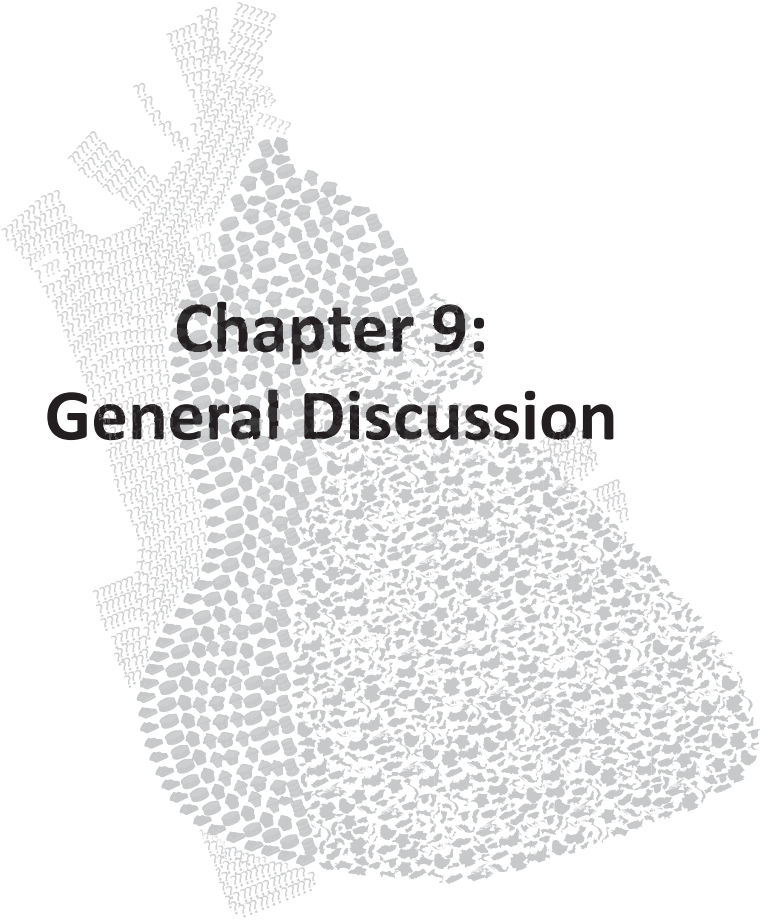

Chapter 9:
General Discussion

Induced Pluripotent Stem Cells

Over the last several years, research on human pluripotent stem cells (both embryonic and induced) has begun to fall into two distinct areas (i) basic stem cell biology, such as understanding the control of differentiation in development and the process of reprogramming, understanding pluripotency and finding ways to improve the reprogramming process itself to obtain a more "perfect" iPSC closer to hESC [1] and (ii) iPSC and genetically modified hESC as a tool to study/model disease [2]. While some aspects of the approaches and methodologies may be similar, their goals are very different so that their requirements of the iPSC and hESC they use may also be different. The work described in this thesis is in the second group: using human (h)iPSC as a model for human (cardiac) diseases so that the primary feature and requirement of the hiPSC used here is that the cell lines form cardiomyocytes efficiently and reproducibly, independent of the way in which the hiPSC were derived.

While it is important to have a fully reprogrammed hiPSC to decrease the variation between the different patients and the control lines, the need for the hiPSC to remain in a ground state of absolute pluripotency is not nearly as important as the ability to differentiate into the desired cell type. In this thesis, cardiomyocyte differentiation is the principle requirement of the hPSC lines. By contrast, those researchers studying the reprogramming process itself [3] or are trying to push hiPSC into a more naïve state rather more like mouse (m)iPSC or ESC [4] may require more stringent reprogramming and pluripotency tests as well as improved reprogramming methods which do not leave any footprint behind in the host DNA [5;6].

For many purposes, the tissue source of somatic cells for reprogramming can be of major importance. Dermal fibroblasts for example are easily derived and grow well in culture so that they are easy to reprogram to pluripotency but in the case of minors or young children, it may not be possible to obtain permission to collect skin biopsies. Some patients may be willing to take part in basic research by donating tissues samples but may be nervous of skin biopsy collection or injections for local anesthetic. In these cases, a non- or minimally invasive source of somatic cells would be preferable. Part of the research described in this thesis concerned selecting the cell sources that could be collected easily and reliably reprogrammed; this work is described in Chapter 3. We found that skin biopsies could still successfully provide cells able to be reprogrammed at about the same efficiency as freshly isolated fibroblasts, even after 14 days of storage in buffered saline solution (a basic salt solution). We also investigated the use of milk teeth and blood as a possible sources of somatic cells for reprogramming and while cells isolated from milk teeth were successfully reprogrammed at similar efficiencies to skin fibroblast, blood proved in our hands to be a more challenging cell source for reprogramming and we needed large starting volumes for success. This was an empirical finding and we did not find a reason for it. Easily accessible and minimally invasive tissue sources, such as hair follicle keratinocytes or small quantities of blood would be allowed for use in both children and adults. Although these tissue sources had been described in the literature as being successfully reprogrammed [7;8], we found it difficult to grow cells from these tissues and to cryopreserve and store them for later use in reprogramming. Banking the isolated somatic cells is a more feasible option when trying to collect a large number of samples either from patients or control samples. Fibroblasts could be dependably isolated from skin biopsies as small as 4mm, were easily stored and recovered well from frozen storage. So while we have successfully generated hiPSC from milk teeth as well as blood (Chapter 3) and others have generated hiPSC from other more easily accessible cell sources like hair keratinocytes [7], urine [9] and T-cells [10], fibroblasts were readily available and most reliable for the studies described in this thesis.

Aside from the tissue cell source, the method used for reprogramming and the technique used to induce differentiation could impact the use of iPSC technology for generating disease models. There has been much development since the first published articles claiming somatic cells could be reprogrammed to embryonic-like stem cells (ESC-like)[11-13]. There has been a variety

of protocols and methods of reprogramming factor delivery reported, including viral vectors (retro-, lenti-,adeno-, Sendai) mRNA, and episomes, with a range of reported efficiencies [14], which could be related their ability to recruit the Mbd3/NuRD repressor complex [15]. However most studies compare experiments with those described in the literature previously, and are not direct comparisons of methods in single reprogramming experiments [16]. This paucity of data that can be directly compared as well as a lack of a consistent method for calculating and presenting efficiencies make statements on efficiencies open to misinterpretation. For example, the total number of cells at the start of the reprogramming is sometimes compared to the number of morphologically identified ESC-like colonies and this is sometimes reported as "efficiency" but the number of cells identified as taking up the reprogramming factors vs number of morphologically identified ESC-like colonies has also been reported as a measure of efficiency. This makes comparing methods difficult and decisions on which methods are 'best' depends highly on the experience of the laboratory performing the experiments and whether it even matters, depends on the purpose for which the reprogrammed cells will be used. At the time at which the work in this thesis was initiated the best method for reprogramming which left only a minimal scar in the genome was using an excisable lentiviral vector. Later it became clear that non-integrating methods such as episomal [17] and Sendai virus-based protocols [6] which leave no trace in the genome at all could be used at a high efficiency and are now being routinely used to produce hiPSC in our lab as well.

With the development of transgene free methods which have been found to be robust and reproducible (e.g. Sendai virus, [6], the question arises if there is a need to develop techniques to identify insert number and locations such as in Chapter 4. In chapter 4 we found that by using a combination of splinkerette-PCR and ion torrent sequencing we could rapidly and accurately identify not only the number of lentiviral inserts in our hiPSC but also the location. While most groups are now implementing transgene free methods, there is a large number of lines which have already been generated and that for various reasons cannot be reprogrammed again (e.g. the loss or lack of source material). To the end of 2013, >90% of the published iPSC lines were generated using viral vectors.

There are several studies that have compared hiPSC lines to hESC to address the question on the extent to which they are similar or differ. There is at present no consensus but it seems the more lines that are compared, the smaller the differences between them become [16]. Some discrepancies have also been attributed to incomplete reprogramming, the insert number or the site in the genome that the reprogramming genes were inserted. Most of these studies have compared undifferentiated hESC and hiPSC lines but reports of characterization of their differentiated derivatives, such as cardiomyocytes, are now becoming more prevalent in literature, allowing comparisons of different lines at a different level. If differences are found, knowing and understanding the insert number and location could help explain some of these controversies.

Cardiac Disease Modeling

Most information on cardiac diseases is from clinical data and transgenic mouse models. While both can provide useful information they both have their disadvantages. Clinical assessment of disease is limited to symptoms (which may be explained by a number of diseases) and genetic testing; information regarding mechanism or cellular functionality is limited or unavailable. Genetically modified mouse models provide a wealth of knowledge by providing a continuous supply of cells as well as providing a full body model in which to study disease development and progression; however the differences between animals and humans, especially with respect to the heart is great [18;19].

There are a number of physiological, metabolic and protein marker expression differences between the human and rodent heart. The most obvious difference between the mouse (or any

small rodents) and human heart is heart rate. The mouse heart beats at 5-600 beats per minute (b/m) while the human heart beats at 60-70 b/m. The high heart rate of the mouse makes studying human arrhythmias caused by specific gene mutations difficult even when the mutated gene is successfully introduced. Furthermore, the differences in ion channels composition and their contribution to the action potential puts channelopathy studies solely carried out in the mouse in question. Another difference is the expression of myosin heavy chain (MHC) isoforms. βMHC is the isoform predominantly expressed in fetal mouse hearts whilst αMHC is predominant in the adult; the reverse is seen in humans [20].

In the past two decades our knowledge of cardiac development and disease has been greatly aided by pluripotent stem cell derived cardiomyocytes (PSC-CM). However there are still many issues which must be addressed before PSC-CM can contribute fully to cardiac research. In this thesis we have attempted to address a few of these points (Chapter 4-8). One of the first factors to consider is which differentiation method to use [21]. In Chapter 4 and 5, we described two different differentiation methods, both using defined medium and specific growth factors, however with striking differences. In chapter 4 we describe a new rapid method for cardiac differentiation of hPSC directly from mechanically passaged colonies on mTESR without the need for adaption to enzymatic passage. We showed this method to produce cardiomyocytes with at least 30% efficiency from 5 established hPSC lines as well as from a newly reprogrammed (less than passage 2) hiPSC line. The cardiomyocytes produced were positive for specific cardiac markers (e.g. α-actinin, troponin T and Nkx2.5) and basic electrophysiological properties (e.g. action potential and calcium transients) could be measured.

The method described in Chapter 5 used an embryoid body (EB) based system. It required optimizing the concentrations and timing of the growth factors, a necessity for many differentiation protocols due to the variation in endogenously produced factors as well as varying kinetics of the differentiation process itself [22]. This method yielded a large number of uniform, beating EBs. It is very suitable when a large number of cardiomyocytes is required from one PSC line, such as for drug discovery or toxicity testing and in combination with bioreactors,to be scaled to produce millions of cells. It is proving especially useful in this context when a cardiomyocyte-expressed promoter driving an antibiotic resistance gene is included that allows selective survival of cardiomyocytes, as described in chapter 8. This allows the large-scale derivation of pure populations of cardiomyocytes. The other differentiation method described in Chapter 4, does not require optimization or adaptation and can result in very rapid production of cardiomyocytes. This method is well suited to/for cases when a large number of different cell lines or clones (for screening after genetic modification) are being studied, for example in disease modeling when it is considered that minimally three clones from three different individuals are required for proper statistical comparison [23;24].

Another important point in phenotypic comparison and functional characterization of cardiomyocytes is the use of proper maintenance media (Chapter 7). Much effort has gone into optimizing the differentiation protocols for hPSC into cardiomyocytes [25-27]; however we are only beginning to analyze in detail the resulting cardiomyocytes that are being generated. In Chapter 7, we examined the effects of fetal calf serum (FCS), commonly used for cardiomyocyte maintenance to support survival and/or attain some degree of maturity, on PSC-CM. We found that treatment of PSC-CM derived using defined medium with FCS not only had hypertrophic effects similar to the addition of the known hypertrophic stimulus phenylephrine which was irreversible but also produced a positive chronotropic response. FCS is a common medium supplement with incompletely known and batch-to-batch variable composition [28]. It has been shown to cause hypertrophy in cultured cardiomyocytes [29;30] and yet is still often used to maintain PSC-CM for extended periods of time [31-33]. By comparing the effects of FCS on PSC-CM derived and maintained in defined medium, we highlighted the importance of the need to understand the effects of FCS components, which we often overlook, can have on results. In

chapter 7, we clearly showed that the disease phenotype that was described in chapter 6 was masked when FCS was present in the culture medium. This brings into question the results and conclusions of other groups (e.g. K. Laugwitz, SE Harding, and C. Denning) that have routinely used FCS or other sera in the cardiomyocyte culture. Lastly,we attempted to develop a generic method for purifying the population of cardiomyocytes among differentiated cells by selection using transiently expressed adeno-associated viruses (Chapter 8). Preliminary data using this construct was promising but the technology needs to be developed further and additional testing is needed before wider use of this virus for selecting cardiomyocytes. Researchers have often used other ways to get around having impure populations by using single cell assays or normalizing population data (for example in Western blots or RT-PCR) for the number of cardiomyocytes using cardiomyocyte-specific gene or protein expression; however this does not negate the need for a ubiquitous selection method which has low toxicity and can be easily applied to multiple PSC lines.

PSC-CM use in drug discovery is increasingly well documented [34;35] and their use in disease modelling has been further developed with the increased use of hiPSC from patients [2;24]. In this thesis we have also shown how hiPSC can be used in modeling hypertrophic cardiomyopathy (HCM). In chapter 6 we describe the reprogramming and basic disease characterization of hiPSC derived from patients with HCM. Fibroblasts from three patients (2 related and one unrelated male) carrying the Dutch founder mutation 2373inG in myosin binding protein C3 (MYBPC3) were collected and reprogrammed into hiPSC. Subsequently cardiomyocytes were derived. We found the cardiomyocytes from the HCM patients contained less MYBPC3 protein and were larger in cell surface area compared to cardiomyocytes from a healthy hiPSC line, comparable to the clinical situation. Also, the HCM-derived cardiomyocytes had an altered response to changes in external calcium as well as a larger caffeine-induced calcium release. Our results were comparable to those found in cardiac samples retrieved from patients [36;37]. However the mechanism of disease is still unknown and is currently being investigated along with more in depth disease characterization by other researchers in the lab.

The research described in this thesis is just the first step in modeling the HCM disease. We showed basic phenotypic characteristics but the exact disease mechanism and the reason for differences in clinical manifestation of the disease in patients are still unknown. However, these hiPSC-CM contain/provide the potential to answer these questions which have eluded research and clinician thus far.

Future Perspective

With the first successful cardiac differentiation of PSC [38], their use for regenerative medicine, drug testing and disease modeling became just a matter of time. The use of PSC-derived cardiomyocytes in cell therapy has unfortunately been fraught with difficulties and has had limited success so far [39]. However, their use in drug testing and disease modeling is a real possibility. Nevertheless there are still many questions that need to be answered to improve their use in drug testing and disease modeling.

In this thesis we have addressed some of these issues but there are still many which need to be investigated. One of the major obstacles to full use of PSC-CM is their immaturity [40;41]. While there have been many developments in improving differentiation procedures, most cardiomyocytes produced, like those generated in this thesis (Chapters 4-8), are still in an immature or fetal-like state based on electrophysiological - [42], physical and gene expression characteristics. While the immature cells have been able to mimic the phenotype of some late onset diseases (Chapter 6 of this thesis and [24] it is still unclear if the current state of maturity is enough to assay all phenotypes. If the field of PSC-CM research is to move forward, understanding of the immature state of PSC-CM and how to mature them in culture is key.

Additionally several reports to date have demonstrated a cardiac disease phenotype in culture [23;24;43] while disease phenotypes have not always been evident in patients[24;33]. This can be intrinsic to the technology or could indicate that patients will develop symptoms only in later life, or that there are lifestyle factors which trigger symptoms (extreme exercise for example). Future development in engineering technology that could modify stress/stretch, pacing or tissue environment combined with increased sensitivity of current assays, may resolve these issues. On the other hand it could be the lack of cellular maturity, mentioned earlier in this section, which has not allowed disease phenotypes to be fully revealed. Lastly it could be the absence of environmental factors, which cannot be replicated in culture that will remain a shortcoming of PSC technology. However which of these possibilities is correct will remain unanswered until the field progresses further and we have the tools that truly mimic all aspects of cardiomyocyte biology in culture.

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