PiPSC derived cardiomyocyte clusters growing on amniotic membrane stained for SMOOTH MUSCLE ACTIN and Nuclei

Chapter 4

GENERATION OF CARDIAC SHEETS WITH PiPSC DERIVED CARDIOMYOCYTES ON HUMAN AMNIOTIC MEMBRANE
**ABSTRACT**

This chapter describes detailed methods to derive robust cardiomyocytes from the PiPSCs and application of PiPSC derived cardiomyocytes in preparation of cardiac sheets on a novel scaffold the human amniotic membrane. It also details the specific methods such as fluorescence imaging, quantitative PCR analysis, calcium flux measurements and mitochondrial complexity determination used for evaluating the enhanced cardiomyogenesis of PiPSC derived cardiomyocytes on the human amniotic membrane.
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REVIEW OF LITERATURE

Somatic cells derived from iPSCs have been extensively applied in the field of tissue engineering and generation of 3 dimensional organoids (Yin et al., 2016) to model complex tissues, their related developmental processes and disease pathologies. iPSC derived cells, particularly cardiomyocytes and hepatocytes, have been used for toxicity testing as an alternative to existing approaches (Grimm et al., 2015; Scott et al., 2013).

In 2014 for the first time a patient received a transplant of iPSC-derived retinal pigmented epithelial cells in a Japanese clinical trial for age-related macular degeneration. This was the first clinical trial which aimed at evaluating the safety and efficacy of iPSC derived cellular therapy (Mandai et al., 2017). Immense research is also focused on deriving cardiac and nervous system cells from hiPSCs to treat diseases of heart such as myocardial infarction and nerve degeneration. These cells could provide more accurate toxicity assessment of new drugs, which could substantially reduce the cost and failure rate of drug development.

hiPSC derived cardiomyocytes (CMs) are gaining recognition for their role in human cardiac disease modeling such as long QT syndrome and Leopard syndrome (Carvajal-Vergara et al., 2010; Gatell et al., 2008; Itzhaki et al., 2011; Rocchetti et al., 2017; Sun et al., 2012), screening of drugs for potential cardio toxicity (Shinozawa et al., 2017; Zhao et al., 2017), cardio active drug discovery (Jung et al., 2012; Yazawa et al., 2011) and their potential clinical applications in repairing an injured myocardium. Cardiovascular tissue engineering has become an intense field of research exploring the combinations of hiPSCs as a source of patient specific cells coupled with diverse biological and synthetic scaffolds to generate engineered heart tissues. Of particular interest is the recent trend of repopulating de cellularized organs with cardiac progenitors to generate heart tissue (Lu et al., 2013). Decellularized animal and cadaveric human hearts have been used by researchers in a quest to create a functional heart worthy of heart transplantation (Guyette et al., 2016).

Patching the human amniotic membrane (hAM) has shown to improve ischemic heart repair in rat and mice models (Cargnoni et al., 2009; Lim et al., 2017; Roy et al., 2016). Recent medical case reports have shown that hAM patching showed anti-inflammatory effects and reduced new onset postoperative fibrillation in patients.
undergoing cardiac surgery (Khalpey et al., 2015; Marsh et al., 2017). The above research activities incited me to test the hAM as scaffold for cardiac tissue engineering. The ease in obtaining placentas and large surface areas of hAMs prepared from a single placenta further encouraged me to generate hAM cardiomyocyte (hAM CM) sheets.

I used the PiPSC derived cardiomyocytes and hAM to generate a functional, patient specific and a clinically relevant cardiomyocyte construct.

The hAM CM sheets were prepared by seeding PiPSC derived cardiac progenitor cells on the hAM surface. It is shown here that the seeded progenitor cells engraft, proliferate and differentiate in situ into functional cardiomyocytes. The hAM scaffold enhanced PiPSC derived cardiomyogenesis compared to the popular basement membrane matrix Matrigel™ suggesting its benefit and potential use in cardiac research.

RESULTS

Generation of contracting cardiomyocytes from PiPSC line

To endorse the translational value of this approach the PiPSC line was used as source of self renewing and individual specific cells for cardiomyocyte derivation. Mesenchymal hiPSC have been shown to differentiate into cardiomyocytes with better efficiency (Buccini et al., 2012; Streckfuss-Bomeke et al., 2013). An embryoid body intermediated differentiation of hiPSCs into cardiomyocytes was carried out (Zhang et al., 2009). Specification towards cardiac mesoderm and cardiomyocytes was given by activin / nodal and BMP signaling (Birket et al., 2015; Freund et al., 2008; Palpant et al., 2016; Yang et al., 2008).

Mesoderm induction of hiPSC was initiated with activin A, bone morphogenetic protein 4 (BMP4), fibroblast growth factor 2 (FGF2) and vascular endothelial growth factor (VEGF) in absence of insulin in embryoid bodies after which the cardiac progenitor spheres were collapsed on a surface of Matrigel™ to facilitate signaling cues for epithelial-to-mesenchymal transition (Zhang et al., 2012). As the protocol progressed past the progenitor cell stage, insulin was included to support cardiomyocyte development. Beating cardiomyocytes were observed by day 14 of differentiation. Cells progressively matured to definitive cardiomyocytes by day 25, at
which point they stained positive for the cardiac developmental marker smooth muscle actin, mature cardiomyocyte sarcomeric proteins cardiac troponin T, myosin ventricular heavy chain α/β, α actinin and gap junction protein connexin 43 (Figure 16A). The gene expression profile of these cardiomyocytes (Figure 16B) showed down regulation of pluripotency marker OCT4 and the epithelial to mesenchymal transition (EMT) signature of low E CADHERIN and high VIMENTIN. The cardiac transcriptional regulators GATA 4 and NKX 2-5 were up regulated as were the sarcomere related transcripts MYH6 (encoding α myosin heavy chain), MYH7 (encoding β myosin heavy chain) and MYL7 (encoding myosin light chain 2a). The hiPSC derived cardiomyocytes demonstrated spontaneous calcium (Ca2+) transients further confirming their functionality (Figure 16C). These phenotypic and functional data suggested that the hiPSC line derived cardiomyocytes were competent and suitable for application in cardiac cell sheet engineering.

The amniotic membrane as a novel scaffold for cardiac sheet engineering

Human term placenta were obtained under ethics review board approval with informed consent. Sterile hAM preparations were made by peeling the amniotic membrane from the placenta and removing the stromal fraction with mild trypsin treatment. The resulting membranes were thin, tough and translucent and used as scaffolds for cardiac sheet preparation.

Seeding of hAM with cardiac progenitors

To test whether hAM matrix can support engraftment and development of cardiomyocytes, amniotic membrane sheets (~ 1 cm²) were seeded with cardiac progenitor spheres as described above. The hAM CM sheets floated in culture medium throughout the process (Figure 17A). The phase contrast micrographs and histological sections revealed the spreading of the cells over the surface of the membrane during culture suggesting that hAM provided an adhesive substrate and mechanical signals for cell migration. (Figure 17B & 17C). hAM CM sheets could be maintained for 60 days in culture resulting in mature elongated and aligned cardiomyocytes traversing the surface of the membrane process (Figure 17D). Seeded cardiomyocytes appeared to demonstrate a stage specific maturity. Early cardiomyocytes (day 25) showed rounded immature phenotypes which progressed to mature cardiomyocytes (day 60) displaying elongated and striated phenotypes (Figure 17D).
Immunohistochemical analysis showed cells positive for the cardiac markers smooth muscle actin and cardiac troponin T throughout the clusters on the membrane suggesting the presence of a uniform cardiac population (Figure 17E). The cells engrafted firmly onto the surface of hAM as no detachment of the spheres was noted during the culture process which included flipping and flushing of the hAM CM sheets for over 60 days.

The hAM CM sheets were further characterized by immunocytochemistry to determine the cardiomyocyte identity of cells on the hAM matrix. The cardiomyocyte layers stained positive for sarcomeric proteins smooth muscle actin, cardiac troponin T, myosin ventricular heavy chain α/β and speckled connexin 43 distribution along the elongated cardiac cells (Figure 18).

The above described data proves that hAM supported engraftment, proliferation and development of induced pluripotent stem cell derived cardiomyocyte development.
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FIGURE 16
CARDIOMYOCYTE DIFFERENTIATION OF PiPSCs
A. A panel of immunofluorescent images of cardiac markers smooth muscle actin, cardiac troponin T, myosin ventricular heavy chain α/β, α-Actin and gap junction protein connexin 43 expressed by PiPSC derived cardiomyocytes cultured on Matrigel™ for 25 days. Scale bars represent 100 microns.
B. Semiquantitative PCR analysis of cardiomyocyte related gene expression by undifferentiated PiPSCs and PiPSC derived cardiomyocytes. B actin was used as the loading control.
C. Series of fluorescent images showing Calcium fluxes of PiPSC derived cardio myocytes on Matrigel™ over time. Scale bar represents 100 microns.
FIGURE 17
AMNIOTIC MEMBRANE CARDIOMYOCYTE SHEETS
A. Cardiac sheets on human amniotic membrane.
B. Phase contrast micrograph of the cardiomyocyte sheet.
C. Hematoxylin and eosin stained section of the cardiomyocyte sheet showing a layer of cells growing on the amniotic membrane.
D. Elongated and aligned cardiomyocytes traversing the surface of the amniotic membrane.
E. Immunohistochemistry of the sectioned cardiac sheet with cardiac specific markers Smooth Muscle Actin and Cardiac Troponin T. Scale bars represent 100 microns
FIGURE 18
PiPSC DERIVED CARDIOMYOCYTES ON THE AMNIOTIC MEMBRANE
A panel of cardiac specific markers expressed by PiPSC derived cardiomyocytes cultured on the human amniotic membrane.
Scale bars represent 100 microns
Amniotic membrane cardiomyocytes express higher levels of cardiac specific genes

To examine whether the hAM scaffold had an advantage over the popular BMM Matrigel™, the expression profiles of a genes ranging from pluripotency to mature cardiac markers on these two matrices were compared. The time points of day 25 and 55 were considered for the comparison. The pluripotency markers OCT4 and NANOG showed a markedly lower expression in hAM CMs compared to cardiomyocytes differentiated on Matrigel™ (Mat CM) indicating a smaller pool of undifferentiated cells in the hAM CMs populations. VIMENTIN was used as an indicator of EMT during the process of differentiation. During the early stages hAM CMs showed a higher VIMENTIN expression suggesting a better transition of these cells into the mesenchymal state. However at later stages Mat CMs over take in the expression of VIMENTIN. A possible reason for this may be emergence of fibroblast population in the Matrigel™ cultures as differentiation proceeds (Hewitt et al., 2011). hAM CMs expressed higher levels of cardiac transcription regulator NKX2-5 and the cardiac chamber protein NPPA. The same pattern was seen with the sarcomeric transcripts MYH6, MYH7, MYL2, MYL7 and TNNT2 (Figure19).
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FIGURE 19
GENE EXPRESSION ANALYSIS OF THE CARDIOMYOCYTES CULTURED ON AMNIOTIC MEMBRANE IN COMPARISON WITH CARDIOMYOCYTES CULTURED ON MATRIGEL™. The genes are categorized as pluripotency markers OCT4 & NANOG, mesenchymal marker VIMENTIN, cardiac gene expression regulators GATA4, NKX2-5 and chamber myocardial marker NPPA. Sarcomere related transcripts MYH6, MYH7, MYL7, MYL2 and TNNT.
Quantitative PCR analysis of hiPSC derived cardiomyocytes was performed by comparative CT method. The CT value of target genes as normalized to CT of reference gene Beta Actin to obtain ΔCT values. The ΔCT values were then normalized to that of Calibrator to obtain ΔΔCT. For pluripotency genes OCT4 & NANOG calibrator samples were placental mesenchymal cells. For all other genes undifferentiated hiPSCs were used as Calibrator samples. Finally the fold changes were calculated using the formula $2^{-\Delta\Delta CT}$. No significant differences were detected in the transcription pattern (Student’s t-test for paired measurements). Error bars ± s.e.m.; n.s. not significant.
hAM CMs exhibit morphological and physiological maturity compared to their counterparts on Matrigel™

Mature cardiomyocytes have an elongated morphology and organized sarcomeres, express higher levels of cardiac markers due to their larger cell volume, are bi or multinucleated, demonstrate faster intracellular calcium transients and have regularly distributed mitochondria that occupy a larger volume of the cell compared to immature cardiomyocytes (Bedada et al., 2016; Robertson et al., 2013; Veerman et al., 2015; Yang et al., 2014). In addition, adult cardiomyocytes in the heart are longitudinally aligned, enabling fast electrical conduction and synchronous muscle contraction. However, fetal cardiomyocytes and monolayers of hPSC derived CMs are chaotically organized. Relative maturity of AM CMs compared to Mat CMs of equal age was shown by studying three parameters – cell shape and organization in culture, spontaneous intracellular calcium flux and the complexity of mitochondrial arrangements. Figure 20A shows representative images of cultures on the two ECMs used in the study. Note the chaotic distribution and mononucleation of cells on Matrigel™. The cells on the amniotic membrane form elongated, bi nucleated aligned cells. The aligned arrangements are also clear in Fluo-4 AM loaded cells (Figure 20B).

The intracellular Ca^{2+} handling properties of cardiomyocytes generated on hAM and Matrigel™ were compared. Loading the CMs with Ca^{2+} indicator Fluo-4 AM demonstrated that both hAM CMs and Mat CMs have large intracellular Ca^{2+} stores which is a hallmark of cardiomyocytes (Figure 20B). Upon comparison of Ca^{2+} handling of hAM CMs with Mat CMs under identical experimental conditions we observed faster calcium oscillations with higher peaks by hAM CMs compared to that of Mat CMs (Figure 20C & D). A higher expression of the calcium handling protein transcripts of Calsequestrin (CASQ2) (Calcium binding protein of Sarcoplasmic reticulum) and sarcoendoplasmic reticulum Ca^{2+} ATPase (ATP2A2) was also seen in hAM CMs compared to Mat CMs. Phospholamban (PLN), an endogenous inhibitor of SERCA showed a lower expression in hAM CMs (Figure 20 E).
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FIGURE 20
MORPHOLOGICAL AND CALCIUM HANDLING MATURITY OF PiPSC DERIVED CARDIOMYOCYTES CULTURED ON AMNIOTIC MEMBRANE AND MATRIGEL™
A. The culture morphology of Matrigel™ CMs and amniotic membrane CMs. Note the chaotic versus aligned arrangements and nucleation of CMs on the 2 matrices shown by arrow heads.
B. Fluo4 AM loading of cardiomyocytes differentiated on Matrigel™ (upper) and amniotic membrane (lower). Note the vertically aligned organisation of cardiomyocytes on the amniotic membrane.
C. The regions of interest selected to analyse the Calcium fluxes.
D. The Calcium oscillations of Matrigel™ CMs and amniotic membrane CMs.
E. The gene expression patterns of Calcium handling genes CSQ, SERCA and PLN in Matrigel™ CMs and amniotic membrane CMs.
Scale bars represent 100 microns
There are three distinct populations of mitochondria in adult cardiomyocytes—perinuclear mitochondria, subsarcolemmal mitochondria, and inter fibrillar mitochondria (Ong and Hausenloy, 2010). The inter fibrillar mitochondria are present in a regular crystal like lattice arrangement. To study the mitochondrial distribution in the cardiomyocytes a live staining of the hAM CMs and Mat CMS with the mitochondrial dye MitoTracker® Red CMXRos was performed. A crystal like lattice arrangement of mitochondria was observed in hAM CMs whereas the Mat CMs had perinuclear distribution of mitochondria (Figure 21A). To demonstrate the inter fibrillar arrangement of these mitochondria the cells were stained with phalloidin post live staining with MitoTracker® Red CMXRos. The hAM CMs showed mitochondria distributed along filamentous actin fibers (Figure 21B). The mitochondrial density was more in hAM CMs and occupied a larger cytoplasmic volume. To better demonstrate the cytoplasmic volume and density of the mitochondria a Sobel filter which enhances the primary edges of image lines was applied to the fluorescence images (Figure 21C). To confirm the higher density of mitochondria a flow cytometry analysis was performed after staining cells with the mitochondrial dye. MitoTracker® Red CMXRos versus a side scatter plot revealed a higher population of cells with more mitochondrial complexity (Figure 21D).
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FIGURE 21
MITOCHONDRIAL COMPLEXITY OF CARDIOMYOCYTES CULTURED ON THE AMNIOTIC MEMBRANE

A. The difference in the mitochondrial distribution of Matrigel™ CMs and amniotic membrane CMs. The distribution is perinuclear in Matrigel™ CMs and crystal lattice like in the amniotic membrane CMs as shown in the boxes.
B. The presence of interfibrillar mitochondria in the amniotic membrane CMs which are not seen in the Matrigel™ CMs.
C. A comparison of the mitochondrial densities of Matrigel™ CMs and amniotic membrane CMs. The visibility of the mitochondria has been enhanced by applying the Sobel filter to the images.
D. Flow cytometry analysis of cardiomyocytes stained with Mitotracker Red CMX Ros. Note the higher side scatter of the amniotic membrane CMs compared to Matrigel™ CMs as expected for higher mitochondrial densities.
Scale bars represent 100 microns.
DISCUSSION

My data shows that hAM supports the development of PiPSC derived cardiomyocytes. The morphological maturation, cardiac related gene transcript expression, functionality in terms of spontaneous calcium oscillations and mitochondrial distribution in hAM CMs appear to be better than development of cardiomyocytes on the routinely used BMM Matrigel™. This finding may be credited to the natural components of hAM and the reduced stiffness of this hAM compared to standard culture conditions provided by Matrigel™ coating of solid surfaces of culture dishes. Also the hAM CM sheets float throughout the culture process. This float property is of value in translation as it makes the picking of the sheets uncomplicated compared to other protocols where an enzymatic or temperature responsive detachment step is required to pick up the sheet from the culture vessel (Masumoto et al., 2015, 2012). Previously it has been reported that reduced stiffness of the substrate enhances maturity of cardiomyocytes (Feaster et al., 2015). The float property also provides a cushioning effect to the cells which could have contributed to the results of our study. However this needs added validation with detailed experiments.

One of the main limitations in cardiac regeneration from pluripotent stem cells the lack of an experimental method that could generate cardiomyocytes with maturity close to adult cardiac cells. Here I have presented a method with a novel scaffold that can generate a relatively mature pool of cardiomyocytes for human heart research. The above described method with refinements is capable of fabricating a cardiac sheets which can be used for transplantation in human patients. These engineered cardiac sheets with patterned cardiomyocytes could be used to study cardiac development, drug toxicity screening and discovery. Most importantly they can be patched on a defective region of a human heart due to the immune privileged and biocompatible nature of hAM. Such a pipeline would be suitable for approaches in personalized medicine. However this requires an intricate and vigilant analysis with animal studies.

A limitation of the hAM CMs presented here, is their inability to spontaneously contract. This could be theoretically explained with regards to inhibition caused by bulk of the membrane and absence of application of external mechanical, electrical or
chemical stimulus which could initiate a contraction provided in other studies (Guyette et al., 2016). Lack of transplantation experiments does not allow us to understand if these cells can integrate and functionally couple with the existing heart parenchyma without causing arrhythmias.

Despite these limitations, the hAM CM sheets presented here along with related data may be an initial step toward fabricating patient-specific cardiac therapy.