providing us with a comprehensive patient population to sample from (**Fig. 4.1A**). The large majority of our patients also have a left ventricular ejection fraction of less than 40%, a feature characteristic of systolic dysfunction (**Fig. 4.1B**). Thus far, we have recruited 228 individuals who consented to be part of the "Heart-in-a-Dish" project. The current patient population includes men and women of different ages and ethnicities as well as healthy individuals that will serve as control subjects (**Fig. 4.1C-F**). Based on available patient data, the family history of cardiac disease is higher in the patient pool compared to control (**Suppl. Table 4.1**). Risk factors associated with HF are also more prevalent in the DCM patient population. Most patients have been prescribed pharmacological agents that target neurohormones; the  $\beta$ -adrenergic or renin-

angiotensin-aldosterone system (RAAS) such as  $\beta$ -blockers, ACE inhibitors, or a combination of angiotensin receptor and a neprilysin inhibitor (**Suppl. Table 4.2**).



## Figure 4.1 Overview of general characteristics of the patient and control population enrolled in the "Heart-in-a-Dish" study.

(A) Bar chart illustrating the percentage per primary suspected etiologies of the registered patient cohort. Heterologous DCM etiologies range from idiopathic, familial, genetic, chemotherapy, alcohol, viral among others. (B) Graph demonstrating the percent of patients with different left ventricular ejection fractions (LVEF). A large fraction of patients fall within a LVEF percentage of less than 40%, a typical feature of systolic dysfunction. (C) Age and (D) Biological sex of patient and control population. Patients of all ages have been recruited in the study as well as a well-balanced ratio of males and females. (E) Patients as well as control subjects have been recruited as part of the study. An approximate 10:1 ratio has been achieved when enrolling patient and control subjects respectively. (F) Various ethnicities of the patient and control subjects when they filled in the questionnaire during enrollment.

To model dilated cardiomyopathy in a dish, patient blood was extracted using veinpuncture and stored in the biobank for subsequent analysis. CD34+ cells were isolated from the buffy coat and reprogrammed into iPSCs using episomal plasmids or nonintegrating Sendai-virus. Successful reprogramming into a primordial pluripotent state was confirmed by immunofluorescence for pluripotency markers, as well as tri-lineage differentiation (Fig. 4.2A-C). Once the "stemness" of the iPSC lines was validated followed by normal karyotype analyses (Suppl Fig. 4.1), hiPSCs were differentiated into cardiomyocytes using a defined protocol that modulates Wnt signaling (Fig. 4.3A) [46]. Spontaneous contracting monolayers were observed for all patient and control lines that entered the cardiac differentiation program. The cardiomyocyte lineage was further validated by gene and protein expression studies (Fig. 4.3B, C). Troponin I3 expression increased with time in culture indicating maturation of the iPSC-CMs and staining with anti- $\alpha$ -actinin2 showed that most of the cells were in fact cardiomyocytes. The expression of the  $\beta$ -adrenergic receptors was increased over time in culture, demonstrating the functionality and ability of iPSC-CMs to respond to external stimuli (Fig. 4.3D). Once cardiomyocyte identity was confirmed and after 4 weeks in culture, we virally introduced optical biosensors using adeno-associated viruses (Fig. 4.3E) as tools to shed light on underlying DCM disease mechanisms, as described below.



Figure 4.2 Reprogramming and validation of patient- and control derived iPSCs.

(A) Schematic illustrating the process required to reprogram PBMCs into iPSCs using either episomal plasmids or non-integrating Sendai virus techniques. The process of reprograming PBMCs into iPSCs takes on average 2-3 months. To some extent, the efficiency of reprogramming was inversely proportional with age, with samples sourced from older individuals exhibiting lower reprogramming efficiency, requiring at times, repeated attempts. (B) Immunofluorescence images depicting protein expression of key pluripotency markers: Nanog, Oct4, SSEA4 and TRA-1-60. As depicted, OCT4 and NANOG co-localize with Hoechst stains and SSEA-4/TRA-1-60 are found at the cell surface. (C) Tri-lineage differentiation of a representative iPSC cell line. Fluorescent microscopy images demonstrate the pluripotential of the iPSC line to differentiate into the three germs layers, ectoderm (Otx2), mesoderm (Brachyury) and endoderm (Sox17). Figure Panel (A) was created with BioRender.com.



Figure 4.3 Differentiation of patient and control iPSCs into functional cardiomyocytes.

(A) Schematic illustration of the differentiation protocol to generate cardiomyocytes from iPSCs. Through the temporal modulation of Wnt signaling, the protocol yields spontaneously contracting cardiomyocytes within 7-10 days. (B) RTaPCR gene expression analysis revealed cardiomyocyte fate of differentiated **iPSCs.** Expression of troponin isoforms relative to housekeeping gene GAPDH of three or more independent differentiations over time. (C) Immunofluorescence images demonstrating protein expression of a sarcomere-related gene. iPSC-CMs stained with  $\alpha$ -actinin2 followed by secondary antibody Alexa-647 and Hoechst nuclear dye validates cardiomyocyte identity. (D) RT-qPCR gene expression analysis demonstrating that iPSC-CMs are signaling competent. RT-gPCR gene expression of  $\beta_1$ - and  $\beta_2$ - adrenergic receptors establishes that cardiomyocytes can transduce signals through cell surface receptors that are also critical targets for DCM pharmacotherapy. (E) iPSC-derived cardiomyocytes are effectively transduced using AAV serotype 6. Fluorescent microscopy images depicting the delivery of eGFP into iPSC-CMs using AAV2/6. The green colour of eGFP is well matched with the AlexaFluor-647 signal when iPSC-CMs are co-stained with sarcomeric marker  $\alpha$ -actinin2. Correspondingly, all biosensors in this study were packaged in serotype 6. Mean  $\pm$  SEM, \* p<0.5, \*\*p < 0.01. Figure Panel (A) was created with BioRender.com.

#### 4.5.2 Biosensors for cardiovascular disease modelling

Over the past decade, genetically-encoded biosensor (GEB) technology has undergone significant development. Biosensors have been designed to probe for the activity of more than 100 different signaling pathways, many of which are cardiac relevant [47,48] (Fig. 4.4A). GEBs typically fall into two broad categories, either being based on resonance energy transfer (RET) or on the intensity of biosensor emission (reviewed in [49,50]). Briefly, RET is a quantum optical process that relies on the non-radiative transfer of energy from a donor to an acceptor. Compatibility between donor and acceptor probes requires the donor's emission spectrum to overlap with the excitation wavelength of the acceptor. This energy transfer event is dependent on distance (up to a maximum of 10 nm) as well as orientation (reviewed in [51-54]). However, certain versions are less dependent on orientation [27]. Addedly, RET biosensors can either have a bioluminescent donor (BRET) or a fluorophore as donor (FRET). BRET biosensors typically suffer from weaker signals but have higher signal to noise ratios compared to FRET equivalents [55]. FRET however is more amenable to single cell imaging and microscopy applications. In contrast, intensiometric biosensors report on analyte concentrations or effector activation in accordance with fluctuations in the magnitude of fluorescence intensity emitted by a single fluorophore. In this context, the fluorescence of intensiometric biosensors is quenched in the absence/presence of the molecule being probed and changes to the degree of pathway activation (increase/decrease) alters the intensity of the fluorescence signal emitted. Since intensiometric sensors monitor signaling by recording the emission of a single wavelength, their main advantage is their ability to be multiplexed.

Biosensors are excellent tools for the detection of numerous biological interactions in real-time and in live cells. When used in human iPSCs and their differentiated derivatives, as well as in patient-derived samples, biosensor methods have the potential to explore disease causative mechanisms. For example, genetically encoded biosensors have facilitated the study of calcium and kinase signaling in iPSC-CMs derived from hypertrophic cardiomyopathy [45] and dilated cardiomyopathy [56,57] patients respectively. GEBs have also helped resolve electrophysiological changes in stem cell derived CMs while revealing their higher throughput capabilities when using GEBs compared to traditional methods [58,59]. However, several studies still rely on indicator dyes such as Fluo-3 and Fluo-4 AM to study calcium handling in patient derived iPSC-CMs [31,60]. Even if the use of indicator dyes does present with certain advantages, such as ease of use without the need of transfection, biosensor technology represents a more versatile approach. Most notably, the catalog of GEBs allows us to dissect changes in the disease- and patient- specific signalosomes investigating the activity of ion channels, protein kinases as well as a long list of protein effectors. Importantly, GEBs tend to result in less cellular toxicity compared to the use of dyes and can be more easily targeted to repeated measures allowing to track cardiomyocyte function in long-term measurements and in response to chronic stressors, a feature that is useful as cardiovascular disease evolves overtime. To our knowledge, biosensor-based studies have only been used in diseased iPSC-CMs with inherited or strong genetic etiologies.

#### 4.5.2.1 Combining optical biosensors with iPSC-CMs

Much evidence points to dysregulated signaling pathways in the development of dilated cardiomyopathy. Considering that DCM shows a phenotypic spectrum with inherited and environmental etiologies, the molecular underpinnings of the disease may be more complex than previously anticipated. To study DCM pathogenesis in iPSC-CM monolayers, we choose genetically-encoded biosensors that interrogate ERK<sub>1/2</sub> and PKA signaling as well as calcium handling as these have been shown to be altered in DCM. We also elected to follow changes in signaling at the single-cell level. Recently single cell approaches have gained prominence as they are being performed at larger scale and tend to generate richer datasets. Bulk analyses may allow for high-throughput biosensor assays and are generally less computationally intensive compared to single-cell analytical approaches. Yet, single cell approaches provide in-depth analysis of the complex behaviours of all cells within the overall population. Individual cells process information differently as their intracellular and extracellular milieus are distinctive, including neighboring cells and growth factor concentrations. Consequently, no cell perceives information the same way and the cellular state has also been reported to impact cellular signaling behaviors of single cells [63].

We hypothesized that by examining heterogenous cell populations, at the single cell level, different cell behaviors and sub-populations would emerge when assessing their functionality using biosensor technologies. Single cell analyses allow to better understand the variability that shapes the population as a whole. In line with this, we applied two single cell analytical pipelines to dissect biosensor data collected using the Opera Phenix automated high content microscope from Perkin Elmer or the Zeiss Axio Observer [43]. The Opera Phenix was used to track ERK<sub>1/2</sub> and PKA signaling overtime while the faster imaging speed of the Zeiss Axio Observer was used to collect calcium handling data. For the ERK<sub>1/2</sub> and PKA assays, the Columbus image analysis software then allowed us to examine the images extracting details relating to each individual cell or nuclei. Integrated within analytical softwares such as Columbus, it becomes possible to find individual nuclei or cells using the donor channel. Further, it is also possible to extract morphological features of individual cells/nuclei. This morphological information can be used in the next steps to correlate signaling with cell- or nuclei-specific morphologies [43]. For example, nuclei size and roundness can be computed in addition to cell size, roundness, length, width, and length-to-width ratio. Extracting this information allows users to deduce whether a correlation exists between these morphological features and the signaling signature of each individual cell [43]. Once these features have been extracted from the collected images, text files can be imported into R for further processing. Here, we used R as it is an open-source free software environment that allows processing of large multivariate datasets. For calcium imaging, raw images were imported into Image J for feature extraction prior to being analysed in Origin Lab software. Below, we explain how our analytical approach previously used on primary neuronal cultures [43] as well as single-cell calcium imaging can help assess signaling in iPSC-CMs derived from control or idiopathic DCM patients.

# 4.5.3 Exploring disease-specific cellular vulnerabilities by probing cardiomyocyte calcium handling

Numerous reports have described impaired calcium handling and corresponding contractile function in DCM. For example, reduced calcium transients were observed in human ventricular muscle strips sourced from DCM hearts compared to non-failing heart samples [64]. In a study using iPSC-CMs derived from a patient carrying a R173W mutation in Troponin T, CMs displayed delayed times-to-peak Ca<sup>2+</sup> transients, showing calcium release from the SR was compromised as well as delayed reuptake kinetics [65]. TnT-R173W iPSC-CMs have also been shown to have reduced calcium transient amplitudes speculated because of reduced SR Ca<sup>2+</sup> storage [66]. In addition to dysregulated calcium handling and contractility, sarcomere misalignment is another common feature observed in DCM cardiomyocytes [66]. In line with this, shorter sarcomere lengths as well as reduced contractile amplitude have been reported in TnT-R173W iPSC-CMs [67]. These data suggest a destabilized calcium-troponin network in DCM. Overall, numerous reports have identified impaired calcium handling as a phenotypic abnormality in iPSC-based models of DCM.

Calcium handling deficits have been linked with numerous mutations associated with the development of hypertrophic and dilated cardiomyopathy with mutations being found in cardiomyocyte cytoskeletal and contractile proteins (Table 4.1) [68]. The role of calcium in the heart is extensive as the heart contracts and relaxes via excitationcontraction, E-C coupling, and involves tight modulation of intracellular calcium concentrations (Fig. 4.4A). To discern molecular level differences between DCM patients as well as in comparison to healthy controls, the assessment of cardiomyocyte calcium handling becomes a significant phenotypic endpoint. To accomplish this, [Ca<sup>2+</sup>], was measured at the myofilament using a red-shifted intensiometric genetically-encoded calcium indicator called RGECO-TnT (Fig. 4.4B) [45]. Our proof-of-concept single-cell results indicate that differences in myofilament-localized calcium handling can be captured between DCM patients and control subjects as well as between DCM patients. Features of calcium transients such as time between peaks, transient frequency, and upstroke velocity displayed a general trend where iPSC-CMs from DCM patients exhibited slower spontaneous calcium transients than iPSC-CMs derived from healthy controls (Fig. 4.4C-J). Furthermore, calcium transients from DCM patients were shown to have lower amplitudes and longer transient durations than healthy controls. Features such as time to 90% baseline, transient duration, and area under the curve exemplified that this biosensor, RGECO-TnT, when expressed in iPSC-CMs can be used to detect differences in myofilament-localized calcium handling between DCM patients, some of which were classified as idiopathic (**Fig. 4.4C-J**). After confirming these results in a larger patient sample, it will be possible to test whether the abnormal calcium handling features observed here can be rescued using pharmacological agents. Overall, calcium is a useful proxy that can be used to assess cellular vulnerabilities and how DCM pathogenesis affects individual cardiomyocytes on a patient-specific basis.



Figure 4.4 Calcium imaging as measured in iPSC-CMs derived from healthy control subjects and idiopathic DCM patients.

(A) Calcium handling in the heart. Since calcium is a critical regulator of cardiomyocyte contractility, abnormal calcium cycling has been observed to result in impaired cardiac functions as seen in the failing heart and dilated cardiomyopathy. (B) Representative fluorescent microscopy image of the red-shifted RGECO-TnT biosensor in iPSC-cardiomyocytes. The myofilament localization of RGECO-TnT is clearly observed with the yellow/orange fluorescence. Several calcium transient properties were measured including (C) calcium transient amplitude dF/F0 which represents baseline fluorescence subtracted from background fluorescence divided by background fluorescence, (D) area under the curve, (E) transient frequency, (F) transient duration, (G) time between peaks, (H) time to 90% baseline, (I) upstroke velocity, and (J) decay tau in both control subjects (gray: HID041004 and HID041101) and idiopathic DCM patients (red: HID041020, HID041019, HID041100, HID041110), n= 9 cells from 1-2 biological replicates. \* =

*p*<0.05, \*\* = *p*<0.01, \*\*\* = *p*<0.001, \*\*\*\* = *p*<0.0001. Figure Panel (A) was created with <u>BioRender.com</u>.

## 4.5.4 Exploring disease-specific signaling signatures in iPSC-CMs through the measurement of PKA activation profiles

Protein kinase A (PKA) is an important effector in regulating cardiomyocyte function and behavior as it is involved in regulating contractility, growth and metabolism. For example, in failing human hearts, PKA-mediated phosphorylation of troponin has been reported to be reduced compared to healthy hearts [69]. A similar observation was reported in  $G\alpha_q$  transgenic mice suggesting PKA has a role in the development of DCM [70]. Cardiac apoptosis driven by norepinephrine has also been shown to be facilitated by a PKA-dependent pathway [71]. Transgenic mice expressing the catalytic subunit of PKA have also been observed to develop dilated cardiomyopathy [72]. The hyperphosphorylation of PKA substrates was also shown to be detrimental to heart function [72]. Inhibiting PKA phosphorylation of RyR2 effectively reversed the DCM phenotype and abnormal calcium handling in mdx mice [73]. Interestingly, in iPSC-CMs, the PKA response to isoproterenol was shown to be attenuated in a genetic DCM line compared to control [74]. Similarly, iPSC-CMs carrying a TnT-R173W mutation were shown to bind PKA at reduced levels, compared to wildtype troponin, supporting the reduced contractility observed in DCM [57]. Further, cAMP levels at sarcomere myofilaments were shown to be increased, indicative of a compensatory mechanism within cardiomyocytes to increase PKA responsiveness [57]. Based on this accumulated evidence pointing towards a role of PKA in DCM progression, we sought to examine whether PKA activation profiles were informative of disease by comparing data gathered from a control male subject and a male patient (Fig. 4.5).

To reveal whether PKA signaling was perturbed in DCM patients, we transduced both patient and control iPSC-CMs using AAV2/6-ExRai-AKAR2-NLS, an intensitybased, single (green) colour biosensor that reports of PKA activity in the nucleus [25] (**Fig. 4.5A-C**). This biosensor has a large dynamic range and its brightness allows for robust responses to be measured. After the recording of a basal measurement, iPSC-CMs were stimulated with saturating doses of agonists that target  $\beta$ -adrenergic (isoproterenol, epinephrine, norepinephrine) and  $\alpha$ -adrenergic receptors (norepinephrine, epinephrine). Agonist-induced changes in PKA activity were subsequently recorded. All nuclei were then run through a clustering algorithm to uncover patterns within the data, that cannot be detected with an untrained eye (Fig. 4.5D). Four clusters were noted, demonstrating four distinct behaviours. Nuclei that exhibited a sustained response to agonists as well as transient responses were observed. Some nuclei did not respond to the drugs tested while other nuclei exhibited a decrease overtime in response compared to baseline. This clustering feature within our analysis demonstrates the ability to identify novel sub-populations of cells that exhibit distinct patterns for further analysis [44]. To visualize agonist induced changes in PKA activity, single nuclei responses were plotted as a heat map (Fig. 4.5E, F). In the control subject, approximately 40% of cells exhibited a sustained or transient response to adrenergic drugs. In the patient nuclei, the ratios of these clusters were evidently distinct in response to isoproterenol (Fig. 4.5F). The two largest clusters observed appeared to represent nuclei populations that exhibited a sustained or transient increase in PKA activity over time. We also observed a distinct pattern in response to norepinephrine, demonstrating that different  $\beta$ -agonists result in different single nuclei signaling signatures. In comparison to the control, the data seems to suggest that the male patient's iPSC-CMs displays a 'hyper-active' phenotype as almost 70% of iPSC-CM nuclei led to a robust measurable response compared to control. Similar observations were observed in response to epinephrine, albeit to a lesser degree.



Figure 4.5 Biosensors to illuminate disease-specific signaling.

(A) Schematic illustration of genetically-encoded biosensor to interrogate nuclear protein kinase A (PKA) activity. PKA is activated downstream adrenergic receptors and regulates numerous aspects of cardiac physiology and pathophysiology including the function of sarcomeric proteins, cardiac contractility, and adverse remodelling. (B) Fluorescent microscopy images of ExRai-AKAR2-NLS. Representative images display nuclear localization of the biosensor in (B) patient and (C) control iPSC-CM nuclei. (D) Summary of distinct clusters identified in response to adrenergic agonists. iPSC-CMs were observed to fall within four different clusters depending on their behaviors. Nuclei were found to exhibit sustained (plum), or transient responses (black) while other nuclei failed to produce a measurable response (light pink) or exhibited a decrease in activity from baseline (yellow).  $\Delta F/F$  (%) represents the percent change in FRET in response to agonist normalized to baseline. (E-F) Representative heatmap representing single cell results of intensity-based biosensor ExRai-AKAR2-NLS in (E) control and (F) patient iPSC-CMs. The signalosome of two male subjects, control and patient were compared post stimulation by either vehicle, isoproterenol, epinephrine, or norepinephrine. Images were collected pre- and post-drug stimulation. Data was collected for 70 minutes post-drug addition. The male patient demonstrated a 'hyperactivated' PKA phenotype as a larger proportion of cells responded to the tested agonists. (G) Response clusters for control and patient iPSC-CMs. When plotted independently, patient clusters are indicative of a 'hyperresponsive' phenotype with a

more robust increase in  $\Delta F/F$  compared to control (see y-axis). Figure Panel (A) was created with <u>BioRender.com</u>.

Here, we demonstrated the capacity of biosensors to uncover distinct PKA activation profiles in a DCM patient. As DCM and HF are associated with impaired calcium handling, this hyperactive phenotype can be speculated to present due to signaling cross-talk. Data collected from this patient demonstrates reduced basal calcium transients and calcium transient amplitudes (**Fig. 4.4**). As cAMP and calcium signals exhibit a certain level of cross-talk, lower basal calcium may also impact the basal cAMP-PKA axis causing the PKA response to  $\beta$ -agonists to appear as hyperactivation. With lower basal cAMP-PKA, there may be a larger 'range' for PKA activity to increase. When sustained, a hyper-adrenergic phenotype can result in increased phosphorylation of PKA substrates resulting in cardiomyocyte remodelling.

#### 4.5.5 Exploring the effect of biological sex on ERK<sub>1/2</sub> activity in DCM iPSC-CMs

Mitogen-associated protein kinases (MAPKs) are important regulators of molecular events in many cells. Myocardial activation of ERK<sub>1/2</sub> is complicated as its activation can result in cellular survival signals as well as maladaptive hypertrophic growth and fibrosis. The nature, kinetics and tone of the signal are important to consider as well as their localization and intensity. MAPK signaling has been identified to be abnormal in cardiomyopathies and HF. For instance, ERK<sub>1/2</sub> has been shown to be abnormally elevated in sections of explanted human DCM hearts measured using a phospho-specific antibody [75]. An ERK<sub>1/2</sub> inhibitor, selumetinib has been demonstrated to prevent cardiac ERK<sub>1/2</sub> activation, which led to positive outcomes on cardiac function in mice [75]. The I61Q cTnC DCM mutation was correlated with cytosolic ERK<sub>1/2</sub> activity and elongated cardiomyocytes while a HCM mutation were associated with nuclear translocation of ERK<sub>1/2</sub> and JNK signaling has been detected in models of LMNA-dependent dilated cardiomyopathy [77,78]. Mitogen activated protein kinase inhibitors have been demonstrated to improve the LMNA dilated cardiomyopathic phenotype [79-82].

However, ERK<sub>1/2</sub> signaling was not altered in guinea pig left ventricular cardiomyocytes modeling DCM mutations of the thin-filament regulatory proteins [83]. Thus, there is a clear phenotypic spectrum in terms of signaling when assessing different mutations and models of dilated cardiomyopathy.

To assess ERK<sub>1/2</sub> activity in iPSC-CMs, we transduced our cultures with AAV2/6-EKAREV-NLS [27] (Fig. 4.6A). As shown for the male patient, the FRET biosensor was well expressed, expressing donor and acceptor fluorophores at comparable intensities (Fig. 4.6B, C) [27]. Upon β-agonist stimulation, a transient ERK<sub>1/2</sub> activity profile appeared in the male patient compared to the iPSC-CMs derived from a female patient (Fig. 4.6D, E). This female patient presented with a larger cluster of nuclei which exhibited a decrease in ERK<sub>1/2</sub> activity post stimulation of adrenergic drugs compared to baseline. With a prominent basal ERK<sub>1/2</sub> signature in females, it can be speculated that females could be more protected from cell death pathways compared to males [84]. Yet, ERK<sub>1/2</sub> has also been associated with adverse cardiac remodelling and may be suggestive of distinct basal disease manifestations. A quantitative analysis of the FRET signals, through the generation of calibration curves, may help further elucidate the differences in basal ERK<sub>1/2</sub> activity. ET-1 treatment led to the most robust increases in ERK<sub>1/2</sub> activation compared to all other drugs tested, in both sexes. A secondary assessment of cell size would help illuminate whether a hypertrophic gene program is underway ensuing an eccentric hypertrophic phenotype. The male patient also displayed a small cluster of iPSC-CMs experiencing a transient ERK<sub>1/2</sub> activation in response to Ang II. We also tested SI and SII, two compounds known for their  $\beta$ -arrestin biased nature. The latter representing ligand properties of interest due to their believed cardioprotective effects [85-87]. Yet, in the male patient, these drugs exhibited a similar response profile compared to Ang II.



Figure 4.6 Biosensors to illuminate how biological sex influences cellular signaling.

(A) Schematic illustration of genetically encoded biosensor to interrogate extracellular signal-regulated protein kinase ERK<sub>1/2</sub> activity. ERK functions downstream the activation of several receptors and growth factor receptors and regulates cardiomyocyte survival and remodelling. Fluorescent microscopy demonstrating the nuclear expression of FRET (B) donor and (C) acceptor fluorophores. (D) Representative heatmap representing single cell readouts of FRET-based biosensor EKAREV-NLS in (D) male and (E) female iPSC-CMs, both diagnosed with dilated cardiomyopathy. The signalosome of two patients, female and male were compared post stimulation by a panel of agonists that target adrenergic, angiotensin and endothelin receptor systems. Adrenergic and angiotensin receptors represent critical pharmacological targets in DCM. Images were collected pre- and post-drug stimulation. Data was collected for 70 minutes post-drug addition. Nuclei from the male patient demonstrated a more pronounced  $ERK_{1/2}$  activation profile compared to those of the female patient assayed. While nuclei derived from the female patient exhibited a robust decrease in ERK<sub>1/2</sub> activity in response to adrenergic drugs. Figure Panel (A) was created with BioRender.com.
Overall, our biosensor platform is sensitive enough to detect signaling differences based on biological sex. As HF and DCM progression is known to be sex-specific, our iPSC-biosensor screening platform can help improve cardiac care for females which currently have worse outcomes as majority of studies focus on males [88]. Besides biological sex, as our DCM patient cohort includes patients of various ethnicities, this initiative will also be able to shed light on how DCM manifests outside the typical 'textbook white male'.

### 4.6 Concluding remarks

The combination of iPSC technology with biosensors provides a unique vantage point geared towards understanding all forms of dilated cardiomyopathy and HF. The data created while assessing how control and DCM patient derived iPSC-CMs behave at the cellular, sub-cellular and transcriptomic levels when stimulated with various stressors will create unprecedented knowledge of the molecular underpinnings of various forms of DCM including idiopathic, chemotherapy-induced and inherited. Translating differences in signal transduction or calcium handling may contribute to the identification of novel mechanisms that may be actionable through pharmacological modulation. Likewise, these differences might be distinct and dependent on the specific patient backgrounds and DCM etiology providing mechanistic information on disease and potential disease specific treatment options. The stratification of patients into distinct clusters may lead to the development of treatment strategies that are tailored to patient profiles as opposed to the non-evidence-based prescribing that is currently being performed in the clinic (Fig. **4.7**) [89,90]. Besides, working with patient-derived iPSC-CMs offers the capacity to discern how biological sex alters dilated cardiomyopathic phenotypes as iPSCs generated from male and female subjects are easily and equally accessible.



Figure 4.7 Summary illustration of how biosensor technologies can assist with the improvement of cardiac care.

The combination of biosensor technology with iPSC-based models of the cardiomyocyte, either in 2D monolayer or in 3D organoid models as an example of future disease modeling experiments using iPSC-CMs derived from control and patient subjects. The use of genetically-encoded biosensors will not only allow for the identification of dysregulated disease-causing pathways but can also facilitate the identification of novel pharmacotherapies to re-establish normal signaling. Figure was created with <u>BioRender.com</u>.

Currently, the immaturity of iPSC-CMs poses certain limitations when attempting to extrapolate data for disease modelling. Yet, several publications have already used non-matured iPSC-CMs derived from patients suffering from DCM and these accounts have shown that the iPSC-CMs derived from these patients recapitulate the disease as observed in the clinical setting (Table 4.1). Our analytical pipeline has the integrated capacity to examine maturity-related concerns as morphological features of cardiomyocytes are distinct depending on the maturation stage; neonatal cardiomyocytes are more rounded while adult cardiomyocytes are bi-nucleated and elongated. Our approach, when combined with whole-cell or cytosolic targeted sensors allows to delineate whether distinct signaling profiles are detected in cardiomyocytes of different morphologies and thus developmental stages or maturity. Now, with patient access and the advent of iPSC technology, we can begin to address questions related to DCM progression at the molecular level in genetic or idiopathic patient contexts. We believe that the deep phenotyping of these patients using biosensor-based strategies will provide novel insight on disease mechanism that may inform therapeutic interventions.

## 4.7 Supplemental Figures, Tables and Legends



Supplemental Figure 4.1 Secondary validation of the induced pluripotent stem cell lines generated. G-band karyotyping analysis of a representative male iPSC cell line. Analysis depicts a normal diploid male karyotype with 23 intact chromosomes. G-banding was performed as means to validate the chromosomal integrity of each iPSC line.

# Supplemental Table 4.1 Patient population.

Characteristic	Patient	Control
Number of participants (n)	209	19
Age (Mean (SD))	54.21 (14.88)	46.74 (17.94)
Male Sex (%)	143 (69.4)	7 (38.9)
Number of risk factors (Mean (SD))	1.8 (1.31)	1.18 (1.07)
Family history (%)	92 (48.9)	9 (52.9)
Ever smoked (%)	96 (50.8)	7 (41.2)
High Cholesterol (%)	49 (25.3)	2 (11.8)
Hypertension (%)	67 (34.5)	2 (11.8)
Diabetes (%)	45 (23.2)	0 (0)

**Supplemental Table 4.2** Summary of heart failure medications prescribed to patient and control subjects.

Category	Number (%) in	Number (%) in	Number (%) of
	Patients	Patients	Controls
	(without cardiac transplant)		- (-)
β-blocker	166 (88)	5(25)	0 (0)
Diuretic	99 (52)	5(25)	0 (0)
Entresto	90 (48)	2(10)	0 (0)
ACE inhibitor	55 (29)	5(25)	0 (0)
Angiotensin II receptor	20 (11)	4(20)	2 (11)
Blocker			

### **4.8 Conflict of Interest Statement**

The authors declare that they have no known competing financial interest or personal relationships that could have influenced the work reported here.

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### 4.10 References – Chapter 4

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# Chapter 5 : General discussion

### 5.1 Chapter 2

5.1.1 Contributions to knowledge & the significance of tool development for basic research applications

In **Chapter 2**, I described the generation and validation of conformation-sensitive biosensors engineered within the human  $\beta_2$ -adrenergic receptor. Stimulation with a saturating dose of isoproterenol led to robust conformational changes in the sensors when the FIAsH tag was introduced in the distal portion of the carboxyl terminus. Transient conformational changes were observed when the FIAsH tag was inserted in the third intracellular loop. These agonist-induced conformational changes were distinct from those assessed in two other GPCRs, notably the hAT<sub>1</sub>R and hFP receptor. In response to their respective agonists, Ang II and PGF2 $\alpha$ , robust conformational changes were seen in the sensors positioned in the third intracellular loop. In contrast to the  $\beta_2$ AR sensors, the sensors built in the C-terminal domain of FP were not robust (unpublished data). This led us to conclude that even if Class A GPCRs share the same seven transmembrane architecture, their conformational dynamics, as measured by our FIAsH-BRET conformation-sensitive biosensors, are distinct.

Beyond the work discussed in *Frontiers in Endocrinology*, follow-up studies led us to hypothesize that the transient conformational changes that were observed in the third intracellular loop of the  $\beta_2AR$  conformational sensors, might be a result of steric hindrance, where  $G\alpha_s$  was constraining the receptor reducing its overall flexibility. This was posited because both AT<sub>1</sub>R and FP are  $G\alpha_q$  coupled whereas the  $\beta_2AR$  is coupled to  $G\alpha_s$ . To test this hypothesis, we generated polyclonal stable cell lines expressing our panel of  $\beta_2AR$  conformation sensors in a HEK 293 background that was CRISPR engineered to no longer express  $G\alpha_s$ ,  $\Delta G\alpha_s$  clone 2. Our first trials were promising as robust conformational changes were observed within all three positions of the  $\beta_2AR$  ICL3 sensors in response to isoproterenol. To further confirm these results, we generated

polyclonal stables in a second clone,  $\Delta G\alpha_s$  clone 4. In the second clone, we no longer observed robust conformational changes in ICL3 (unpublished data). Instead, the conformational outputs observed in these cells, resembled that of the unedited parental cell line where  $G\alpha_s$  was still expressed. It is thus possible that the CRISPR editing affected more than  $G\alpha_s$ , altering the cell context of clone 2 that may have led to a 'clonal' effect.

A second hypothesis at the time was the thought that the three receptors that we probed exhibited differential interactions with  $\beta$ -arrestin. The AT<sub>1</sub>R is classified as a Class B GPCR, strongly interacting, and internalizing with  $\beta$ -arrestin in endosomes. The  $\beta_2$ AR, on the other hand, is a class A GPCR, transiently interacting with  $\beta$ -arrestin and recycles back to the cell surface more rapidly while FP does not typically recruit  $\beta$ -arrestin [742]. To address if these differential interactions could be influencing  $\beta_2AR$  dynamics, we designed chimeric receptors by swapping the C-tail of the AT<sub>1</sub>R onto the  $\beta_2$ AR and vise versa. Truncated AT<sub>1</sub>R-(Met<sup>1</sup>-Tys<sup>302</sup>)-FIAsH sensors were combined with the carboxyl terminus of the β<sub>2</sub>AR-(Cys<sup>327</sup>-Leu<sup>413</sup>) and β<sub>2</sub>AR-(Met<sup>1</sup>-Lys<sup>348</sup>)-FIAsH sensors were also fused with the C-tail of the AT<sub>1</sub>R-(Ala<sup>324</sup>-Glu<sup>359</sup>). Indeed, the β<sub>2</sub>AR+AT<sub>1</sub>R-C-tail FIAsH sensors now responded robustly to isoproterenol and the presence of the  $\beta_2$ AR C-tail silenced the AT<sub>1</sub>R sensors (unpublished data). This was an interesting observation that was also confounded by the idea that the lengths of the C-terminal regions between the  $\beta_2$ AR and AT<sub>1</sub>R differed substantially as the AT<sub>1</sub>R C-tail (~35 a.a), in our chimeric models, was significantly shorter than the  $\beta_2$ AR C-tail (~86 a.a). Thus, the lengthier  $\beta_2$ AR carboxyl terminus may position the donor outside the RET range, >10 nm, rendering only the  $\beta_2$ AR C-tail sensors, where the FIAsH tag acceptor is closer to the donor moiety, capable of reporting on conformational changes due to distance constraints.

At the same time, we were also trying to understand how nanobodies, single chain antibodies that were a "hot topic" due to their success as crystallization chaperones, influenced GPCR conformation. We began working with nanobodies raised against the h $\beta_2$ AR. We had access to five nanobodies that were either believed to stabilize the inactive (Nb60, Nb74) or active (Nb71, Nb80, Nb84) conformation of the  $\beta_2$ AR. Nanobodies also carry the nickname 'G protein mimic' as they *frequently* bind GPCRs at similar positions as do G proteins. However, nanobodies are not functional mimics of G proteins as when they are introduced in a Gas null cellular background, they do not rescue cAMP activity [743, 744]. Thus, these inactive/active classifications are more nuanced than previously anticipated. Besides, we were interested in testing whether our conformation-sensitive biosensors could capture the distinct conformations stabilized by these five nanobodies. To this end, we co-transfected nanobodies with our β<sub>2</sub>AR conformational sensitive biosensors and our data demonstrated Nb60's ability to alter the conformational change observed when stimulated with isoproterenol (unpublished data). This observation suggested that Nb60 stabilized the h $\beta_2$ AR in a conformational state that was less conducive to signaling via the full agonist isoproterenol. However, cotransfection of other nanobodies did not reveal significant changes in  $\beta_2$ AR conformation. Also, as nanobodies interfere with signaling, shown with Nb80's ability to reduce cAMP accumulation due to steric hindrance and competition with  $G\alpha_s$ , it became difficult to interpret these experiments as well as extrapolate how our experiments related with  $\beta_2 AR$ biology. Expressing nanobodies at lower levels, may reduce the competition with Gas and consequently interfere less with endogenous signaling. Besides our unpublished work using nanobodies, Nb80 has been demonstrated to function well as an 'active state sensor'. As Nb80 recognizes active conformations of the  $\beta_2AR$ , it has been used as a surrogate to probe for active receptor pools at intracellular locations. When fused to GFP, Nb80 has revealed that the  $\beta_2$ AR is functional/active and can signal from endosomes and well as in the Golgi [745, 746]. Based on the success of the Nb80 active state sensors, we wanted to test whether we could adapt nanobodies as tools to detect homomers and heteromers in living cells. This idea was prompted by the fact that most tools for GPCR dimer studies relied on overexpression of tagged receptors as well as the shortage of studies investigating dimers in their native context. Nanobodies could potentially be repurposed to probe for  $\beta_2AR$  and  $AT_1R$  dimer species at the endogenous level as nanobodies have been developed to recognize both receptors [747]. Nanobodies were cloned in viral expression vectors fused with YFP/mTurg or split Nanoluc, SmBiT/LgBiT. By designing these biosensors, we wanted to test if we could detect FRET/BRET between two  $\beta_2AR$  recognizing nanobodies or signals between AT<sub>1</sub>R and  $\beta_2AR$  nanobodies suggestive of  $\beta_2AR$  homodimers or AT<sub>1</sub>R/ $\beta_2AR$  heterodimer species, respectively. As different nanobodies recognize distinct activation states, R and R\*, these studies could

potentially be informative of the activation state of the higher order species. Pilot studies were conducted in HEK 293 cells and interrupted by a global pandemic. Yet, similar tools were developed for the metabotropic glutamate receptors demonstrating the feasibility of the approach [748, 749]. If the project were to get revived, these tools are portable for expression in primary or iPSC-derived cardiomyocytes and  $\beta_2AR$  dimer studies can be reevaluated in more relevant contexts. The occurrence of dimer species can also be examined in patient iPSC-CMs as disease has been reported to alter the balance of certain dimeric species.

Altogether, Chapter 2 resulted in the generation and validation of tools to study  $\beta_2AR$  biology. Several versions were designed to address various hypothesis that emerged based on in-house observations. Below, I discuss how I transitioned to working with biosensors in more physiologically relevant cellular contexts.

# 5.1.2 Beyond Chapter 2: the portability of the FIAsH-BRET method allows to gain a glimpse into the functional importance of cellular context

The hAT<sub>1</sub>R is an equally interesting GPCR target, as HF and DCM therapeutics are designed to affect AT<sub>1</sub>R signaling. In Devost et al., the AT<sub>1</sub>R FIAsH-BRET sensors built in our lab demonstrated their full potential revealing their ability to capture distinct conformational signatures based on the agonist's chemical nature [715].  $\beta$ -arrestin biased agonists stabilized unique AT<sub>1</sub>R conformations compared to balanced ligands (Ang II), an observation that exposed the power behind the technology. As such, FIAsH-BRET driven drug screening campaigns can assist the identification of novel and chemically distinct compounds based on the conformation, FIAsH-BRET technology has also been applied to monitor the conformational dynamics of the signaling effector;  $\beta$ -arrestin. In this report, the FIAsH tag was inserted in the sequence of  $\beta$ -arrestin to monitor the range of its conformational outputs in response to different agonists [750]. Thus, the FIAsH method has the capacity to uncover biased drug candidates based either on distinct conformational signatures within GPCRs or effectors like  $\beta$ -arrestin. The latter statement

being relevant as biased agonists represent contenders to improve cardiovascular disease.

Within the scope of the work performed using the AT<sub>1</sub>R conformation-sensitive sensors, Dr. Devost introduced the AT<sub>1</sub>R sensors in HEK 293 cells but also in rat vascular smooth muscle cells (VSMCs). The experimental rationale here reiterated the idea of cellular context. As the AT<sub>1</sub>R is expressed in the vasculature as well as fibroblasts, cardiomyocytes and endothelial cells, the Hébert lab wanted to test how these biosensors would behave in a more physiological context, progressively moving away from the prototypical HEK 293 cell. This also highlights the portability of the biosensors being amenable to be assayed in different cellular contexts. Lenti-virus mediated transduction of the AT<sub>1</sub>R sensors in VSMCs demonstrated that in response to Ang II, sensors built in the second intracellular loop (ICL2) now led to a measurable conformational change while ICL2 did not move in HEK 293 cells. This observation, along with those made in CRISPR G protein null cells highlighted the importance of cell context when studying GPCR conformational dynamics. Additionally, since the AT<sub>1</sub>R is expressed in endothelial cells as well, Dr. Devost is currently testing how the AT<sub>1</sub>R sensors are behaving in immortalized human aortic endothelial teloHAEC cells. Preliminary data has revealed distinct conformations that were not previously appreciated in HEK 293 background.

With Dr. Devost's success at exposing cell type specific conformational determinants by transitioning towards the use of physiologically relevant cell types, VSMCs and ECs (in progress), I also opted to test the AT<sub>1</sub>R sensors in rat neonatal cardiomyocytes (RNCMs). AAV mediated transduction of RNCMs demonstrated that in response to Ang II, the FIAsH ICL3 p3 sensor no longer reported on receptor conformation. This observation could be suggestive of a cell context specific effect. However, these experiments suffered from low expression. To circumvent this issue, I replaced *Renilla* luciferase with nano-luciferase, a small but brighter donor, exhibiting a 150-fold increase in luminescence with the help of an undergraduate student, Lidia Avvakoumova [751]. Unfortunately, this did not improve our suboptimal expression profiles. This may also have been caused by the fact that RNCMs can only be maintained

*in vitro* one week post isolation from neonatal pups combined with weaker BRET signals, compared to FRET for instance. The short time in culture to allow for significant biosensor expression may have been limiting our experiments as the luminescence emitted by our sensors was flirting with the limit of detection of our plate reader. Besides, another plausible explanation may point to the notion that the AT<sub>1</sub>R is expressed at higher levels in the VSMCs and well as in endothelial cells compared to substantially lower levels in cardiomyocytes. To what extend this may present as a contributing factor, still needs to be elucidated. However, repeating these experiments in iPSC-CMs, where time in culture is no longer an issue, may prove fruitful.

Circling back the major theme of this thesis, cell context, the FIAsH method can further discern the impact of intracellular (allosteric) players on GPCR conformation and function. This is critical as cells within the body are never isolated, rather they are embedded in tissues composed of a plethora of other cell types within their immediate surroundings. Realistically, paracrine signals emanating from these surrounding tissue resident cells may impact receptor biology including conformation and subsequent activation parameters. The co-culturing of cardiac myocytes with endothelial cells for instance, combined with the use of cell-type specific promoters driving the conformation-sensitive biosensors in a specific cell type, could allow to address questions of how neighboring cells influence receptor conformation and function. Besides, the AT<sub>1</sub>R is known as a mechano-sensor and, realistically, contact with other cells may be able to influence corresponding receptor functions.

Related to investigations centered on cell context in various cell types, HEK 293, VSMCs, RNCMs, endothelial cells, the FIAsH method represents an important technique to help illuminate physiologically relevant receptor partners. In Dr. Sleno's, a previous PhD student, publication, both AT<sub>1</sub>R and FP FIAsH-based conformation sensors were used in HEK 293 cells. This report demonstrated that the stimulation of biosensors engineered within the F Prostanoid receptor generated measurable conformational outputs when stimulated by Ang II. This result essentially suggested that the binding of Ang II to AT<sub>1</sub>R was allosterically transferred to the F Prostanoid receptor and

subsequently measured using FIAsH-BRET. The transfer of allosteric information was also shown to be dependent on a third partner,  $G\alpha_q$ . The allosteric interaction was revealed to be asymmetric as the stimulation of sensors built within the AT<sub>1</sub>R by PGF2 $\alpha$ , FP's agonists, did not lead to measurable conformational changes in AT<sub>1</sub>R. Overall, this publication revealed the power of the FIAsH method to identify novel allosteric GPCR modulators.

Moreover, FIAsH can be used in the context of large-scale screening campaigns to identify novel drug candidates as well as putative dimer partners that modulate receptor conformation and possibly function. Within this framework, compound libraries consisting of thousands of biologically active agents can be screened to discover 'hit' molecules that influence the GPCR target conformation. This could result in the identification of novel dimer partners. When applied in patient and control samples, the FIAsH method can provide information on novel signaling entities that may be important in the context of human disease. In essence, nothing about the biological system needs to be known, allowing novel partner receptors to be identified without prior knowledge of the receptor biology. Besides knowing the GPCR sequence as means to generate the biosensor construct, introducing the donor and acceptor moieties, this approach can also be used on orphan GPCRs. By screening thousands of ligands, it becomes possible to find the ligand that induces a conformational change via binding events, thus de-orphanizing the GPCR.

The limiting factor in terms of the establishment of high throughput automation for the FIAsH method is the fact the EDT<sub>2</sub>, a reagent required during the FIAsH labeling procedure has a foul smell. Also, the labeling steps involve lengthy incubation times, over one hour as well as numerous washing steps. In view of this, the rhodamine-derived bisboronic acid, RhoBo, acceptor presents as a potential alternative strategy [752]. This dye has nanomolar affinities for tetra-serine tags, such as Ser-Ser-Pro-Gly-Ser-Ser [752]. The RhoBo labeling simply requires 20 minutes followed by a single wash step in comparison to the FIAsH labeling which requires 1 hour incubation followed by two wash steps with the odorous BAL buffer, an additional 10-minute incubation and two washes with the assay buffer. Further, the RhoBo acceptor has no odor making it more compatible with high-throughput and automated systems. To my knowledge, this has not been used in the context of RhoBo-BRET, yet the opportunity is present.

A second limitation of the FIAsH-BRET approach is that it requires the overexpression of the receptor under study. As this thesis is pushing the importance of cell context, the mere fact of overexpressing a biosensor that encodes a functional receptor may be altering the cell context and the balance of available receptor for G protein-coupling for example. To bypass this issue, while unfortunately reducing the portability of the approach, genome engineering techniques can be applied to genetically modify the endogenous receptor introducing the CCPGCC motif as well as a compatible donor, both being driven under the endogenous promoter. This would remove any confounding factors resulting from the overexpression of the receptor, creating what some may consider as an 'artificial system'.

All things considered; Chapter 2 instilled in me that tool development should not be undervalued. Even if the tools I developed did not prove fruitful in the cellular context that they were assayed in, there are still a plethora of discoveries to be made using the FIAsH-BRET approach as well as with adjacent biosensor-based technologies.

### 5.2 Chapter 3

# 5.2.1 Contributions to knowledge & the significance of using the *right* cellular model for research use

In **Chapter 3**, we used more *passive* biosensors that report on effector and pathway activation. These biosensors are referred to as *passive* in the sense that they do not encode for proteins within the endogenous signaling machinery but represent downstream *reporters* that can be modified/phosphorylated by effector kinases and consequently report on their intracellular activities. In this manuscript, we captured distinct signaling signatures when assessing pathway activation in primary rat neonatal cardiomyocytes and human iPSC-derived cardiomyocytes. Agonists tested drove

pathways activation to varying degrees in the two cardiomyocyte models assayed. Bulk transcriptome profiling also exposed differential gene expression patterns between neonatal rat and iPSC-derived cardiomyocytes. Overall, the deep profiling of both cells revealed appreciable species differences that need to be considered when translating experimental data.

During the conceptualization of the project, the overall goal was to develop tools and relevant model systems that would allow us to fully discern the impact of cellular context on GPCR signaling modalities. Originally, we wanted to assess GPCR signaling in human derived VSMCs, cardiomyocytes, endothelial cells and neurons. Neurons were only of interest as a post-doc at the time was working with them and a non-mesodermal cell type could have been a good *external* control. Within a year, I successfully differentiated cardiomyocytes as well as VSMCs. This process took longer than anticipated as my cell cultures suffered with mycoplasma contaminations which negatively impacted my differentiation attempts. As we were not able to free ourselves of the mycoplasma contamination, the issue was resolved by receiving a new iPSC line from a trusted iPSC biobank.

Further, as we experienced a 'clonal' effect in the FIAsH-BRET experiments summarised earlier, we discussed the use a 'landing pad' system to stably introduce our biosensors in a genomic safe harbour locus (GSH). Safe harbour sites represent intragenic or extragenic regions that permit the stable integration of a transgene at a predictable location ensuring *definite* expression while safeguarding against malignant insertional genome effects. These regions are thus generally located far (300 kb) from oncogenic genes to prevent insertional oncogenesis [753]. Several GSH have been identified thus far with AAVS1, hROSA26, and CCR5 being most studied. These regions are desirable for human gene therapies [754] but can also be quite useful for the stable integration of biosensors in iPSCs. For example, in rhesus iPSCs, the AAVS1 site was chosen to stably introduce, via CRISPR technology, a calcium indicator, GCaMP6s, for the study of calcium handling properties in these cells after differentiation into cardiomyocytes [755]. This has also been done in human iPSCs and can be used for

cardiomyocyte as well as pharmacological characterizations [756]. These landing pads inserted within safe harbours of iPSCs would enable the introduction of countless biosensors through cassette-mediated recombination using Cre-Lox or similar systems [757]. Cellular signaling events can then be probed for in the same isogenic background of iPSC-differentiated derivatives simply by swapping out one sensor for the next. Addedly, the expression level would be constant between experiments decreasing variability and removing potential confounding factors especially when using intensitybased sensors. However, one significant disadvantage of the so-called 'landing pads' is their scalability. These GSH systems are ideal for small biosensor-based studies, probing one or two iPSC lines, with specific questions that focus on cellular functions and behaviors. Scalability does become increasingly challenging as the stable introduction of these landing pads, followed by single colony selection and validation, into 10 or more patient derived iPSCs as well as age-matched controls for disease modelling is time consuming and consequently becomes less efficient. As Chapter 4 was developing during this time, predicated on the generation of around 100 patient iPSCs, the 'landing pad' strategy became less appealing, therefore we opted for using adeno-associated viruses instead. I also realigned my project objectives and conducted a head-on comparison of two cardiomyocyte models.

Since cellular context can affect biological outputs, one significant aspect behind Chapter 3 was the importance of understanding the strengths and weaknesses of the experimental model system selected. As George E.P. Box once said, "all models are wrong but some are useful" [758]. Working with iPSC-CMs not only provides access to a human background for studying biology and pathology but also offers the opportunity to keep cells in culture for longer than one week. This is significant as heart disease is complex and continuously evolves overtime. The ability to perform repeated measures, over weeks or even months presents itself as a unique opportunity when working with iPSC-CMs. However, if experiments need to be accomplished in a timely manner, it is much quicker to sacrifice neonatal rat pups and isolate their cardiomyocytes as the generation of functional CMs from iPSCs requires a minimum of 30 days. Another factor that should be considered is the funds required to culture either rat or human derived cardiomyocytes. When considering manpower and reagent costs, it is less expensive to work with rat neonatal cardiomyocytes compared to differentiating iPSCs into CMs. Thus, culturing iPSC-CMs may not be feasible in laboratories with limited funding. Lastly, and perhaps most notably, the genome of iPSCs can be modified to introduce diseasecausing mutations or for the generation of isogenic controls for comparative studies. With limited time in culture, this is less feasible with RNCMs unless transgenic animals are being sacrificed. However, in that case, transgenic animals need to be housed in animal care facilities significantly increasing the cost of working with RNCMs with the added drawback of working with the rat species as opposed to human. As our study revealed species differences, these distinctions should no longer be ignored. Besides, iPSCs can either be engineered or can be generated from patients, permitting to study human diseases in vitro. Lastly, when working with neonatal rat pups, the entire litter is often sacrificed without separating female and male pups thus these studies are not amenable for the in vitro assessment on how biological sex alters the phenotypic outcomes measured. All in all, we believe that working with hiPSC-CMs offers more advantages compared to RNCMs, one of which is reducing the use of animals in research.

## 5.2.2 Beyond Chapter 3: improving cardiac models for disease modelling

When comparing our RNCM cultures to our iPSC-CMs cultures, there was more than their respective species that differentiated them. To elaborate, RNCMs are *in vivo*, 5 days prior to being assayed. Yet, iPSC-CM cultures are in a petri dish for 30 days or more. To test whether this can be a contributing factor leading to signaling differences, rat iPSCs can be differentiated into cardiomyocytes and assayed in parallel with hiPSC-CMs. In effect, we tried to culture rat iPSCs however, the cells obtained were not viable post-thawing. To re-assess this question, cells from another laboratory can be obtained or mouse iPSCs can perhaps be used as a rodent iPSC surrogate.

Further, even if our iPSC-CM cultures are predominantly ventricular, there are also some atrial and nodal cells as well. The contribution of these other CMs should be evaluated to assess whether these cells cluster together in our signaling profiles. A single cell RNA sequencing experiment, scRNAseq, would complement our analyses as means to further elucidate the percentage of cells belonging to different cardiac lineages within our overall heterogenous population. A scRNAseq may help us further dissect the different response clusters observed. More recently, it has been shown feasible to use publicly available scRNAseq data to deconvolute and identify distinct cell populations from bulk transcriptomics [759]. This can also be applied to single nuclei data sets. By applying this method, as described in [759] or similar approaches, it may be possible to deconvolute our bulk RNAseq data set to uncover the percentage of ventricular, atrial and pacemaker cells and assess whether a correlation emerges within our response clusters. However, this represents a post-hoc analysis and does not allow to track and correlate the identity of a given cell/nuclei to a signaling response in a microwell. Yet, this could be accomplished by combining spatial transcriptomics to our single cell FRET pipeline allowing us to elucidate whether ventricular, atrial or nodal cells of the heart exhibit unique signaling signatures that are lineage specific. Addedly, the spatial transcriptomics datasets would be used to further investigate transcriptomic profiles of these cells and clusters.

Likewise, with my expertise, a post-hoc immunohistochemistry analysis may be more feasible, yet less informative, allowing to detect the identity of individual cells using ventricular- and atrial-specific antibodies. In theory, X, Y coordinates of each cell can be matched associating FRET responses to a specific myocardial lineage. These approaches can also be applied to our RNCM cultures as a small percentage of cardiac fibroblasts are also present. A post-doc in our lab, Dr. Étienne Billard, is currently developing R code with this intention. However, to obtain a higher purity of primary rat neonatal cardiomyocytes within the overall isolation procedure, a percoll gradient separation-based method can be used. Additionally, to side-step the need for post-hoc analyses, fluorescence-activated cell sorting or FACS can be performed to sort our pool of cells allowing to seed ventricular and atrial cardiomyocytes in separate microwells and subsequently measure our single cell FRET responses in segregated myocardial populations. Equally, lineage specific promoters can be used to drive the expression of our biosensor toolbox. For example, MLC-2v, ANF can drive expression in ventricular and atrial CMs respectively while cTnT, MHCα, NKX2.5 target cardiomyocytes regardless of lineage [760]. This methodological approach has been used to drive the expression of an optical voltage sensitive biosensor to study electrophysiological properties in hiPSC-CMs. *The MLC2v, SLN*, and *SHOX2* promoter elements were used for subtype specific expression in ventricular-, atrial-, and nodal-like cardiomyocytes respectively [761]. However, some reports have served to suggest that these types of promoters do not lead to strong expression when compared to ubiquitous promoters. Rather, robust biosensor expression with cell-type specificity is attainable through the incorporation of FLEx vectors and Cre-lox technology [762, 763]. In these systems, Cre recombinase expression is driven by a constitutive promoter, is positioned in an inverse orientation and can only be *flipped* into the correct orientation in cardiomyocytes due to cTnT-Cre. With the use of cell type promoters for cardiomyocytes, endothelial cells or fibroblasts, this system permits the interrogation of cell type-specific signaling in co-cultures and is also amenable to 3D organoid models.

The question of how maturation impacts our data is continuously present. In Chapter 3, we used iPSC-CMs between 40 and 50 days in culture taking advantage of 'time in culture' as a *maturation* technique. Yet, to tackle the concern of maturation, in a more integral manner, a master's student in the lab, Alyson Jiang, is attempting to drive the maturation of our iPSC-CMs cultures. Alyson is testing two metabolic maturation medias that require low glucose formulations as well as a combination of fatty acids, dexamethasone, PPAR $\alpha$  agonist and thyroid hormone supplements [644, 650]. To date, we have noticed that high fatty acid concentrations result in CM cell death. An observation that has been previously reported in the literature [764]. We are thus troubleshooting the Funakoshi et al. protocol and titrating the dose of fatty acids to find the optimal concentration that does not negatively impact CM viability. So far, concentrations ranging between 20 to 50  $\mu$ M are conducive to CM survival. We believe this was a necessary reduction in fatty acid concentration, from an initial 200  $\mu$ M, as we adapted a protocol for embryoid bodies to monolayer CM cultures. Besides, when using the Feyen et al. protocol, visual inspection of our iPSC-CM cultures under a brightfield microscope

revealed CMs with elongated morphology. A promising observation that requires further validation. Regardless of these issues encountered, we are moving forward whilst optimizing the protocols and we anticipate combining our *matured* cultures with our signaling sensors to assess how cellular signaling changes as a response to cardiomyocyte maturation.

Further, I have also described a method of how to study cardiac hypertrophy in ratand human-based systems [765]. However, in contrast to other publications, our microscopy-based data failed to show hiPSC-CMs capacity to undergo a hypertrophic response to known inducers. Yet, more recently, we have been able to demonstrate hypertrophy in response to urotensin II in hiPSC-CMs. Nonetheless, it is possible to upgrade our hypertrophy analysis pipeline with a single cell approach. A 24-hour experiment can be conducted where single cells are tracked overtime in an automated microscope. Basal as well as post-stimulation images would be captured where the same cell can be tracked overtime due to it fluorescing via the transduction of a fluorescent tag, example RFP. In addition, CMs can also be co-transduced with a CFP/YFP biosensor to track signaling for a 24-hour period. Performing spatial transcriptomics would provide insight on the transcriptomic landscape at a single cell level allowing us to combine transcriptomics with signaling as well as a phenotypic endpoint. It would provide transcriptome-level information on the cells that did not experience an increase in size while also demonstrating the gene programs activated in cells that hypertrophied. In supplement, this method would also provide insight on the identity of neighboring cells. A correlation may arise in terms of the distinctiveness of neighboring cells surrounding those cells that failed to respond versus those that did respond to the hypertrophic inducers. However, might there be a direct correlation between hypertrophy and the maturation status of our iPSC-CMs? To test this hypothesis, matured iPSC-CMs cultures can be used. Likewise, we can incorporate morphological features in our hypertrophy analyses. Following the 24-hour proposed experiment, as outlined above, it may be possible to assess whether elongated iPSC-CMs experience a hypertrophic response compared to cells that are more rounded at baseline. Similar analyses can be done comparing the cell size of iPSC-CMs that are bi-nucleated to those with single nuclei.

Perhaps the new Live-seq method [766], which demonstrated the ability to couple baseline transcriptomics with a downstream phenotype, can prove useful.

Further, in Chapter 3, we used targeting sequences to study nuclear kinase activity however, there are a plethora of targeting tags that can be used to study localized signaling. This is essential as intracellular signaling occurs differently in distinct compartments or microdomains (reviewed in [198]). For example,  $\beta_2$ -adrenergic receptor-mediated cAMP signaling has been shown to be more pronounced at specialized anchoring sites compared to whole cell readings [767, 768]. As the concentration of regulatory proteins or enzymes, phosphatases/phosphodiesterase's, is distinct at these microdomains, it can shape distinct signaling hubs within the same cell. As these microdomains have been shown to be altered in disease, in correlation with altered cardiomyocyte architectures, studying signaling at specific compartmentalized locations can assist in the generation of a comprehensive 'image' of how signaling modalities change and correspondingly contribute to disease [769]. To demonstrate case and point in iPSC-CMs, a targeted cAMP sensor, CUTie, helped reveal impaired local microdomain signaling in troponin T-R173W DCM iPSC-CMs [678]. An increase in cAMP activity measured at the myofilament was speculated as means to re-establish the decreased PKA activity, myofilament phosphorylation and unstable troponin complex. Interestingly, sarcomeric PDE activity was also observed to be different in DCM compared to control iPSC-CMs [678]. Equally, are these signaling hubs different in rat versus human cardiomyocytes? Further, the use of targeting sequences has demonstrated distinct signaling patterns when measuring whole cell versus myofilament-localized calcium transients [730]. For example, a myofilament-localized biosensor detected changes in calcium handling not observed using cytoplasmic RGECO including increased concentration of calcium at the myofilament in systole, lengthened time to peak, and delayed calcium release. Minor variations in signals may be captured in localized microdomains as opposed to whole cell recordings. This exemplifies the strengths of assessing localized cellular events and can provide supplemental information on how signaling modalities change in disease especially diseases of idiopathic nature as described in Chapter 4.

#### 5.3 Chapter 4

5.3.1 Contributions to knowledge, areas for future action and the significant growing role of patient access in translational studies

In **Chapter 4**, I developed a platform for the patient-specific modelling of dilated cardiomyopathy using hiPSCs. We demonstrated the feasibility of recruiting and enrolling patients diagnosed with DCM, the reprogramming of PBMCs into iPSCs and subsequent differentiation into cardiomyocytes as well as preliminary phenotyping studies. Cellular signaling was used as an endpoint to assess the disease- and sex-specific signalosomes between patient and control subjects. We also integrated a single cell analytical approach to decipher whether a sub-set of iPSC-CMs exhibited distinct signaling behaviours. We applied a clustering algorithm which revealed clusters of cardiomyocytes based on their drug response profiles. In our PKA profiles, iPSC-CMs exhibited either no response, a decrease from baseline or responses that were either transient or sustained in nature. In our ERK<sub>1/2</sub> profiles, iPSC-CMs were clustered in three response profiles. As a whole, our case studies demonstrate the power of uniting our single cell analytical approach with patient and control iPSC-CMs for disease modelling.

As this project is still in its infancy, conceptualized two years ago, there are several directions that I can see this project taking over the next few years. Firstly, our initial analyses investigating ERK<sub>1/2</sub> and PKA activation can and will be complemented with supplemental biosensor-based assays that assess PKC, p38, JNK, β-arrestin and calcium signaling. With my guidance, a master's student in our lab, Cara Hawey, has developed a pipeline for assessing myofilament-localized calcium handing in control and patient derived iPSC-CMs. Using RGECO-TnT, a red-shifted calcium indicator, Cara demonstrated that iPSC-CMs derived from patients exhibited smaller calcium transient amplitudes and overall impaired calcium handling compared to control iPSC-CMs. As RGECO is a red-shifted biosensor, it will soon be possible to multiplex our calcium handling assays with intensiometric-based PKA assays as the ExRai-AKAR2 biosensors are green. This will allow us to assess several signaling networks in the same cell with the potential to reveal crosstalk between signaling pathways. These studies will be

facilitated with Perkin Elmer's Opera-Phenix Plus, an automated high-content microscope that our lab will soon acquire which has the capacity to image up to 100 frames per second, a speed amenable for calcium imaging. By combining automated microscopy with liquid handling options, it will be possible to study calcium and other signaling modalities in the same cell, enriching our datasets while utilizing the generated iPSC-CMs to their full potential. Beyond our toolbox of signaling sensors, we can also expand on our expertise and use biosensors that measure autophagy and apoptosis to assess cellular vulnerabilities when exposed to acute or chronic drug stimulations.

In a sense, untransformed biosensor data is *qualitative* informing us on relative changes and fluctuations within a biological system. The degree of activity of the analyte/effector under investigation after drug stimulation is often normalized by means of a percentage over baseline or fold change over the vehicle condition. However, it is also possible to convert these datasets to obtain molar concentrations of the analyte/effector under study. This can be accomplished by benchmarking the data to a standard or calibration curve. For instance, calibration curves have been produced for calcium biosensors by combining solutions of varied calcium concentrations with EGTA-Ca<sup>2+</sup>-buffered solutions followed by the application of specialized equations, summarized in [770]. Similarly, other techniques have relied on plotting FRET ratios as a function of analyte (cAMP) concentration generating a calibration curve that allows the extrapolation of cAMP concentrations [771]. However, this method can be associated with certain confounds including intracellular factors, such as pH and anion concentration [772]. To compensate for this, other mathematical expressions have been developed and require the determination of FRET efficiency, fraction of sensors in the bound-state amongst other values [772]. All in all, the ability to generate quantitative data increases the robustness of biosensor outputs as we can quantify disease signaling in a more exact manner. These quantified calcium or cAMP concentrations, for example, can be compared to the known steady state range of healthy cells determining the scale of change as a consequence of disease. Besides, quantitative measurements allow to compare differences in basal concentrations as opposed to exploring differences relative to a normalized baseline. This can be applied to further elucidate basal ERK activities in

our patient pool as well as in repeated measures experiments or when assessing how age or cardiomyocyte maturity impacts basal or ligand induced signaling. It may also be possible to uncover pharmacotherapies that re-establish these '*normal* analyte ranges' in diseased CMs.

Moreover, it may be possible to associate signaling profiles with clinical responses as previously done for the hMOR [773]. By assembling concentration responses curves for a panel of full, partial, inverse, and neutral antagonists and calculating  $E_{max}$ ,  $EC_{50}$ ,  $Log(\tau)$  as well as other parameters, it becomes possible to infer translatable and clinically relevant associations from the data [773]. Instead of combining our data with pharmacovigilance records as done for the hMOR [773], we can make use of cardiac imaging data, blood tests, etc, a topic that will be revisited below.

To complement our signaling assays, bulk or single cell RNA (scRNA) sequencing of hiPSC-CMs and other cardiac resident cell types will assist the elucidation of disease pathways. Performing RNAseq at basal conditions, independent of ligand stimulation would permit the identification of gene sets that are down- or up-regulated because of patient-specific disease. Additionally, gene ontology and pathway enrichment analysis would provide mechanistic information on the differentially expressed gene sets identified. The effect of neurohormonal modulation can also be assessed through the stimulation of iPSC-CMs with adrenergic and angiotensinergic drugs. This framework would allow to uncover patient vulnerabilities revealed at the level of the transcriptome to known endocrine stressors in heart failure. The in-tandem pre-treatment with ARBs and βblockers, alone or in combination, would expose whether these vulnerabilities may be diminished or reversed. This would also help correlate which medications may be most optimal to improve patient care in a clinical setting. The benefit of scRNAseq above the more conventional bulk methods would be the supplementary ability to identify whether distinct populations of cardiomyocytes respond differently to stressors and known HF medications. This would provide unprecedented insight on disease and actions of drugs used to mitigate HF. Separately, some patients enrolled in our project have received heart transplants. This provides a unique opportunity to assess the degree of baseline

similarities between atrial and ventricular tissues extracted from patients compared to our *in vitro* iPSC-CM patient-derived models. Single cell resolution of the proteome may also be useful strategy to phenotype our patient population [774].

In parallel, exome sequencing would allow to identify underlying genetic mutations occurring in exons within the overall idiopathic DCM patient pool with previously unknown etiology. Blood-derived DNA, currently available through our biorepository, as additional blood samples have been collected and stored, can be used to assess whether a patient's genetic variations are drivers of their disease phenotype. Exome sequencing would provide a deeper understanding of our patient disease profiles.

Nevertheless, how will the maturation of iPSC-CMs impact disease modelling? Many publications centered on iPSC-CM disease modelling are investigating diseases with strong genetic influence. For example, several iPSC lines being used in these studies carry a genetic mutation in TTN, TNNT or RBM20. With strong genetic and disease penetrance, the maturation status of the iPSC-CMs may not be as relevant, in a sense, as the disease phenotypes observed in the clinic are being relatively well recapitulated in a dish. However, to what extend will this translate to diseases of idiopathic nature or those due to the sum of numerous low penetrance mutations or sporadic disease? Direct conversion of somatic cells into cardiomyocytes has been demonstrated to generate more matured CMs compared to CMs that journeyed through an iPSC stage. As iPSCs are proliferative, the iPSC-CM route provides large CM numbers and is favoured. Thus, as mentioned earlier, we are focusing on adapting maturation medias as a method for maturing our iPSC-CM cultures. Besides, to what extent does the reprogramming stage, from PBMCs to iPSCs, alter the epigenome of an individual, erasing epigenetic marks that are contributing to the disease as seen in the patient? With access to atrial and ventricular tissues derived from patients included in our project, it may be possible to examine how the epigenetic landscape changes as a consequence of reprogramming.

However, it is difficult to answer these questions without first having tested the questions *in vitro*. With the patient population described in Chapter 4, we will continue by

modelling DCM not only in idiopathic patient lines but also in iPSC lines derived from patients with known genetic/familial causes. In tandem, our team will work with iPSCs derived from patient that experienced doxorubicin toxicity as the literature serves to suggest that this type of DCM is also well recapitulated in a dish. Beyond these, we will assess the idiopathic group of patients and examine whether their clinical features reported in their patient files will be perceived at the molecular level and further identified through our phenotypic strategies. Using this approach, it will allow us to test whether genetic DCM is *easier* to model compared to idiopathic DCM.

Reasonably, 3D organoid models may contribute to resolving concerns regarding CM maturation as well as the elucidation of disease mechanisms underlying idiopathic DCM that are driven by paracrine mediators. As HF is often accompanied by fibroblast activation and extracellular matrix deposition, a master's student in the Hébert lab, Grace Mazarura, has been working to differentiate hiPSCs into quiescent fibroblast cultures [775] for eventual co-culture and organoid-based assays. We anticipate that co-cultures will increase our understanding of the paracrine effects influencing or aggravating DCM. The latter statement is supported by observations made from LMNA DCM cultures, where co-cultured fibroblasts were shown to negatively impact CMs [776]. Non-cardiomyocyte contributions were also observed in patients with arrhythmogenic cardiomyopathy [671]. In the same vein, endothelial dysfunction has also been observed in HF. Our collaborators at the MUHC Glen site are thus working towards the differentiation of endothelial cells from iPSCs for a critical overview of how cell-cell interactions affects DCM. This is relevant as in LMNA cardiomyopathy, endothelial cell dysfunction was shown to be improved due to lovastatin treatment of co-cultures composed of iPSC-CMs and iPSC-ECs, improving overall cardiomyocyte function [777]. Moving beyond monolayer-based and unicellular models will undoubtably increase the richness of the data collected. Homogenous monocellular cardiomyocyte cultures may not have the capacity to expose certain key phenotypic hallmarks of DCM without the paracrine influence of contributing neighbors.

Supporting the need to elucidate paracrine regulation of dilated cardiomyopathic phenotypes, there are numerous 3D heart models that can be used [778]. Current heart organoids do not accurately reflect the four chambers of the heart but rather represent
3D structures assembled from cells of cardiac origin (reviewed in [778-780]). Some organoid models have inner cavities developed as rudimentary chambers while other organoids models lack these inner cavities. Cardiac organoids have been shown to be spherical/spheroid in shape however, cardiac microtissues or engineered heart tissues (EHTs) in the form of bands or rings, are also considered as 3D models of the heart. When using cardiac organoids to model disease, beat rate and other phenotypic endpoints can be extracted. Organoids facilitate the modelling of how fibroblasts and ensuing fibrosis for example affects calcium handling properties and signal propagation as reported in infarct organoids [696]. Further, the use of specialized culture devices can be used to seed cells around micropillars to assess contractility and force measurements. Therefore, to enhance our DCM model, we have been attempting to engineer cardiac organoids based on the temporal modulation of Wnt signaling [781, 782]. Under my supervision, an undergrad summer student, Hanwen Wang, has successfully generated cardiac organoids ranging between 1.5 to 2 mm in size. Once validated, we will combine these structures with our biosensor platform to study signaling signatures in three dimensions.

### 5.3.2 Beyond Chapter 4: looking towards the future of cardiovascular medicine

# 5.3.2.1 Drug discovery, cardiotoxicity, and regenerative therapies: the future of cardiovascular care

Chapter 4 demonstrated the capacity of leveraging patient-derived hiPSC-CMs for disease modelling. Beyond this role, hiPSC-CMs can also be employed in the context of drug discovery and cardiotoxicity screening as cardiovascular safety concerns are everpresent for pharmaceutical companies [783]. Clinical trials in a dish (CTiD) using hiPSC-CMs have been proposed considering that drug responses, as occurred in a clinical setting, have been well recapitulated *in vitro* [680, 784]. In this context, hiPSC-CMs would be representative of trial *participants* and used as proxies. Large scale iPSC-CM drug screening has been accomplished in a diabetic cardiomyopathy model where 480 compounds were screened to *rescue* the diabetic iPSC-CM phenotype [785]. Tyrosine kinase inhibitors were also screened to determine their cardiotoxic effects using multiple endpoints including viability, contractility, and electrophysiology [786]. The *in vitro* results were shown to correlate well with clinical toxicity profiles of the drugs tested [786]. Several other reports have described the use of iPSC-CMs to screen for drug activity or toxicity [787-789]. iPSC-CMs have also been combined with medicinal chemistry to reengineer compounds [790]. The above-mentioned studies used a range between 1 and 11 iPSC lines when performing their drug screens. With large enough numbers of iPSC-CMs derived from independent individuals, it should, in theory, be possible to conduct a clinical trial in a dish that equates to those conducted in consenting adults [791]. It has been proposed that a sample size of 100 iPSC-CM lines would achieve 88% probability of predicting events in 2% of the population, while a CTiD would need 250 lines to have a 90% probability of foreseeing events in 1% of the population [792]. With robotics and assay automation, this seems *reasonable* in the pharmaceutical setting. If properly designed, the use of 'state-of-the-art' iPSC-CMs can uncover whether females, different ethnicities or other sub-populations that are currently underrepresented in clinical trials, fare differently when administered new candidate drugs.

Moreover, safety pharmacology is a critical component in drug discovery and new candidate drugs are continually screened against their potential predisposition to block the hERG potassium channel. Even if the integration of hiPSC-CMs for use in drug screening to reduce cardiovascular safety liabilities has been recognized at a 2019 FDA workshop, there is still a requirement for more predictive and standardized assays [793]. The current screening strategies most probably overestimate the number of potentially cardiotoxic drugs as it is estimated that 70% of drugs tested may block hERG, using dose response curves that go beyond doses that would be useful in the clinic [794, 795]. More innovative assays are thus being tested in conjunction with iPSC-CMs to develop a more predictive model for cardiotoxicity screening which is being done under the Comprehensive In Vitro Proarrhythmia Assay (CiPA) initiative. A private and public initiative that also promotes the integration of hiPSC ventricular cardiomyocytes in cardiotoxicity screens for human relevance as one of its four main components [796]. In time, it will be interesting to uncover the true usefulness of genetically encoded biosensors as tools for disease modelling but also in drug discovery and toxicity screens. However, the standardization of assays may prove more difficult than previously anticipated as even if iPSC-CMs suffer from an inherent immature phenotypes [797], other factors such as media formulation [798] have also shown to impact the evaluation of pro-arrhythmic risks. The lack of iPSC standardization, reprogramming techniques and colony picking, for instance, may also be a contributing factor [799]. Nonetheless, even if iPSC-CMs represent a physiologically human alternative to other model systems, there is still work that needs to be done to standardize the cultures for preclinical assessment, yet their promise remains.

iPSC technology comes with the inherent ability to leverage the uniqueness of each individual. As such, there can be significant patient benefit to creating biobanks of iPSCs derived from 'at risk' individuals. For instance, if two generations within a family are diagnosed with DCM, then the third generation may undergo genetic testing to determine whether they also carry the faulty gene. If they do, their iPSC-CMs would be available in a biobank, and a tailored drug regimen would be obtainable. However, instead of DCM, if the disease in question is breast cancer, then biobanking followed by iPSC-CM generation and drug screening, may result in the development of a tailored therapeutic strategy, one that is not cardiotoxic. This is important as anthracyclines, first-line chemotherapy agents, can result in DCM, even after the survival of the initial cancer. This principle is known as n=1 clinical trials [800]. Even if the upfront costs may be high, due to costs associated with iPSC generation etc, it may be less expensive in the long term as if two drugs are shown to be efficacious, the least expensive drug can be prescribed. This can represent significant cost-savings as heart failure is a chronic disease and patients can be prescribed drugs for decades.

Besides the standardization of assays, another limitation of using iPSC-CMs, is that the monolayer of cells are exposed to a uniform concentration of drug. This contrasts with drug diffusion patterns seen in tissue and organ systems as drug diffusion gradients result in greater drug exposure for the outer tissue layer with less drug penetrating the deep inner layers of the organ. Traditionally, clinical trials took advantage of *in vivo* animal models to allow for organ system integration and the ability to measure pharmacokinetic parameters to assess drug safety. With animal models, it becomes possible to assess whether drugs cause toxicity at the level of the liver, kidney or the myocardium. Conventionally, this is not possible using 2-dimensional *in vitro* culture systems. To replace 2D cardiomyocyte models and prior to testing drugs in *in vivo* animal models, 3D human derived organoid systems can be incorporated in drug screening campaigns and should better reflect drug absorption properties. Beyond 3D, 4D systems integrating microfluidic technology also known as human-on-a-chip are being developed as state-of-the-art approaches for toxicity screening [801]. With multiple organ compartments, it becomes possible to use a *human* system to assess where drugs accumulate and predict toxicity signatures in response to candidate drugs. These systems allow for pharmacokinetic measurements while reducing the number of animals used. Taken together, iPSC-CMs and related technologies may be the missing link required to address the drug discovery crisis driving high attrition rates.

However, there is a limit of what pharmaceuticals can do to improve heart function particularly in the event of cardiomyocyte death. To this end, cell-based therapies are being developed to improve myocardial function. Regenerative medicine is being evaluated due to donor heart shortages and high demand for heart transplantation procedures. The link between regenerative medicine and hiPSCs stems from the theory that somatic cells that are reprogrammed and further differentiated into CMs should not result in an immune response as they originate from the patient's body. To test the viability of this approach, cell-based therapies have been investigated in numerous animal models. In a coronary artery ligation rat model, the injection of millions of iPSC-CMs improved heart function, reduced fibrosis and reversed ventricular remodelling [802]. Intramyocardial transplantation of hiPSC-derived cardiomyocytes, endothelial cells, and smooth muscle cells along with a IGF loaded fibrin patch resulted in modest success in an infarct porcine model. [803, 804]. Yet, in one study, conducted in non-human primates, a subset of animal experienced graft-associated ventricular arrhythmias [805, 806]. The direct reprogramming of cardiac fibroblasts into cardiomyocytes using transcription factors, Gata4, Mef2c, and Tbx5 have also demonstrated some success at improving heart function [807]. Even if these select examples do convey the promise of using iPSC-CMs for regenerative medicine, there are still concerns that need to be addressed. The

percent survival of injected iPSC-CMs are very low and there is a need to develop strategies or cocktails to improve these numbers [808, 809]. The maturation status of iPSC-CMs remains an unsolved issue as well as the propensity to cause ventricular tachyarrhythmias. Regardless, of these concerns, hiPSC-CM transplantations have entered human clinical trials in Japan [810] and in China [811]. Caution is of course warranted but the promise of regenerative medicine remains.

## 5.3.2.2 Bridging the disconnect between scientists and clinicians while integrating machine-learning, phenomapping and clustering algorithms in cardiovascular medicine

For years, researchers have been trying to assess and understand human disease without an open line of communications with clinicians. However, the future of cardiovascular medicine should foster the integration of basic science data with clinical data. There is decades worth of information being collected by nurses, residents and cardiologists that is not being shared with scientists at the bench. There is a wealth of data: cardiac imaging, lab results, patient testimonies that can help better frame heart disease for scientists. Do the clinical manifestations of heart failure differ in a 20-year-old patient versus a 40- or 80-year-old individual? Does the heart adapt differently, or recover better in younger individuals? Do we know why certain individuals recover while others do not? The answers to these questions may be hidden in patient files. Access to patient charts through the creation of an anonymized patient database that can be shareable across hospitals and research labs within the same or across different institutions would allow scientists to better understand the manifestation of disease. It may also provide insight on which patients may respond better when prescribed different pharmacotherapies. There are clinician scientists, but missing are 'scientist-clinicians' who would bridge this gap and bring patient information to the benchtop.

It is quite possible that the wealth of clinical data, embedded within hospital databases and patient files is not being taken advantage of due to its complex and unstructured formats. Yet, these datasets represent an untapped valuable resource and information can be extracted using artificial intelligence (AI) and machine-learning algorithms. Machine learning is a branch of AI that can be used to deconvolute and

provide structure to multidimensional and multiparametric datasets of complex syndromes (reviewed in [812-814]). Large sums of data can be fed into algorithms as means to identify novel patterns [815]. Previously, machine learning has been used to probe datasets originating from COVID19 [816], breast cancer [817], cognitive impairment [818, 819], HFpEF [820], as well as DCM [392, 821]. More specifically, machine learning has been used to identify patterns within simple ECG readouts to identify individuals with asymptomatic left ventricular dysfunction (reviewed in [814]). The convolutional neural network developed was able to identify a LVEF  $\leq$  35% with 86.3% sensitivity, 85.7% specificity, and 85.7% accuracy [822]. As heart failure is a progressive disease beginning years before the appearance of symptoms, the development of an algorithm being fed heart rhythm data from a smartwatch may be able to predict atrial fibrillation or other heart-related diseases [823, 824]. Earlier identification of 'at-risk' individuals may allow a prophylaxis medicine to be administered reducing the burden of cardiovascular disease. Current smartwatch devices may not synthesize enough high-quality data, lacking some sensitivity and specificity, but the idea remains encouraging nonetheless [823, 825]. Relatedly, ECGs have been tested as means to diagnose hypertrophic cardiomyopathy (HCM) [826, 827]. The rational being that most HCM patients have abnormal ECGs thus there was interest to see whether patterns could be uncovered. As DCM patients also have abnormal ECG recordings, it may be interesting to apply this approach to our patient cohort while also feeding in echography and blood test result data. If ECG metrics prove useful for DCM diagnosis, with reasonable accuracy, conducting an ECG-based longitudinal study using smart watch or 12-lead ECG tracking of individuals with a strong family history of DCM/HF may allow to reveal when abnormal recordings begin. This data can then be useful for earlier diagnosis purposes and potentially decreasing mortality. Upon diagnosis, the effectiveness of medications can be monitored as well. AI most likely has the potential to find patterns in datasets that took a trained cardiologists or principal investigator an entire career to learn. Junior scientists and clinicians would be able to use these algorithms giving them access to unprecedented knowledge that may otherwise take them years to acquire.

Furthermore, the unsupervised clustering of clinical data from electronic health records can aid to reduce the dimensionality of clinical information permitting the grouping/clustering of patients into distinct 'phenogroups' that can be used to inform clinical decisions and improve patient care. Phenogrouping refers to 'grouping' patients based on similarities, both apparent and those that require AI to unearth. Notably, this becomes increasingly important when faced with heterogeneous clinical syndromes such as heart failure [828]. Secondly, numerous drug classes are available for the treatment of HF however, without a logical guide correlating patient- and disease-oriented phenotypes with medication use including dose and sequencing, it is difficult to design rational treatment options. For instance, the AI-assisted identification of patient 'improvement scores' linking how disease phenotypes evolve over time with medication use would undoubtedly aid patient outcomes, perhaps reducing hospitalizations. It may also have the power to predict adverse patient outcomes or provide a *risk* score for the eventual need of cardiac transplantation. For example, a post-hoc analysis of non-ischemic dilated cardiomyopathy (NIDCM) correlated patient genetic profiles with drug responses [829]. This example serves to suggest that Al-driven post-hoc analyses of patients enrolled in clinical trials may help uncover novel responding or non-responding subgroups in clinical trials previously marked as failures. These unbiased clustering methods may result in significant patient benefit being able to unearth data that was previously unappreciated prior to the AI era [820].

Using multivariate datasets, including, cardiac echography, cardiovascular magnetic resonance, LVEF, genetic disease contributors, comorbidities, biomarker analysis, among others, can all aid in stratifying large patient populations informing on patient prognosis and for therapeutic counselling [821]. It may provide the ability to generate decision trees for clinical use. Phenomapping conducted on a patient population with HF with preserved ejection fraction generated three distinct 'pheno-groups' [820]. As reported, these three groups were '(1) younger patients with moderate diastolic dysfunction who have relatively normal BNP; (2) obese, diabetic patients with a high prevalence of obstructive sleep apnea who have the worst LV relaxation; and (3) older patients with significant chronic kidney disease, electrical and myocardial remodeling,

pulmonary hypertension, and RV dysfunction' [820]. The ability to cluster patients based on clinical presentation of the disease represents a significant advancement in cardiac patient care. Further, in a DCM population, differences in patient characteristics, including clinical data as well as transcriptomic datasets, were able to uncover four distinct patient clusters [392]. It was also demonstrated that feeding these informations into a decision tree algorithm allowed the clustering of an independent patient cohort into these four subgroups [392]. Similarly, in another DCM patient cohort, the multiparametric clustering algorithms permitted the detection of 3 clinically distinct subgroups [821]. Differences were uncovered in regard to profibrotic metabolic, mild nonfibrotic, and biventricular impairment and each cluster was associated with a specific prognosis [821]. Their strategy also led to the identification of interleukin-4 receptor alpha (IL4RA) as a prognostic marker for DCM [821]. As our patient data is spread across different medical records software, we currently have a team of medical students compiling patient information onto one centralized platform which will eventually enable AI-assisted pattern recognition and the generation of phenogroups.

Beyond clinical data, machine learning can also be used for the deconvolution of large complex imaging datasets derived from biosensor-based experiments, for example. Raw fluorescence images be fed into algorithms to deconvolute can cellular/morphological features while finding 'grouping' patterns amongst patient and control images. For instance, this can be applied to the immunohistochemistry staining of patient- and control-derived iPSC-CMs by multiplexing spectrally distinct dyes that stain various intracellular compartments, otherwise known as cell painting [830]. The morphological profiling of the plasma membrane, Golgi, ER, mitochondria, actin filaments, nucleus, nucleoli, and cytoplasmic RNA, provides a framework to illuminate vulnerabilities at the level of certain organelles which can be investigated further. This may be exceptionally relevant for idiopathic DCM as scientists may not always know where to begin their investigations. Cell painting can also be used in the context of iPSC-CM driven drug discovery through morphological screening of compound libraries to identify drug candidates that can revert diseased morphological phenotypes into a benchmark normal

phenotype. This can be combined with CRISPR genome engineering techniques when working with research questions focused on diseases with genetic etiologies.

All in all, it is reasonable to assume that machine learning may be useful to deconvolute the complex nature of idiopathic DCM. Several groups believe that AI marks the beginning of a new era in science and health care considering that complex numerical and categorical data obtained from patient records or basic science experiments can be deconvoluted to achieve a wholesome overview of the varying presentations of disease [392, 820, 821]. This is exceptionally true for dilated cardiomyopathy, as even if some may consider DCM as *homogenous*; clinically presented as LV dilatation, it also incorporates a heterologous etiological spectrum causing significant challenges when designing treatment plans to address the syndrome. However, human oversight is still needed as the degree of accuracy of AI-driven approaches still need to be confirmed. Ethics will also need to be considered as transformative progress may at times be daunting.

### 5.4 Concluding remarks

As a whole, this thesis goes beyond the use of heterologous cell systems and integrates human iPSC-CMs for disease modelling. The cumulation of my work exposes how GPCR signaling modalities are altered because of cell context and disease. My findings support the use of patient derived samples to extrapolate disease-relevant conclusions from the data. The chapters embedded within this thesis describe tool development as well as the establishment of "disease in a dish models" of the heart. The unity of biosensors and *in vitro* cardiovascular models provides a framework for future studies and expands on our current knowledge of DCM. Our biorepository also provides a valuable resource for the cardiovascular field. By pioneering the first steps of our '*bed to bench*' approach, it is my hope that my work will, in a small way, result in tangible outcomes that will help improve the lives of patients diagnosed with dilated cardiomyopathy. These findings, including both knowledge and methodological contributions, add another piece to the puzzle, allowing us to get one step closer at solving the complex puzzle that is heart failure.

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I, <u>Terence Hébert</u> consent to the incorporation of the following material(s) in the doctoral thesis prepared by Kyla Bourque.

Bourque, K., Pétrin, D., Sleno, R., Devost, D., Zhang, A., and Hébert, T.E. "Distinct conformational dynamics of three G protein-coupled receptors measured using FIAsH-BRET biosensors." *Frontiers in endocrinology* 8 (2017): 61. https://doi.org/10.3389/fendo.2017.00061

Bourque K, Jones-Tabah J., Pétrin D, Martin R.D., Hébert T.E. "Comparing the signaling and transcriptome profiling landscapes of human iPSCderived and primary rat neonatal cardiomyocytes." Manuscript in preparation.

Bourque K, Derish I, Hawey C, Jones-Tabah J, Khan K, Alim K, Jiang A, Sadighian H, Zwaig J, Gendron N, Cecere R, Giannetti N, Hébert T.E. "Effective use of geneticallyencoded optical biosensors for profiling disease signalosomes in iPSC-CMs derived from idiopathic dilated cardiomyopathy patients". Manuscript in preparation.

Jengther

Signature:

Date: \_\_\_\_\_September 7<sup>th</sup>, 2022\_\_\_



I, \_\_\_\_\_Darlaine Pétrin \_\_\_\_\_\_ consent to the incorporation of the following material(s) in the doctoral thesis prepared by Kyla Bourque.

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Signature: Darlaine Petrim Date: Sept. 7, 2022



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Date:	September	2022



I, <u>Rory Sleno</u> consent to the incorporation of the following material(s) in the doctoral thesis prepared by Kyla Bourque.

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Date: <u>31 August 2022</u>



I, <u>Jace Jones-Tabah</u> consent to the incorporation of the following material(s) in the doctoral thesis prepared by Kyla Bourque.

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I, <u>\_\_\_\_\_Ryan Martin</u> consent to the incorporation of the following material(s) in the doctoral thesis prepared by Kyla Bourque.

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I, <u>IDA DERISH</u> consent to the incorporation of the following material(s) in the doctoral thesis prepared by Kyla Bourque.

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I, <u>Cara Hawey</u> consent to the incorporation of the following material(s) in the doctoral thesis prepared by Kyla Bourque.

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I, <u>Kashif Khan</u> consent to the incorporation of the following material(s) in the doctoral thesis prepared by Kyla Bourque.

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I, am Dr. Karima ALIM consent to the incorporation of the following material(s) in the doctoral thesis prepared by Kyla Bourque.

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Date: \_2022/09/06\_\_\_\_\_



I, <u>Alyson Jiang</u> consent to the incorporation of the following material(s) in the doctoral thesis prepared by Kyla Bourque.

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I, <u>Jeremy Zwaig</u> consent to the incorporation of the following material(s) in the doctoral thesis prepared by Kyla Bourque.

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I, <u>Hooman Sadighian</u> consent to the incorporation of the following material(s) in the doctoral thesis prepared by Kyla Bourque.

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Signature: Hooman Sadighian

Date: 2022-09-07



I, \_\_\_\_\_Natalie Gendron\_\_\_\_\_ consent to the incorporation of the following material(s) in the doctoral thesis prepared by Kyla Bourque.

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# Peeking at G-protein-coupled receptors through the molecular dynamics keyhole

Giuseppe Deganutti <sup>™</sup>, Stefano Moro & Christopher A Reynolds

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#### Figure 1. Structure of a G-protein-coupled receptor.

(A) Snake plot (modified from **www.gpcrdb.org**) showing the main structural features of a GPCR. The protein single-chain spans the cytoplasmic membrane via seven transmembrane helices (TM1-7), and it is characterized by three ECLs (ECL1-3) and three ICLs (ICL1-3), as well as a helical intracellular C terminus (H8). (B) Ternary organization (ribbon representation) of the structural elements.

ECL: Extracellular loop; GPCR: G-protein-coupled receptor; ICL: Intracellular loop; TM: Transmembrane.



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Regular Issue

Phosphodiesterases Catalyze Hydrolysis of cAMPbound to Regulatory Subunit of Protein Kinase A and Mediate Signal Termination \* "

### Signal Termination PDE Regulation cAMP PKA

Balakrishnan Shenbaga Moorthy ‡, Yunfeng Gao ‡, Ganesh S. Anand ≈‡⊠



Fig. 1. Overview of cAMP signaling and regulation of PKA. Adenylyl cyclases catalyze synthesis of cAMP from ATP whereas phosphodiesterases (PDEs) catalyze hydrolysis of cAMP to 5' AMP. cAMP activates PKA by mediating dissociation of the PKA holoenzyme to release R and Csubunits via a largely well-understood mechanism. Little is known, on how cAMP-bound Rsubunits reassociate with C-subunit to generate inactive PKA, and the potential role of PDEs in cross-talk with the R-subunits leading to signal termination.

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# MAPK/ERK Pathway as a Central Regulator in Vertebrate Organ Regeneration

by 🙁 Xiaomin Wen 🖂, 🙁 Lindi Jiao 🖂 and 🙁 Hong Tan \* 🖂 💿

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Figure 2. Simplified schematic of the regulatory mechanism and functions of the MAPK/ERK1/2 pathway.

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# Stimulated nuclear import by $\beta\mbox{-like}$ importins

Karen Flores and Rony Seger

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# Abstract

Classic nuclear shuttling is mediated by an importin- $\alpha$ · $\beta$  heterodimer that binds to cargoes containing a nuclear localization signal, and shuttles most nuclear proteins immediately after their translation. Aside from this canonical mechanism, kariopheryn- $\beta$ s or  $\beta$ -like importins operate by binding to non-canonical nuclear localization signals to mediate translocation without the assistance of importin- $\alpha$ . The mechanism by which these components operate is much less understood and is currently under investigation. Recently, several  $\beta$ -like importins have been implicated in the stimulated nuclear translocation of signaling proteins. Here, we propose that this group of importins might be responsible for the swift nuclear shuttling of many proteins following various stimuli.

# Mechanisms of stimulated nuclear import

Intracellular signaling pathways transmit signals of various extracellular stimuli to their cytosolic and nuclear targets in order to induce biological responses, such as proliferation, differentiation, cell death and migration. When needed, the signals are transmitted from the cytoplasm to the nucleus via translocation of one or more components of each of the signaling pathways involved. Thus, after stimulation, a large number of signaling proteins are rapidly translocated to the nucleus to induce and regulate many nuclear processes. However, despite the importance of stimulated nuclear signaling, the mechanisms by which these components reach the nucleus upon stimulation have been elucidated only for a few signaling pathways.

Classic nuclear shuttling is mediated by an importin- $\alpha \cdot \beta$  complex that binds to cargoes containing a nuclear localization signal (NLS), consisting of mono- or bi-partite clusters of basic amino acids [<u>1-3</u>]. This importin- $\alpha \cdot \beta$  complex often acts as a housekeeping mechanism that shuttles most nuclear proteins immediately to the nucleus after their translation [<u>4</u>]. The relocalization of cargoes

#### Stimulated nuclear import by $\beta$ -like importins - PMC

is followed by the dissociation of the proteins from the importins upon binding to RanGTP [5], which exports the importins back to the cytoplasm, while the cargo remains in the nucleus [6]. However, only a limited number of signaling proteins, such as NF $\kappa$ B [7] and ERK5 (extracellular signal-regulated kinase 5) [8-10], use this machinery for their stimulated nuclear shuttle. Aside from this canonical mechanism, importin- $\beta$  [11] or similar karyopherins, termed  $\beta$ -like importins [12], operate by binding to non-canonical NLSs to mediate translocation without the assistance of importin- $\alpha$ . The mechanism by which these components operate is much less understood and is currently under investigation. Recently, several  $\beta$ -like importins have been implicated in the stimulated nuclear translocation of signaling proteins. Here, we propose that this group of importins might be responsible for the swift nuclear shuttling of many proteins following various stimuli.

# The mechanism of ERK1/2 translocation to the nucleus

ERK1/2 are important signaling proteins that translocate to the nucleus upon stimulation. The rapid and robust activation of ERK1/2 allows the phosphorylation and modulation of the activity of more than 300 proteins, which are localized either in the cytoplasm or the nucleus [13-15]. These substrates are important for the induction and regulation of cellular processes, including proliferation, differentiation, and migration amongst others [16-19]. The sub-cellular localization of ERK1/2 plays an important role in its regulation and physiological functioning [20,21]. Interestingly, it was shown that the nuclear accumulation of ERK1/2 is important primarily for the induction of proliferation [22,23], while other ERK-dependent processes are mostly regulated by cytosolic molecules [24].

ERK1/2 localization, as well as the mechanisms that govern it, has been elucidated over the past decades. In resting cells, all components of the ERK1/2 cascade are localized primarily in the cytoplasm due to their interaction with different anchoring proteins [25-28] (see Figure 1). Upon stimulation, MEK1/2 phosphorylates ERK1/2 in their TEY motif, thereby inducing a conformational change resulting in the activation of ERK1/2 and detachment from their anchors [28]. This detachment exposes ERK1/2 to an additional phosphorylation on two Ser residues (an SPS motif) within a nine amino acid sequence, termed nuclear translocation signal (NTS) [29]. This phosphorylation can be mediated by both stimulated and constitutively active protein kinases, including protein kinase CK2 and auto-phosphorylation by active ERK1/2 molecules to the nuclear pores, inducing nuclear sliding. Once in the nucleus, RanGTP dissociates importin-7 from ERK1/2, and consequently, induces their nuclear accumulation [29]. It was also shown that ERK1/2 may interact directly with the nuclear pores, and it is possible that these direct interactions are able to facilitate the ERK1/2 translocation [31]. In addition, this process may be regulated by calcium, as a reduction in intracellular calcium concentrations was shown to induce faster nuclear shuttling [32,33].



### Figure 1.

### Schematic representation of the mechanism of stimulated ERK1/2 translocation to the nucleus

The following steps are illustrated: (A) Binding of ERK1/2 with anchor proteins in resting cells; (B) stimulation is followed by phosphorylation of the TEY motif of ERK1/2 by MEK1/2, and detachment of ERK1/2 from their anchors; (C) phosphorylation of ERK1/2 on its SPS motif by CKII. (D) Binding of phosphorylated ERK1/2 to importin-7 and nuclear sliding through the NUPs; (E) Dissociation of ERK1/2 from importin-7 by RanGTP, and nuclear accumulation of ERK1/2. For more details, see text.

Interestingly, these results in mammals were consistent with findings in *Drosophila* [34], where DIM-7 (the ortholog of importin-7) was identified as the carrier of D-ERK to the nucleus [35,36]. Once in the nucleus, ERK plays a critical role in the development of eyes and wings in *Drosophila* [37,38]. Moreover, while comparing the mechanism of nuclear translocation of components of the ERK cascade with other proteins, we established that the NTS might act as a specific stimulus-induced and importin-7-dependent nuclear translocation signal for some signaling proteins lacking



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# Excitation-contraction coupling of human induced pluripotent stem cell-derived cardiomyocytes

Christopher Kane (https://www.frontiersin.org/people/u/236085),

Liam Couch (https://www.frontiersin.org/people/u/240982) and

Cesare M. N. Terracciano (https://www.frontiersin.org/people/u/225521)\*

Laboratory of Cell Electrophysiology, National Heart and Lung Institute, Imperial College London, London, UK

Induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) hold enormous potential in many fields of cardiovascular research. Overcoming many of the limitations of their embryonic counterparts, the application of iPSC-CMs ranges from facilitating investigation of familial cardiac disease and pharmacological toxicity screening to personalized medicine and autologous cardiac cell therapies. The main factor preventing the full realization of this potential is the limited maturity of iPSC-CMs/fcelli2019.0009/pdf)

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Therefore, understanding the current electrophysiological properties of hiPSC-CMs will be crucial for proper interpretation of investigations into diseases modeled in this way.

# Calcium Handling in Induced Pluripotent Stem Cell-derived Cardiomyocytes

Ca<sup>2+</sup>-induced Ca<sup>2+</sup> release (CICR) is the key link between electrophysiological stimulation and the production of mechanical force as well as a feature that distinguishes cardiomyocytes from other types of muscle cells. Cardiomyocyte depolarization causes Ca<sup>2+</sup> entry via voltage-sensitive L-type Ca<sup>2+</sup> channels, triggering the release of Ca<sup>2+</sup> from the sarcoplasmic reticulum (SR) via ryanodine receptors (RyR), raising [Ca<sup>2+</sup>]<sub>*i*</sub> and causing contraction. Relaxation is achieved by removing the Ca<sup>2+</sup> from the cytosol, either by resequestering it in the SR via the sarco-endoplasmic reticulum ATP-ase (SERCA), or extruding it into the extracellular space through the Na<sup>+</sup>-Ca<sup>2+</sup> exchanger (NCX) or Ca<sup>2+</sup> ATPase pump (Bers, 2002; Figure 1).





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**Keywords:** induced pluripotent stem cell-derived cardiomyocytes, excitation–contraction coupling, stem cell maturation, disease modeling, pharmacological screening

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# A Brief Review of Current Maturation Methods for Human Induced Pluripotent Stem Cells-Derived Cardiomyocytes

Razan Elfadil Ahmed (https://www.frontiersin.org/people/u/903527)<sup>1</sup>,

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Cardiovascular diseases are the leading cause of death worldwide. Therefore, the discovery of induced pluripotent stem cells (iPSCs) and the subsequent generation of human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-12445) and the subsequent generation of human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-12445) and the subsequent generation of human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-12445) and the subsequent generation of human induced pluripotent stem cells (iPSCs) and the subsequent generation of human induced pluripotent stem cells (iPSCs) and the subsequent generation of human induced pluripotent stem cells (iPSCs) and the subsequent generation of human induced pluripotent stem cells (iPSCs) and the subsequent generation of human induced pluripotent stem cells (iPSCs) and the subsequent generation of human induced pluripotent stem cells (iPSCs) and the subsequent generation of human induced pluripotent stem cells (iPSCs) and the subsequent generation of human induced pluripotent stem cells (iPSCs) and the subsequent generation of human induced pluripotent stem cells (iPSCs) and the subsequent generation of human induced pluripotent stem cells (iPSCs) and the subsequent generation of human induced pluripotent stem cells (iPSCs) and the subsequent generation of human induced pluripotent stem cells (iPSCs) and the subsequent generation of human induced pluripotent stem cells (iPSCs) and the subsequent generation of human induced pluripotent stem cells (iPSCs) and the subsequent generation of human induced pluripotent stem cells (iPSCs) and the subsequent generation of human induced pluripotent stem cells (iPSCs) and the subsequent generation of human induced pluripotent stem cells (iPSCs) and the subsequent generation of human induced pluripotent stem cells (iPSCs) and the subsequent generation of human induced pluripotent stem cells (iPSCs) and the subsequent stem cells (iPSCs) and the subsequent stem cells (iPSCs) and the subsequent stem cells (iPSCs)

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# **FRET Microscopy in Yeast**

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## Figure 2.



#### Fluorescent biosensors for ERK activity.

(A) Schematics of the EKAREV-based FRET biosensor (upper) and the probe distribution in a cell (below). The ERAREV comprises a CFP variant, a YFP variant, a substrate peptide domain, a linker, and the WW-phosphopeptide binding. Subcellular localization of the fluorescent proteins depends on localization signals, including the NLS or the NES, in the probe. The bottom panel shows a case with the NLS-type FRET biosensor. (B) Schematics of the KTR-based biosensor (upper) and the probe distribution in a cell (below). The KTR comprises a fluorescent protein, a substrate recognition motif domain, the NLS, and the NES. The FP-fused KTR is localized in the cytoplasm when the ERK is active while it is in the nucleus in the case of inactive ERK.

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