Using RNA-Sequencing to Improve Characterisation and Production of iPSC Induced Cardiomyocytes for Heart Failure

By

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This thesis is dedicated to Snoopy!

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LIST OF ABBREVIATIONS

cGMP current good manufacturing practices

CVD Cardiovascular Diseases

EHM Engineered Human Myocardium

hESC Human Embryonic Stem Cells

HF Heart Failure

hIPSC Human Induced Pluripotent Stem Cells

NCD Non Communicable Diseases

NGS Next-Generation Sequencing

qPCR Quantitative Polymerase Chain Reaction

RNA-Seq RNA-Sequencing

scRNA-Seq Single-cell RNA Sequencing

CHAPTER 1

INTRODUCTION

There is growing scientific evidence and recognition that human actions both directly and indirectly have profoundly changed the Earth system, in a continously accelerating process, commonly called as the "Anthropocene". Human mortality, one of the very few certainities of human life has also not been spared by this radical change (Moysés and Soares 2019). People are living much longer across socities in the world, in 2017 almost 49% of deaths belonged to the 70s+ age bracket, while in 1990 this percentage was around 30%. In the same time frame, there has been a significant increase in the proportion of deaths caused by non communicable diseases (NCD). Taken together, we see an aging population strained by NCDs of which cardiovascular diseases (CVD) are the most pronounced (see 1.1). Almost half of the deaths attributed to CVDs are caused due to heart failure (HF). Despite impressive improvements in modern medicine, pharmacological interventions are capable of only alleviating the symptoms of HF which then renders it a progressive, terminal disease. It is estimated that 1-2% of the healthcare budget is spent on HF (Liao, Allen, and Whellan 2008), while the global economic budern is estimated at $108 billion per annum (Cook et al. 2014) and in Germany the annual prevalence-based costs for heart failure patients are around €25,532 (Lesyuk, Kriza, and Kolominsky-Rabas 2018). Increasing proportion of elderly in western societies and with developing nations following suit, it is only expected that the incidence of HF would be on the rise. Yet, this debilitating and expensive disease's only viable treatment in terms of long-term life quality and mortality is a heart transplant. As per one study (Trivedi et al. 2016), 15% of patients died while waiting for a donor heart (at 180 days after listing), elucidating the severity of shortage of viable donor hearts. As of February 2020, there are a total of 1082 people on the heart transplant waitlist within the EuroZone as per Eurotransplant statistics ("Eurotransplant - Statistics," n.d.).

Although there are myriad causes of HF, such as isichemic heart disease, aortic or mitral regurtitation (volume stress), aortic or mitral stenosis (pressure stress), congenital cardiomyopathy, constrictive pericarditis, alcohol excess, anemia, thyrotoxicosis, septicemia, acromegaly, they commonoly operate through the central mechanism of

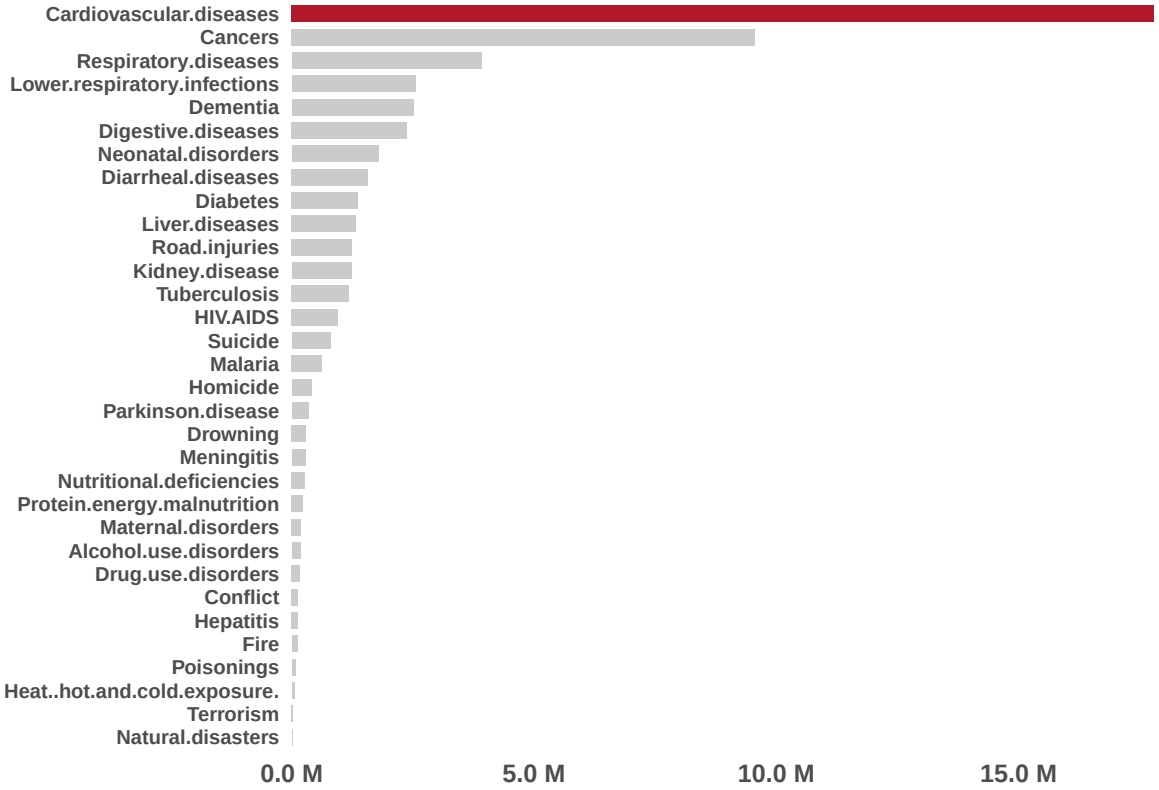

Figure 1.1: Number of Deaths by Cause in the world in 2017

reduced ventricular function. Consequently, the heart is unable to adequately perfuse the tissues, resulting in a wide variety of clinical symptoms including but not limited to the ones shown in Table 1.1. These can be considered as compensatory measures, for example, an initial phase of cardiac hypertrophy is seen to compensate for the loss of viable cardiomyocytes resulting in a transient maintenance of the ejection fraction, sustainance of heart rate and blood pressure and thereby maintaining organ perfusion. Over time, these remodelling mechanisms become detrimental and end up worsening the left ventricular function. In effect, a negative feed-forward pathophyiological loop governed by a dissonant neurohormonal system and imapaired calcium signalling is established in late-stage HF. Most of the pharamacological treatments currently available for HF (diuretics, beta blockers, angiotensin receptor blockers, angiotensin converting enzyme inhibitors aldosterone antagonists, etc) are symptomatic treatments and do not halt or address the underlying pathophysiology. Device therapies are currently the alternatives to pharmacological drugs. These include cardioverter-defibrillator (ICD) which are implanted in severe cases as a means of primary or secondary prevention of sudden cardiac death. The last in line prior to a heart transplantation which is considered the only viable option for treating end-stage HF in terms of long-term

quality of life and mortality, is a ventricular assist device which acts as a bridge to transplant. Given this current scenario, it is vital to explore other avenues for the treatment and management of HF.

Table 1.1: Systemic effects of Heart Failure

System	Changes
Heart	• Left ventricular hypertrophy • Left ventricular dilatation • Changes in calcium-cycling proteins • Switch to fetal isoforms of contractile proteins • Atrial natriuretic peptide release • Brain natriuretic peptide release • Release of proinflammatory cytokines
Lungs	• Increased dead space • Excessive ventilation
Kidneys	• Erythropoietin release • Renin release
Posterior pituitary	• Antidiuretic hormone(vasopressin) release
Adrenal Glands	• Aldosterone increase • Catecholamine release
Autonomic nervous system	• Activation of sympathetic system
Arteries	• Vasoconstriction • Endothelin release

1.1 Need for better therapeutics

Although modern medicine has vastly improved the management of heart failure it still remains a debilitating disease which would immensely benefit from newer

therapies. One of the most straight-forward approaches would be to simply address the underlying pathology of HF, wherein "vital/fresh" cardiomyocytes can be supplemented to counteract the progressive loss of cardiomyocytes in a terminally-differentiated, post-mitotic heart (Bergmann et al. 2015). This has been made possible largely due to the introduction of human embryonic (Thomson et al. 1998) and induced pluripotent stem cells (Takahashi et al. 2007) along with defined protocols of directed differentiation to a cardiac lineage/cell fate, covered in the review (Burridge et al. 2012). However, this is fraught with its own key limitation: lack of long term engraftment of cardiomyocytes (Nguyen et al. 2016). Several other stratergies to strenghten/remuscularize the heart such as, converting scar into healthy heart muscle (Inagawa and Ieda 2013),inducing endogenous cardiomyocyte regeneration and proliferation (Kubin et al. 2011), and methods to save the remaining cardiomyocytes from cell death by modulating paracrine factors (Gnecchi et al. 2005) have been investigated. Despite the limitation in long term engraftment, cardiomyocyte implantation remains the most plausible option in a translational and mechanisitic stand point. It is currently known that cardiomyocytes supplemented as a cell injection have the worst retention and epicardial delivery of cardiomyocytes as tissue engineered patches show an improved retention (Sekine et al. 2011). Animal studies indicate that transplantation of engineered heart muscle (EHM) , made from human induced pluripotent stem cells (hIPSCs) , to a failing heart as a means of remuscularization showed improved cardiomyocyte proliferation, vascularization, unimpaired electrical coupling and improved left ventricular function. Additionally, these engineered patches have also not shown to be associated with an increased propensity for arrhythmia (Weinberger et al. 2016; Yang et al. 2015; Zimmermann et al. 2006). More recently a macaque model of heart failure (with human-like cardiovascular physiology) studied by (Liu et al. 2018), showed near normal levels of contractile function after 3 months of transplantation of cardiomyocytes derived from human embryonic stem cells (hESCs). Collectively, these preclinical studies hold promise for the utilization of cardiomyocytes and EHMs thereby derived as a potential therapeutic source for failing human hearts.

1.2 Engineered Human Myocardium

Translation to clinics require a production protocol that is compliant with current good manufacturing practices (cGMP). Ths has already been established and optimized by the working group over the years (Tiburcy et al. 2017). Briefly, iPSCs are differentiated to not only cardiomyocytes, but also supportive stromal cell population

using serum-free, GMP-friendly media and protocols. These differentiated cells are then combined in a fixed, optimized ratio within a collagen matrix. This mixture is then pipetted onto circular casting molds to make EHM rings or onto other shapes of molds to obtain the desired output form of the EHM see 1.2. Ideally, several such EHMs would be stacked to make a muscle layer of optimum thickness and sutured onto the failing myocardium, which would then integrate and assist mechanically in pumping. > write about characteristics of EHM > maturity/ immaturity? more on protocol? > get more images for patches

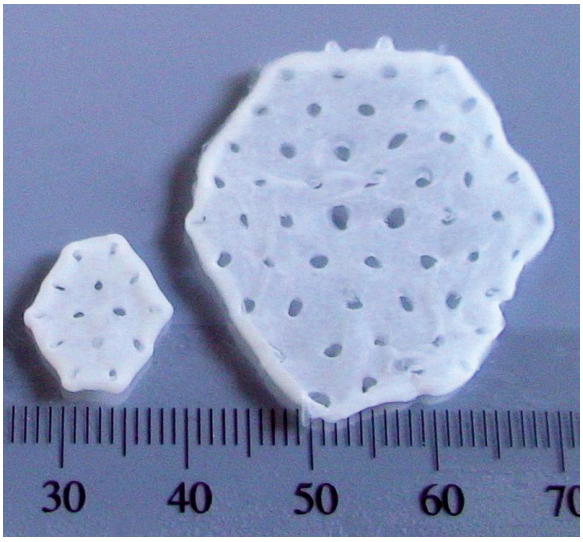

Figure 1.2: Various shapes and forms of EHMs for clinical and experimental applications

1.3 RNA Sequencing

Information stored in genes as DNA is transcribed into RNA and ultimately translated into proteins, this is the central dogma of biology. The transcription of a subset of genes into RNA molecules gives a cell it's specificity and identity, along with regulating its activities. 'Transcriptome' is a term that refers to the total RNA, whether from a population of cells or a single cell. It is also used to refer to the total mRNA, which focuses on gene expression. Previously, gene expression studies relied on techniques such as northern blots and quantitative polymerase chain reaction (qPCR) which are low-throughput and are limited to measuring a single transcript. The last two to three decades has seen the evolution of assays to quantify genome-wide gene expression, better known as transcriptomics. Microarrays, a hybridization based approach, were the main-stay of such transcriptomics until the recent revolution of high-throughput next-generation sequencing (NGS). It has revolutionized transcriptomics by enabling

RNA analysis via the sequencing of complementary cDNA (Wang, Gerstein, and Snyder 2009). This is termed as RNA sequencing (RNA-Seq) which has distinct advantages over the former approaches, such as its ability to detect transcripts that are not yet annotated, low background signal (in comparison to DNA microarrays), a large dynamic range of expression level, higher sensitivity, higher reproducibility, all of which allow for understanding the dynamic and complex nature of the transcriptome.The type of information that RNA-Seq provides can be broadly classified into two categories: - Qualitative data which includes identifying transcripts, identifying intron/extron boundaries, poly-A sites and transcriptional start sites (TSS) which in RNA-Seq terminology is commonly referred to as "annotation". - Quantitative data which includes measuring differences in expression, alternative TSS, alternative splicing, alternative polyadenylation between two or more treatments or groups. This power of sequencing RNA has led to RNA-Seq not only being limited to the genomics community but has also led to it becoming a main-stay in the toolkit of all life science research communities. A typical RNA-Seq experiment can be split into three parts (Conesa et al. 2016):

1. Pre Analysis
    - Experimental Design (choosing the library type, sequencing legth, the number of replicates and sequencing depth)
    - Sequencing Design (spike-ins, randomization at library prep, randomization at sequencing run)
    - Quality Control (raw reads, read alignment, quantification, reproducibility)
2. Core Analysis
    - Transcriptomic Profiling (read alignment, transcript discovery, quantification level, quantification measure)
    - Normalization (Z-scale, variance stabilized transformation, etc)
    - Differential Expression
    - Interpretation (functional profiling)
3. Advanced Analysis
    - Visualization
    - Integration (eQTL, ATAC-seq, ChIP-Seq, proteomics/metabolomics)

The sucess of an RNA-Seq study depends on the choices and decisions made at each of these steps. Naturally, the real-world analysis of RNA-Seq data has as many variations as there are applications of the technology.

1.3.1 Bulk RNA Seq

Generally, unless otherwise specified RNA-Seq refers to **bulk** RNA-Sequencing. Here, the RNA is collected from an entire tissue (biopsy), or a group of cells and thereby the sequenced data represents the *average expression level* for each gene across the large population of input. This bulk RNA-Seq which is the main work horse of gene expression studies is adequate for comparative transcriptomics, wherein samples of the same tissue are compared across species, or for quantifying expression signatures from ensembles, such as in disease studies. However, it falls short in its ability to be an effective tool for studying heterogeneous systems, such as complex tissues (brain, heart, etc) or early developmental studies. It also fails to capture the stochastic nature of gene expression and spatial resolution can not be obtained. An ilustrated, simplistic example is shown in 1.3.

1.3.2 Single-cell RNA Seq

Single-cell RNA-Seq (scRNA-seq) is a new and active field of RNA-seq which arose to fulfill the unmet needs of bulk RNA-Seq. Despite being introduced in 2009 (Tang et al. 2009), it did not gain popularity until the advent of newer protocols and reduced sequencing costs much later. It measures the *distribution of expression levels* for each gene across a population of cells. It has revealed new, unknown cell types in what were considered to be well-studied and established diseases, such as the discovery of ionocyte cells, in cystic fibrosis (Montoro et al. 2018). Spatially resolved scRNA-Seq holds similar promises, revealing novel information on the extent of fetal marker gene expression in small populations of adult heart tissues (pubmeddev and al et, n.d.). Thus, novel biological questions addressing cell type identification, heterogeneity of cell responses, stochasticity of gene expression and inference of gene regulatory networks across cells can be studiesd. The applications of scRNA-Seq to novel biological questions and the computational and laboratory methods catering to it are advancing at such a rapid pace that even recent reviews (Stegle, Teichmann, and Marioni 2015; Svensson, Vento-Tormo, and Teichmann 2018) are becoming outdated.

1.4 Computational deconvolution

It is established that scRNA-Seq allows for unbiased transcriptional profiling of thousands of individual cells. Yet, the usage of this powerful technology is limited by its cost and impracticality with respect to analyses of large sample cohorts. Also, most clinical specimens are fixed, for example in formalin or embedded in paraffin,

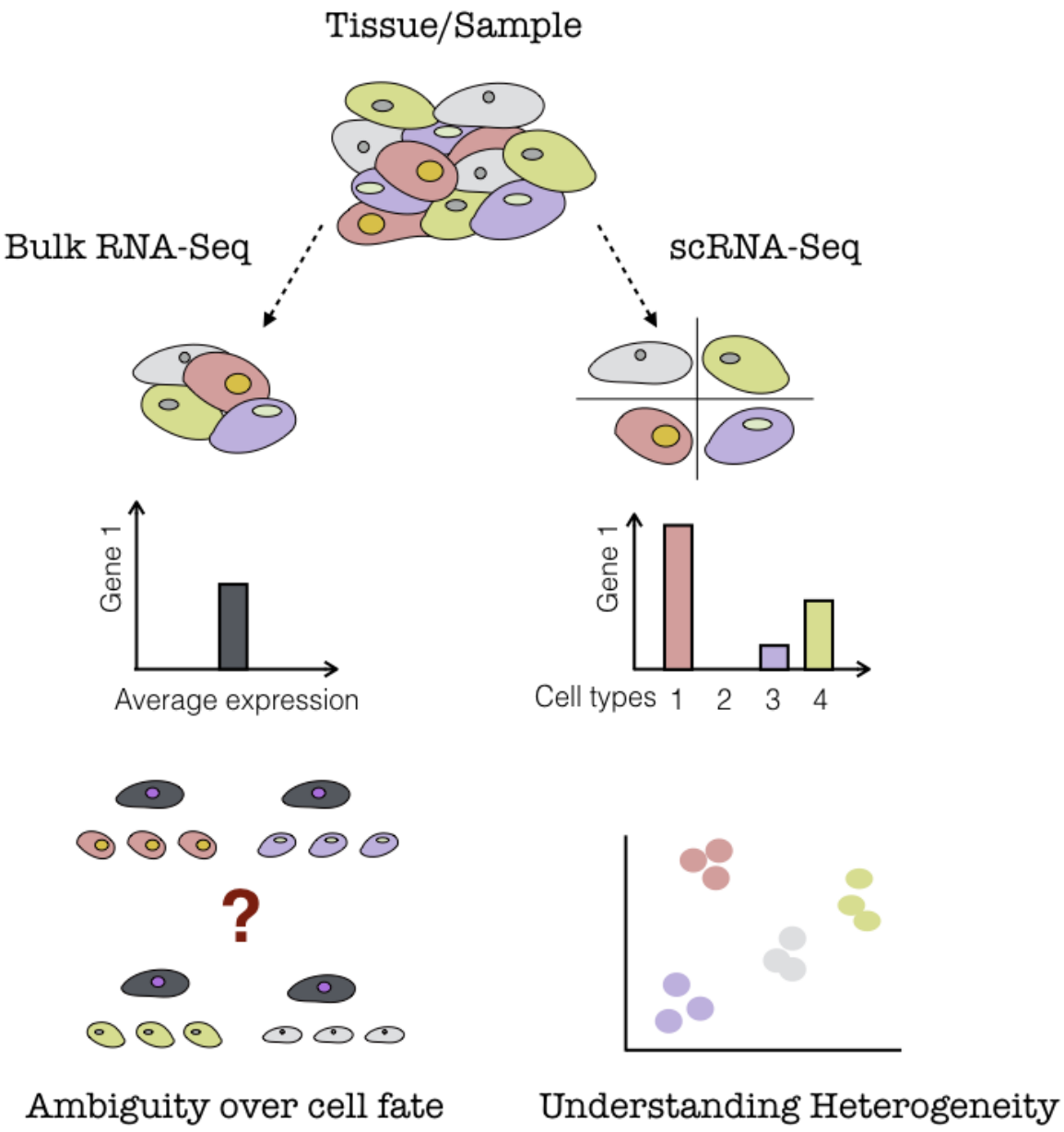

Figure 1.3: A caption

which renders its dissociation into intact single-cells impossible. To circumvent these limitations and utilize the specificity and accuracy of scRNA-Seq along with the ease of bulk of RNA-Seq, several groups around the world have developed *deconvolution* computational techniques (Aran, Hu, and Butte 2017; Becht et al. 2016; Dong et al., n.d.; Kang et al. 2019; Newman and Alizadeh 2016; Quon et al. 2013; Racle et al. 2017; Shen-Orr and Gaujoux 2013). Deconvolution, in the realm of a sequencing, is a common umbrella term for a procedure that estimates the proportion of each cell type in a bulk sample. Flow cytometry and scRNA-Seq are experimental methods of deconvolution. Computational deconvolution leverages scRNA-Seq reference sets (or fluorescence-activated cell sorting (FACS)-sorted, purified bulk sets) for bulk gene expression deconvolution. A basic comparison of the available tools soon led to CIBERSORTx ((Newman et al. 2019)) as being currently at the forefront of deconvolution because unlike other methods it can: 1.Leverage scRNA-Seq derived reference profiled for bulk tissue dissection 2. Overcome technical variation arising from different platforms (eg., bulk RNA-Seq, scRNA-Seq, microarrays) and tissue preservation techniques 3. Digitally "purify" cell-type specific expression profiles from bulk tissues without physical cell isolation. Briefly, most deconvolution algorithms, including CIBERSORTx, work to solve the following linear equations for $\mathbf{f}$:

$$m = Hf$$

m: mixture gene expression profile (GEP) (to be deconvolved)

f: a vector of fraction of each cell type in a signature matrix (the unknown)

H: a *signature matrix* containing signature genes for cell subsets of interest

Both m and B are input requirements. Further analytics of deconvolution are outside the scope of this thesis and be found at (Chen et al. 2018; Newman et al. 2019). So with this framework, a relevant single-cell or bulk-sorted RNA sequencing data can be used to tease out molecular signatures of distinct cell types and these signatures can then be used to characterize cellular heterogeneity from bulk tissue transcriptomes without physical cell isolation, see 1.4.

1.5 Principal Component Analysis (PCA)

High-dimensional data are common in today's biology as they arise when several features, like the expression of many genes, are measured for multiple samples. This kind of data holds several challenges — high computational demand, an increased

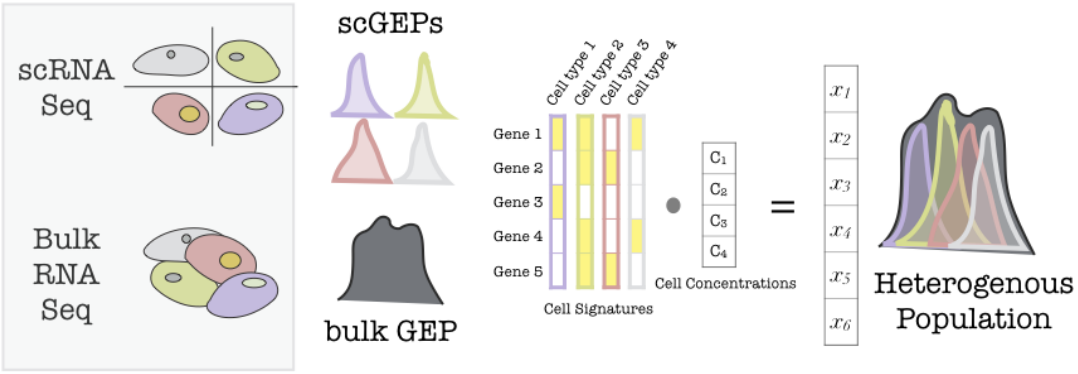

Figure 1.4: A caption

error rate due to multiple test corrections when testing each feature for association with an outcome. PCA is an unsupervised dimension reduction technique, that on any given dataset performs linear transformation and fits the data to a new coordinate system in such a way that maximum variance is explained by the first coordinate, and each subsequent coordinate is orthogonal to the last and explains progressively lesser variance. Each principal component thus sums up a certain percentage of the total variation in the dataset. In this way, a set of x correlated variables over y samples is transformed to a set of p uncorrelated principal components over the same samples. Where many variables correlate with one another, they contribute strongly to the same principal component. PCA can find patters without prior knowledge about whether samples come from different treatment groups or have phenotypic differences. PCA also allows for low-dimensional representation (eg, bi-plot) of the data, while retaining as much information as possible as most of the noise in the dataset is pushed to the last few principal components. The goal is to reduce the features' dimensionality while only loosing a small amount of information.

1.6 Rationale for the current work

Omics technologies — such as transcriptomics, genomics, proteomics and metabolomics — are shaping modern medicine and biology at an extraordinarily detailed molecular level. Cardiovascular sciences is a field that stands true to the previous statement. For example, an integrated omics approach was used to identify genes associated with isoproterenol-induced hypertrophy and resulting heart failure in the Hybrid Mouse Diversity Panel (HMDP) (Chella Krishnan et al. 2018; Lin et al. 2018; Lusis et al. 2016; Park et al. 2018; Rau, Civelek, et al. 2017; Rau, Romay, et al. 2017; Santolini et

al. 2018; Shu et al. 2017). Several candidate causal genes that determined the extent of cardiac hypertrophy were identified by integration of the cardiac transcriptome and genomic information. *Hes1* was particularly predicted to be causal in the progression to cardiac hypertrophy after heart damage. This study also showed that knocking down *Hes1* in ventricular myocytes had a 90% reduction in hypertrophy, confirming its role in hypertrophy (Santolini et al. 2018). This is just one example of many where newer technologies are spearheading research in the cardiovascular field, detailed reviews of the topic can be found at (Lau and Wu 2018; Leon-Mimila, Wang, and Huertas-Vazquez 2019). Knowledge about differentiation and differentiation protocols are other areas relevant to this project, that have been vastly improved by the usage sequencing technologies (Burridge et al. 2012; Cuomo et al. 2020; Han et al. 2018; McCracken et al. 2019; Müller et al. 2012; Wesolowska-Andersen et al. 2020). This project is a starting point to produce a work similar to that of Wu et al (Wu et al. 2018) reviewd in (Freedman 2019), where current protocols to generate kidney organoids from hiPSCs (meant as tissue replacement source) were evaluated using scRNA-Seq. The study showed that the organoid-derived cell types were immature, and contained a significant, 10-20%, percentage of cells which were non-renal. Overall, the study was a proof-of-concept of the power of scRNA-Seq technologies to characterize and improve organoid differentiation. Prof.Zimmermann's research group has developed GMP compliant protocols for the differentiation of hiPSC to cardiomyocytes and stromal cells which are then used to make EHMs using optimized tissue engineering techniques. These are also intended to be a tissue replacement stratergy. Unlike Wu et al, currently scRNA-Seq is not available but multiple bulk RNA-Seq data across several differentiation runs has been performed by the group. Also, unlike Wu et al, this project aims at first characterizing the hiPSC induced cardiomyocytes and not the entire organoid/EHM, which would ideally be a continuation of the current work. Taken together, the availability of bulk RNA-Seq data and a relevant scRNA-Seq data set like that of (Friedman et al. 2018) along with accessible deconvolution techniques, like CIBERSORTx, the project aims at an in-depth characterization of hiPSC induced cardiomyocytes.

CHAPTER 2

AIMS AND OBJECTIVES

CHAPTER 3

MATERIALS AND METHODS

Here is an example citation (**???**). Here is an example abbreviation:

Here is an example table.

Table 3.1: Wookiees and their homeworld.

name	homeworld
Chewbacca	Kashyyyk
Tarfful	Kashyyyk

Here is an example plot.

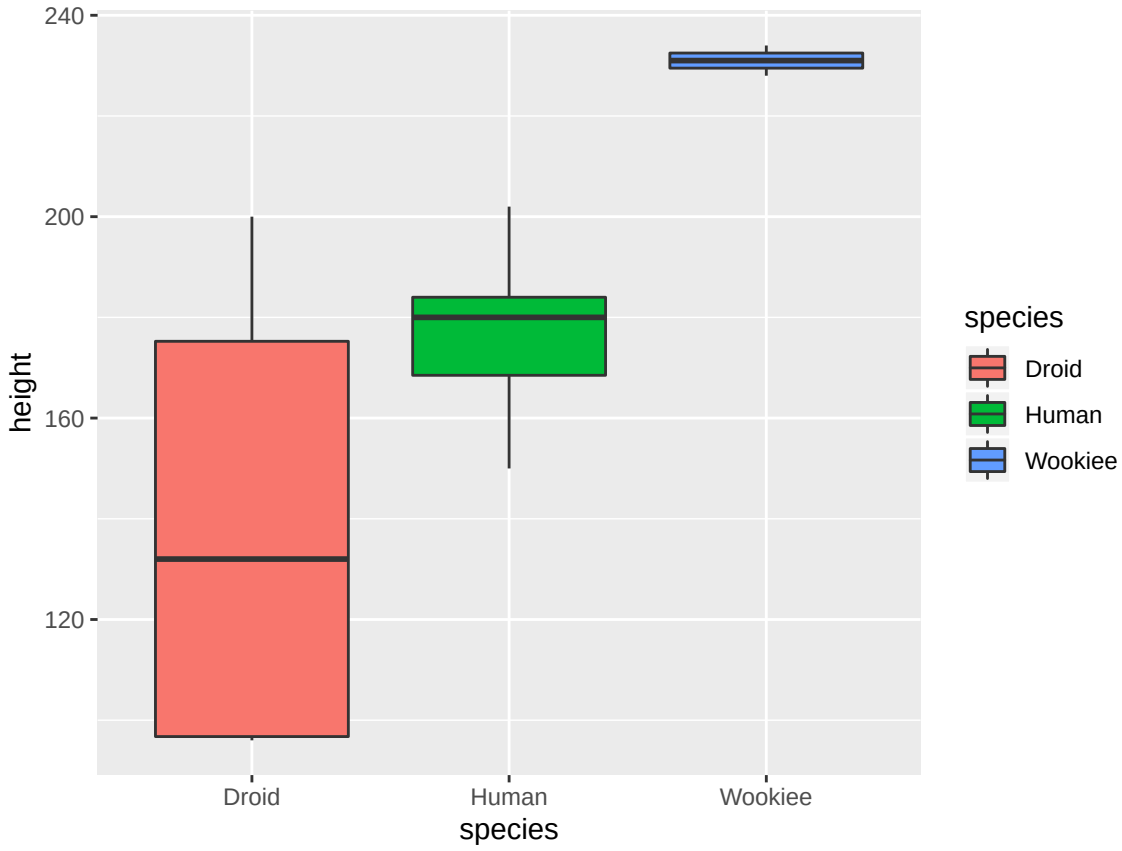

Figure 3.1: Starwars distribution of heights by species

CHAPTER 4

RESULTS AND DISCUSSION

CHAPTER 5

CONCLUSION AND FUTURE WORK

CHAPTER 6

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