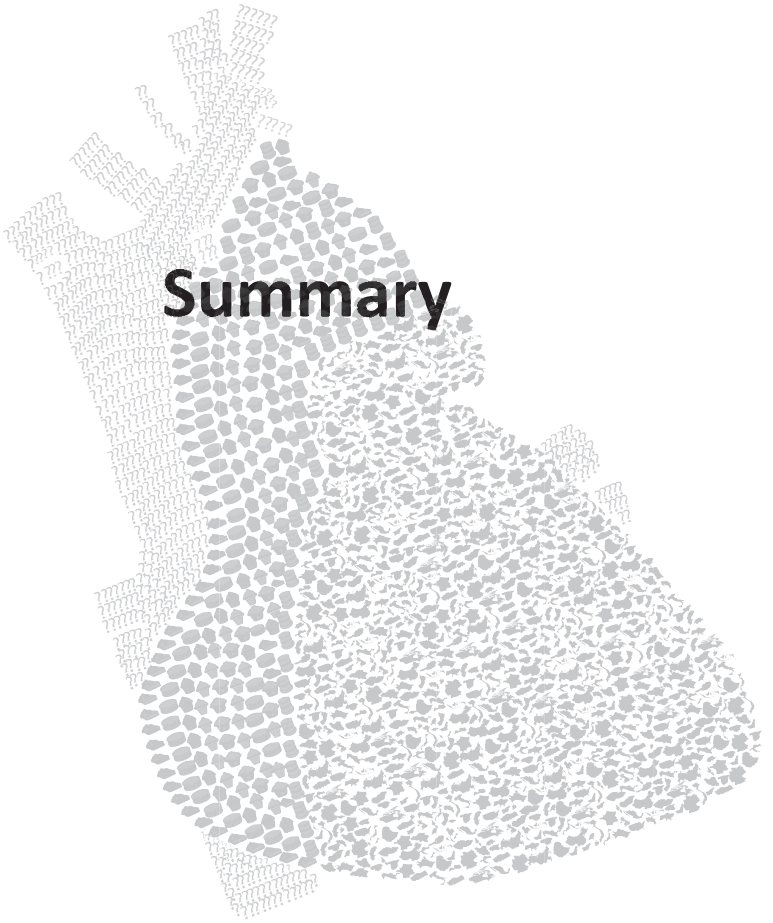
From teeth, skin, blood to heart : induced pluripotent stem cells as an in vitro model for cardiac disease

Dambrot, C.S.



Summary

Since the first reports of human induced pluripotent stem cells (hiPSC), the field of pluripotent stem cell (PSC) research has grown in leap and bounds, particularly in the area of (cardiac) disease modeling. This is in part because it is fairly easy to produce cardiomyocytes from hPSC and also there are now more assays available which can determine phenotypic behavior. This thesis describes and discusses improvements to reprogramming technology as well as its use in cardiac development and disease modeling. **Chapter 1 and 2** give a general background on PSCs, covering embryonic stem cells (ESC) and the discovery and development of induced pluripotent stem cells, with a particular focus on their use in studying cardiac development and disease modeling.

In **chapter 3** we examined some practical aspects relevant to the collection of tissues from patients in the clinic. We compared (i) skin biopsies stored in standard physiological salt solution for up to 2 weeks (ii) blood outgrowth endothelial cells (BOECs) isolated from fresh peripheral blood and (iii) children's milk teeth lost during normal replacement, for their ability to form somatic cell cultures suitable for reprogramming to hiPSCs. All hiPSC lines were derived using the same reprogramming method (a conditional (FLPe) polycistronic lentivirus) and under similar conditions (same batch of virus, fetal calf serum and feeder cells). Skin fibroblasts could be reprogrammed robustly even after long-term biopsy storage. Generation of hiPSCs from juvenile dental pulp cells gave similar high efficiencies but that of BOECs was lower. In terms of invasiveness of biopsy sampling, biopsy storage and reprogramming efficiencies, skin fibroblasts appeared best for the generation of hiPSCs but where non-invasive procedures are required (e.g. for children) dental pulp cells from milk teeth represent a valuable alternative.

In **chapter 4** we described a useful combination of procedures that enables the simultaneous screening of multiple iPSC clones to determine their genomic integrity as well as their cardiac differentiation potential within two weeks of the putative reprogrammed colonies initially appearing in culture. By coupling a splinkerette-PCR method with Ion Torrent sequencing, we could ascertain the number of and map the proviral integration sites in lentiviral-reprogrammed hiPSCs, enabling identification of clones containing single copy integrations in genomic regions devoid of known genes or transcriptional control elements. In parallel, we developed an effective cardiac differentiation protocol that generated functional cardiomyocytes within 10 days without requiring line-specific optimization for any of 6 independent human pluripotent stem cell lines tested. Finally, to demonstrate the scalability of these procedures, we picked 20 nascent iPSC clones and performed these independent assays concurrently. Before the clones required passaging, we were able to identify clones with a single integrated copy of the reprogramming vector and robust cardiac differentiation potential for further analysis.

Chapter 5 describes another cardiac differentiation method. Starting from mechanically passaging PSC grown on mouse embryonic fibroblasts, this chapter describes adapting PSC to enzymatic passaged bulk cultures in order to generation spin EBs for directed defined cardiac differentiation. This method is useful for the generation of large numbers of uniformly produced cardiomyocytes from a single cell line, as each line would most likely require optimization of the growth factors used (also described in this chapter).

In **chapter 6** we derived hiPSC from three hypertrophic cardiomyopathy (HCM) patients by reprogramming fibroblasts from skin biopsies; two related patients and an unrelated individual who also had the same Dutch founder mutation (2373insG) in the myosin binding protein C type 3 (MYBPC type 3) gene. MYBPC type 3 protein levels were significantly reduced by Western blotting in HCM-iPSC cardiomyocytes compared to the control, consistent with reduced MYBPC3 levels previously found in heart biopsies from patients. Dissociated and replated single HCM-iPSC derived cardiomyocytes had a cell surface area more than 2-fold greater than control cardiomyocytes. In addition, calcium signaling was altered in the disease cardiomyocytes compared with controls, consistent with previously published data. Cardiomyocytes from HCM-

iPSC thus show essential features of the disease and provide a basis for further studies on the nature of HCM caused by mutations in MYBPC3.

In **chapter 7** we examined the direct effects of serum on cardiomyocytes from hESC, hiPSC and hiPSC from patients with HCM linked to a mutation in the MYBPC type 3 gene to understand how they might affect interpretation of phenotypes in culture. We first confirmed previously published hypertrophic effects of serum on cultured neonatal rat cardiomyocytes evidenced by increased cell surface area and beating frequency. We then found that serum induced similar and poorly reversible increases in the cell surface area of hESC- and hiPSC-derived cardiomyocytes and significantly increased their rate of contraction. Phenylephrine, which normally induces hypertrophy in cultured cardiomyocytes, had no additional effects under these serum conditions. We further showed that hiPSC-derived cardiomyocytes from 3 MYBPC3 patients had a greater surface area than control cardiomyocytes in the absence of serum, indicating hypertrophy as predicted by their genotype, but this difference was not evident in the presence of serum. Serum can significantly alter the phenotype of human PSC derived cardiomyocytes under otherwise defined conditions such that the effects of drugs and gene mutations affecting hypertrophy may be underestimated. It is pertinent therefore to examine cardiac phenotypes, whenever possible, in fully defined culture media without or in low concentrations of serum.

In **chapter 8** we developed a universal selection system for PSC-CMs with high cell survival and low toxicity based on adeno-associated virus (AAV) vectors. The generated AAV serotype 2 (AAV2) vector had a reduced susceptibility to proteasomal degradation and carried a striated muscle-specific promoter (i.e. MHCK7)-driven puromycin resistance gene for selecting PSC-CMs. We showed that this vector transduced PSC-CMs better than fibroblasts. In initial experiments, puromycin selection resulted in ≥97%, pure cardiomyocyte populations but in later experiments it proved difficult to reproducibly replate the cardiomyocytes following puromycin treatment. Suggestions are given for further improvement of the vector and selection procedure, which could ultimately result in an efficient AAV vector-based PSC-CM selection method.

Finally **chapter 9** is a general discussion linking findings in the different chapters in this thesis as well as discussing future perspectives and obstacles which still limit some uses of PSC technology principally in regards to obtaining information on diseases from disease bearing hPSC lines e.g. immaturity of PSC-CM and disease phenotype in clinically asymptomatic patients.