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Human Induced Pluripotent Stem Cell-derived Beating Cardiac Tissues on Paper

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

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There is growing interest in using paper as a biomaterial scaffold for cell-based applications. In this study, we made the first attempt to fabricate a paper-based array for the culture, proliferation, and direct differentiation of human induced pluripotent stem cells (hiPSCs) into functional beating cardiac tissues and create "beating heart on paper." This array was simply constructed by binding a cured multi-well polydimethylsiloxane (PDMS) mold with common, commercially available paper substrates. Three types of paper material (print paper, chromatography paper and nitrocellulose membrane) were tested for adhesion, proliferation and differentiation of human-derived iPSCs. We found that hiPSCs grew well on these paper substrates, presenting a three-dimensional (3D)-like morphology with pluripotent property. The direct differentiation of human iPSCs into functional cardiac tissues on paper was also achieved using our modified differentiation approach. The cardiac tissue retained functional activities on the coated print paper and chromatography paper with a beating frequency of 40–70 beats/min for up to three months. Interestingly, the human iPSCs could be differentiated into retinal pigment epithelium on nitrocellulose membrane under conditions of cardiac-specific induction, indicating the potential roles of material properties and mechanical cues involved in regulating stem cell differentiation. Taken together, these results suggest that different grades of paper could offer great opportunities as bioactive, low-cost, and 3D in-vitro platforms for stem cell-based high-throughput drug testing at the tissue/organ level and for tissue engineering applications.

1. Introduction

Human induced pluripotent stem (iPS) cells are unique pools of cells that have shown great promise for embryonic development, regenerative medicine, and drug research. [1-3] These cells have similar desirable properties to embryonic stem cells due to their inherent ability to propagate infinitely while maintaining the potential to differentiate into multiple types of cell lineage and offer new opportunities and perspectives for personalized medicine. [4,5] At present, several differentiation protocols using two-dimensional cell culture methodologies have been developed for a variety of key cell types, such as the in-vitro differentiation of cardiomyocytes, neural stem cells, or osteoblasts from iPS cells; [6-8] however, challenges still remain for the achievement of higher differentiation efficiencies and the representation of native tissue architecture or extracellular microenvironments with specific cellular phenotypes across the developmental spectrum.

Recently, significant efforts have been made in the development of microengineering techniques, such as photolithography, replica molding and microcontact printing, for fabricating biocompatible materials to create 3D culture microenvironments with a functional phenotype and improve the regenerative potential of

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Electronic Supplementary Information (ESI) available: Supporting video1, 2,3,4, Fig. S1, S2. See DOI: 10.1039/x0xx00000x

stem cells. [9-11] However, the polymers tailored to each technique are very limited and often require complicated fabrication processes and expensive instruments, impeding the manufacture and mass production of the scaffolds. [12-14]

Paper is a flexible and porous material that offers an interesting alternative to the conventional substrates, such as polystyrene, glass and PDMS, commonly used in cell studies. It is mostly composed of a bundle of cellulose microfibers and inherently forms a porous 3D architecture, providing a diversity of properties in surface topography, internal microstructure and mechanical features. Moreover, paper produced from natural sources is biocompatible and can be supplied in large quantities at low cost using well-established fabrication processes. Hence, paper may offer an alternative cell culture platform and scaffold for cell-based applications. Over the past few years, several studies have highlighted the feasibility of paper materials as a substrate for cell culture. [15-18] Mosadegh et al.[19] reported the construction of a tissue-like structure using multilayer stacking papers seeded with rat cardiomyocytes to mimic cardiac ischemia *in vitro*. Park et al. [20] reported the use of a paper scaffold produced by chemical modification for implantable bone tissue engineering using human adipose-derived stem cells. Mosadegh et al. [21] presented a paper-based invasion assay to study the chemotaxis of cancer cells in gradients of oxygen. Human derived iPS cells are known to be very sensitive to biophysical and biochemical cues within extracellular microenvironments supported by various scaffold materials; however, no study has yet explored the growth and differentiation capabilities of human-derived iPSCs on paper materials.

In this study, we developed a straightforward approach to fabricate a paper-based array for the culture, proliferation and direct differentiation of human iPS cells into beating cardiac tissues for the first time. The paper array was simply constructed by binding a cured multi-well PDMS mold with common paper substrates using pre-cured PDMS glue. Three types of commercially available paper material (print paper, chromatography/filter paper, and nitrocellulose membrane) were tested for cell growth, proliferation and differentiation of human iPSCs. The different paper materials were characterized using scanning electron microscopy (SEM) images. The differentiation capability of human iPS cells into cardiac tissues on paper substrates was also studied.

2. Materials and methods

2.1 Fabrication of paper-based array

The paper-based array was prepared from three types of commercial paper materials, including print paper, chromatography/fliter paper, and nitrocellulose membrane, that are commonly available in the laboratory. Initially, the Polydimethylsiloxane (PDMS ordered from Sylgard 184, Dow Corning, USA,) block with structures was initially produced and replicated based on polymethylmethacrylate (PMMA) or SU8 molds with multi-well configurations (PDMS base and curing agent mixed thoroughly at ratio of 13:1 by mass). The pre-polymer PDMS was used as a glue to bind the PDMS layer with the paper substrate. The permeability of the PDMS glue into the paper decreased by spin-coating a thin layer of pre-polymer PDMS onto a clean glass and pre-curing it at room temperature. This allows to maintain an adequate blank space on the paper array for cell culture. The PDMS block with multi-well configuration was dipped in pre-cured PDMS glue and bound with the paper substrate to produce a hybrid paper device with micro-well configuration. After curing at 80°C for 20–30 min, the paper array was sterilized with ultraviolet radiation for 30–60 min followed by cell culturing.

2.2 Characterization of paper materials

The architectural properties of the different grades of paper were characterized by SEM. Prior to this assay, all samples were dehydrated using a graded series of ethanol (25%, 50%, 75%, and 100% ethanol for 5 min per concentration). The samples were air dried on a clean bench for 1 h. After sputter-coating with gold (~10nm), the surface morphology of all samples was monitored by SEM (Hitachi, S4800).

2.3 Human iPSC culture and identification on paper

The human induced iPSCs were kindly provided by professor Ning Sun (Fudan university, China). In order to culture the hiPSCs on the paper array, all of the paper substrates were pre-coated with gelatin and Matrigel to facilitate the cell proliferation and differentiation. Briefly, 0.1% sterile gelatin in water was added into the paper arrays at room temperature and removed after 1 h. The paper arrays coated with gelatin were air-dried on a clean bench for 1 h and placed in a refrigerator. After cooling, the gelatin-coated paper arrays were then coated with Matrigel (diluted 1:40, BD Bioscience) overnight at 4 °C. And after incubation at 37 °C for 1 h to solidify the Matrigel, the paper arrays were ready for use. The human iPSCs were prepared and characterized as described previously.[22] The human iPS cells were cultured on Matrigel-coated dishes in mTeSR1 medium until fully confluent, and then subcultured on the paper arrays to test their proliferation ability using hematoxylin & eosin (H&E) staining.

Immunofluorescence and immunocytochemistry staining were used to identify the pluripotent properties of the human iPSCs. Briefly, the cells on the paper were fixed with 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) for 15 min at room temperature. After permeabilizing cells with 0.1% (v/v) Triton-100 (Sigma) in PBS for 10 min at room temperature, the samples were washed with PBS for three times. To block non-specific binding of the antibodies, the samples were incubated with normal goat serum (Beyotime Company, China) at room temperature for 1 h. The human iPS cells were then incubated with primary antibodies, SOX2 and OCT4 (diluted 1:300, Cell Signaling Technology (CST)) at 4°C overnight. Alexa 594-conjugated and horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibodies (diluted 1:100, Beyotime Company, China) in PBS were added to the cells for 1 h at room temperature, after which the samples were washed with PBS for three times with each time for 5 min. For immunofluorescence staining, the cell nuclei were counterstained with 40, 6-diamidino-2-phenylindole (DAPI, Sigma) for 5 min at room temperature. For immunocytochemistry staining, HRP-conjugated secondary antibody was used and diaminobenzidine (DAB) served as the chromophore substrate. The samples were photographed under a fluorescence microscope or photomicrograph (Olympus). Cells cultured on a conventional culture plate were used as a positive control.

2.4 Human iPSC-derived cardiomyocytes cultured on paper

To check whether the hiPSCs derived cardiomyocytes can grow on the paper substrates, we initially differentiated the human iPSCs into cardiomyocytes according to a protocol reported previously.[23] Briefly, human iPSCs were seeded on the six well culture plate pre-coated with Matrigel (1:60 diluted) for 4-5 days in mTeSR1 medium. When the stem cell clusters reached 90% confluent, the human iPSCs were induced by adding RPMI1640/B27-insulin (Life Technologies) medium with 6 μM GsK3 inhibitor CHIR99021 (Selleckchem) for 24 h. It was named as the first day of differentiation. On day 2, the cells were treated with RPMI1640/B27-insulin for two days; on day 4, the medium was replaced with RPMI1640/B27-insulin containing 5 μM IWR1 (Stemgent) for two days; on day 6, the medium was replaced with RPMI1640/B27-insulin. The medium was changed to RPMI1640/B27 supplement on day 7. After day 7, the medium was changed every two days and maintained in RPMI1640/B27 supplement.

The cardiomyocytes derived from human iPS cells were then seeded on the paper arrays and their growth and function on different paper substrates were tested. After growing for 3 days, the cells on the paper were identified by a specific cardiac marker, cardiac Troponin T (cTnT, mouse antibody against human cTnT, diluted at 1:150; Thermo, USA) using an immunofluorescence assay. The procedure of immunofluorescence assay was similar to that described for the identification of human iPSCs. Cardiomyocytes cultured on a plate well were used as a positive control.

2.5 Direct differentiation of human iPSCs into cardiac tissues on paper

To test the direct differentiation capability of human iPSCs into a cardiac lineage on paper, we modified a previously reported protocol[23] and performed the experiment as follows. Briefly, we seeded human iPSCs on the paper arrays at 1 ×10^4 cells/mm^2 for 2 h with mTeSR1 containing 5 μM ROCK inhibitor (Y27632, Selleckchem) and then initiated the induction of cardiomyocytes directly. After

cell seeding for 2 h, the human iPSCs were directly induced by adding RPMI1640/B27-insulin (Life Technologies) medium containing 6 μM CHIR99021 for 24 h. On day 2, the medium was replaced with RPMI1640/B27-insulin for 2 days; on day 4, the medium was replaced with RPMI1640/B27- insulin and 5 μM IWR1 for 2 days; on day 6, the medium was changed with RPMI1640/B27-insulin; and on day 7, the medium was replaced with RPMI1640/B27 supplement. After day 7, the medium was changed every 2 days. The cells were dyed with a live cell membrane dye (PKH26, Sigma) to visualize the cardiac tissues using fluorescence microscopy. The beating cardiac tissues could be observed within 7–14 days.

3. Results and discussions

3.1 Preparation and characterization of paper array

To characterize the paper materials from different resources (print paper, chromatography/filter paper, and nitrocellulose membrane), we evaluated their properties using SEM measurements as shown in Figure 2A. The nitrocellulose membrane exhibited a spongy structure with pore diameters of 0.4–0.5 μm. The structure of the print paper was knitted by a bundle of porous microfibers, and was similar to that of filter paper. The fiber arrangement appeared to be more compact in print paper than in filter paper.

The paper-based array prepared from three types of paper was used for the culture and differentiation of human iPSCs into cardiac-specific tissue. The schematic procedure used to fabricate this paper array is shown in Figure 1. We initially prepared a PMMA or SU-8 mold fabricated with a multiple-post structure. A layer of PDMS block with multi-well configuration was then replicated from the PMMA/SU-8 mold followed by binding with different paper substrates to produce the paper-based array. We used pre-cured PDMS as glue to bind the PDMS block with paper materials. To produce the shaped culture zone on paper during the binding process, a thin layer of pre-polymer PDMS was spin-coated onto a clean glass and pre-cured at room temperature for 30 min to increase the viscosity and decrease the permeability of the PDMS glue into the paper, as shown in Figures 2B and 2C. The optimized experimental conditions not only facilitated a tight binding between the PDMS layer and the paper, but also allowed sufficient space for cell culture on the paper array. In contrast to the conventional paper patterning methods, such as wax printing or patterning instruments, the proposed method is very simple and well suited for cell-based high-throughput assay on localized paper materials.

3.2 Culture and identification of human iPSCs on the paper array

To improve the stability and control the surface properties of the paper substrates for stem cell culture, bare papers were simply pre-coated with a thin layer of gelatin and Matrigel. To test the cell proliferation ability on different papers, the human iPSCs at a density of 3 $\times 10^4/cm^2$ were seeded and cultured on the modified paper and stained on the day 1, 3 and 5 after culturing. Using H&E staining, the clones of human iPSCs gradually became larger with increasing culture time on the three types of paper grade (Figure 3A). In addition, the number of human iPSCs on the nitrocellulose membrane and print paper was greater than that on filter paper. Because the pore diameter of the filter paper we used was 15–25 μm, which is bigger than the size of the human iPSCs (5–10 μm diameter), we assume that the smaller number of cells on the filter paper might be due to its large pore size allowing some cells to filter out from the substrate.

To check the pluripotency of hiPSCs when cultured on paper, we identified the cells properties with stemness markers (SOX2 and OCT4) using immunofluorescence and immunocytochemistry staining, respectively. The cells with positive expression of SOX2 and OCT4 in plate culturewere used as a control group (supplementary 1A, B). As shown in Figure 3B, most of the cells grew well and exhibited strong expression of SOX2 and OCT4 on the three types of paper material on day 3, but the pluripotency of human iPSCs was gradually lost on day 5 (data not shown). We further characterized the morphology of the seeded cells on paper using SEM imaging. From the SEM images (Figure 3B, bottom), we observed that the cells on the nitrocellulose membrane were spread loosely with no tight cell-cell junction after 3 days of culture, while those on the print paper and filter

paper exhibited close cell-cell contact, forming a tight cell sheet. These results indicated that the structure of the paper materials with inherent properties may affect the self-renewal ability of human iPSCs and facilitate the differentiation of stem cells. These findings also inspired us to further investigate the differentiation capability of human iPSCs via cells proliferation on paper substrates in the following study.

3.3 Characterization of human iPSC-derived cardiomyocytes on paper

Human iPSCs are known to be able to differentiate into a variety of cell types in different tissues, including cardiomyocytes, neural stem cells, and osteoblasts. Cardiomyocytes derived from human iPSCs are an essential cell resource for drug toxicity testing in various cardiac-specific diseases. We initially evaluated the growth ability of cardiomyocytes differentiated from human iPSCs on different paper materials. The human iPSCs were differentiated into cardiomyocytes according to the protocol reported previously, [22] and the cardiomyocytes were identified by a cardiac-specific marker, cTnT, using immunofluorescence staining. As shown in Figure 4A, cardiomyocytes derived from human iPSC exhibited good growth with strong expression of cTnT marker on the three types of paper substrates. However, the cardiomyocytes exhibited a diversity of morphological characteristics on different papers materials. It is noted that the cells grew and spread loosely on the nitrocellulose membrane, similar to those cultured on plastic plates (supplementary 1C), while the cardiomyocytes were prone to form aggregates and exhibited spheroid-like feature on printed paper and filter paper after three days of culture. Cell features were further characterized on paper using SEM imaging (Figure 4B). The cells exhibited similar morphological features with those findings from immunofluorescence staining. In particular, most of the cardiomyocytes tended to form clusters in 3D structures surrounded by fibers on the print paper and filter paper.

In addition, the cardiomyocytes on print paper and filter paper demonstrated spontaneous beating functions in fluorescence microscopy (supplementary video 1), but not those on nitrocellulose membrane. It appears that the microstructures shaped by fibers on the printed paper or filter paper are beneficial for maintaining the 3D structure of cardiomyocytes and supporting active beating functions of cardiac tissues. Paper material is mostly composed of cellulose microfibers with a porous 3D architecture. In this work, we pre-coated the paper with Matrigel, which provides a native cellular microenvironment containing extracellular matrix and 3D architecture features. These features might determine the cell morphology and maintain the tissue-specific differentiation function of stem cells, thus providing an alternative and low cost 3D scaffold to support human iPSCs in high-throughput drug testing.

3.4 Direct differentiation strategy of human iPSCs into beating cardiac tissue

As described above, both human iPSCs and human iPSC-derived cardiomyocytes grew well on the three types of paper, but it was unclear whether the human iPSCs could be differentiated into cardiomyocytes directly on paper. We previously demonstrated that human iPSCs were prone to lose their pluripotency on paper after long-time culture (5 days), indicating the potential of these paper materials to facilitate differentiation of stem cells. Conventionally, the cardiac differentiation of iPS cells requires the formation of embryoid body (EB) or cell proliferation for 4-5 days in 2D dish culture. [22, 23, 24] We therefore modified the cardiac differentiation procedures by omitting the step of cell proliferation and further explore the feasibility to differentiate hiPSCs into cardiac tissue directly on paper. We named it as direct induction strategy (DIS) as shown in Figure 5.

According to this new strategy, the human iPSCs could be differentiated into cardiomyocytes directly on the three types of papers coated with Matrigel (Figure 6). The differentiated cells were round in shape and strongly expressed the cardiac-specific marker cTnT on printed paper and filter paper, but only expressed cTnT very weakly on the nitrocellulose membrane. However, we observed cardiac tissues with a strong beating function on print paper 7–14 days after differentiation using live cell staining. The beating frequency of this cardiac tissue was about 40–70 beats/min, similar to that observed on dish culture, suggesting the feasibility of this paper material to support the direct differentiation of cardiac tissue

(supplementary video 2). Although we did not observe strong beating cardiac tissue on the filter paper, we thought this might be due to the low cell density and large pore size on this substrate (cells filtered out). Myers et al.[25] previously reported that the differentiation of human iPSCs into cardiomyocytes was typically initiated on the second day after the single cells were seeded at a high density. Combining this information with our findings, we think that higher cell density and sparse human iPSCs colonies are essential factors in promoting the loss of pluripotency and spontaneous differentiation in stem cells. In addition, from the SEM images in Figure 6C, the differentiated cardiomyocytes exhibited cluster shape similar to that on print paper suggesting the inherent knitted microstructure with porous fibers was beneficial for differentiating human iPSCs into cardiac-specific tissue. Different from conventional approaches, this direct induction strategy doesn't require initial formation of embryoid body or cell proliferation in 2D culture as reported previously, [23, 26] thus simplifying the differentiation procedures and saving much time. In addition, the paper scaffold coated with Matrigel allows to create a physiologically relevant cell microenvironment consisting of native extracellular matrix and 3D architecture with internal structural and mechanical properties. This can not only facilitate the maintaining of tissue-specific cell phenotype, but also the tissue-specific differentiation of stem cells. Moreover, as higher cell density is essential factors to promote the spontaneous differentiation of iPS cells, the pre-coated paper array allows to maintain the parallel 3D cell culture at high density in compartmented region, facilitating the differentiation of human iPSCs into cardiac lineage and further applications in drug testing.

It was noted that the cardiac tissues were able to maintain long-term spontaneous beating activity for up to 3 months on paper (supplementary video 3). After that, the cardiac tissues differentiated from human iPSCs on paper were digested with collagenase and subcultured on plastic plates to demonstrate cardiomyocytes activity. The subcultured cells also maintained good activity and stable contractile frequency on plastic plates (supplementary video 4).

Interestingly, it was found that the cells on the nitrocellulose membrane could be differentiated into a retinal pigment epithelium (RPE)-like lineage after 2 months using our cardiac induction method (supplementary 2). The pigmented clusters on the membrane could be visualized with the naked eye. Both light microscopy and SEM images revealed the typical hexagonal cell shape relevant to RPE-like cells (supplementary 2A-D) and the SEM images showed the presence of characteristic microvilli at the apical of the RPE. We also observed that the microvilli of the primary RPE were thicker and shorter than those of mature RPE, the morphology of which was more like "corals" (supplementary 2E and 2F).[27] The findings suggested that these RPE-like cells were possibly in a state of immaturity because most microvilli of the immature RPE were dimly visible under the cellular membrane. However, this phenomenon rarely occurred on the two other types of paper during the cardiac differentiation process in human iPSCs. These new findings indicated that the microstructure of the nitrocellulose membrane was favorable for differentiation of hiPSCs into the RPE lineage. This also hinted the possible mechanical and structural cues of materials in regulating stem cell differentiation. Further work is undergoing to elucidate the detailed mechanism behind this membrane-supported differentiation of human iPSCs into RPE.

Conclusions

In this study, we presented a straightforward approach to fabricate a paper-based array for using in the culture, proliferation and direct differentiation of human iPSCs into cardiac tissue for the first time. The human iPSC derived cardiac tissues were able to maintain long-term spontaneous beating activity and stable contractile frequency on print paper for over three months. The established approach was very simple with low cost, enabled the functional representation of human cardiac tissue in conjunction with biological readouts and was compatible with high-throughput analysis. In particular, it was suitable for relevant human cell types and could facilitate the engineering of human iPSC-derived organs on paper. By integrating patient-specific iPSCs with other electrical sensor elements, the established paper-based array could serve as an in-vitro system relevant for humans and provide great opportunities for developing

further flexible, low-cost, and 3D high-throughput platforms that could predict drug cardiac toxicity and efficacy more accurately.

Acknowledgements

This research was supported by International Science & Technology Cooperation Program of China (2015DFA00740), Key Laboratory of Separation Science for Analytical Chemistry (Dalian Institute of Chemical Physics, Chinese Academy of Sciences), National Nature Science Foundation of China (Nos. 81273483, 81201689), Special Fund for Agro-scientific Research in the Public Interest (No. 201303045).

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Figure and figure legends

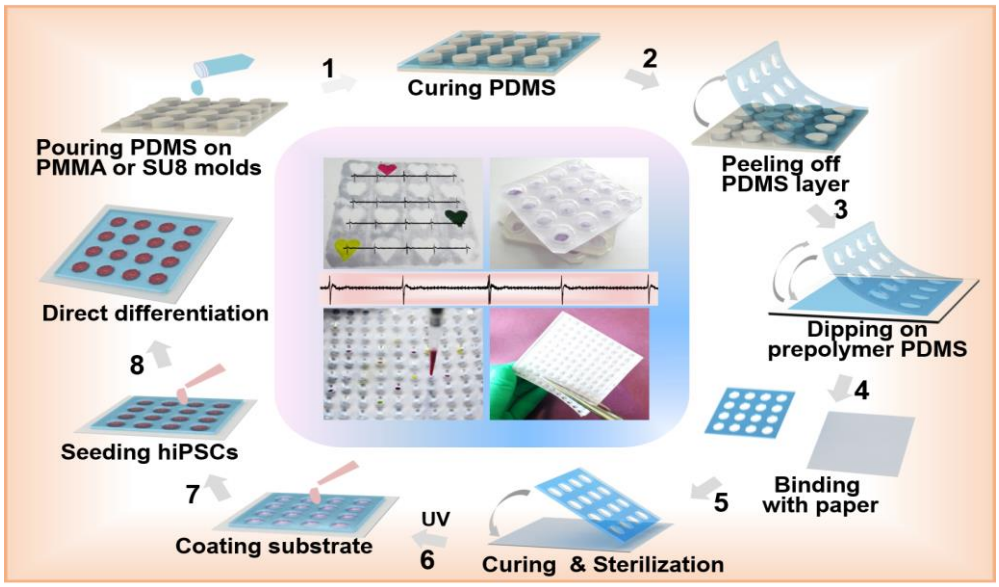

Figure 1: The procedures for fabricating a paper-based array

Schematic diagram of the procedure for fabricating a paper-based array. The central panel shows photographs of the multi-well paper array.

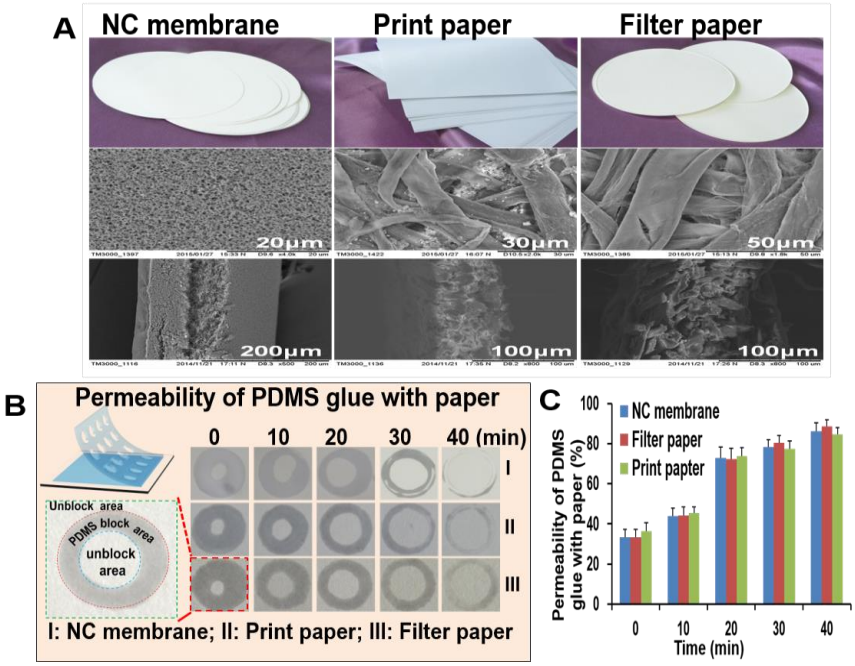

Figure 2: Characterization of the three types of paper materials used for cell culture. A: SEM images of the microarchitecture of the three types of paper –nitrocellulose membrane, print paper and chromagraphy/filter paper. B and C: Optimizing the permeability of PDMS glue binding with paper substrate. The area ratio indicates the ratio of unblocked area in the center of the paper to the original circle area in the PDMS layer. A higher ratio indicates a larger unblocked area.

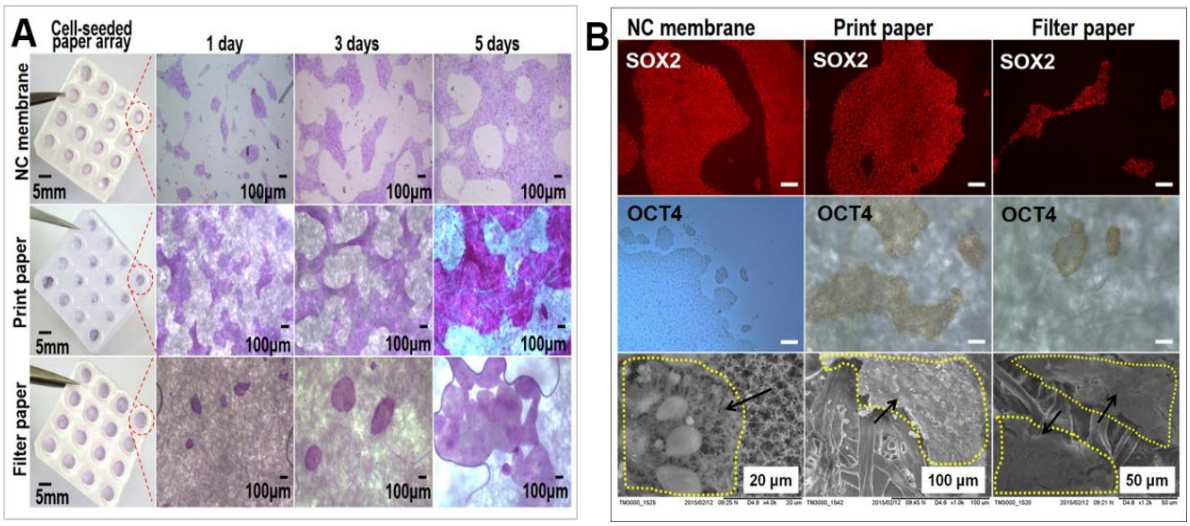

Figure 3: Proliferation and identification of human iPSCs on the different papers. A: The human iPSCs cultured on pre-coated three kinds of paper for different lengths of time in mTeSR1 medium. The human iPSCs were seeded at the same initial cell density of 3 × 104/cm2. The cells were stained with H&E. B: Top: staining of a pluripotency marker (SOX2) in immunofluorescence assays; middle: staining of a pluripotency marker (OCT4) in immunocytochemistry; bottom: SEM images of human iPSCs on paper. The regions surrounded by a yellow dotted line indicate human iPSCs. Scale bar: 100 μm.

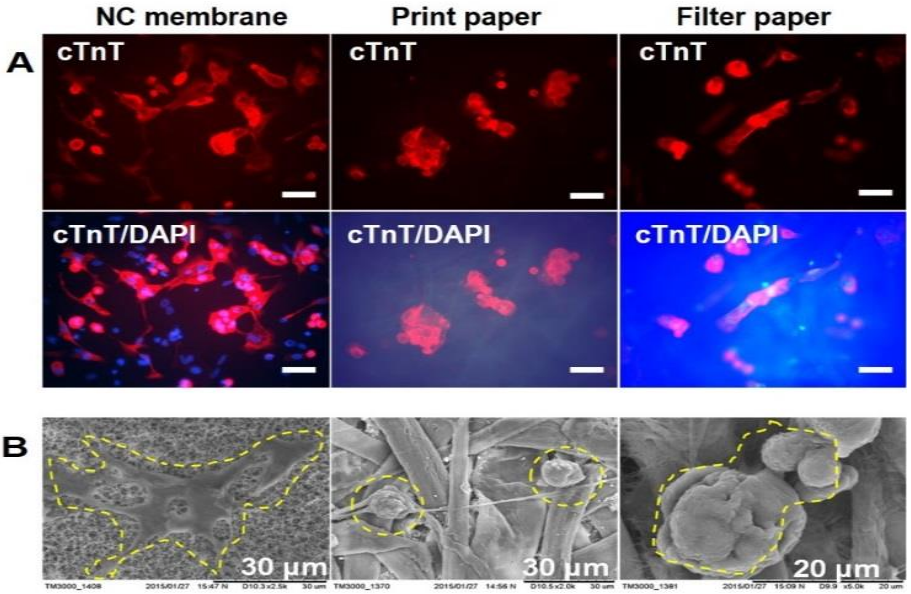

Figure 4: Identification of cardiomyocytes derived from human iPSCs on paper. A: Identification of cardiomyocytes on three kinds of paper substrate with cTnT antibody after 3 days' culture; bottom images: cTnT staining merged with DAPI staining. B: SEM images of cardiomyocytes on different paper substrates. The regions surrounded by a yellow dotted line indicate the cardiomyocytes. Scale bar: 50 μm.

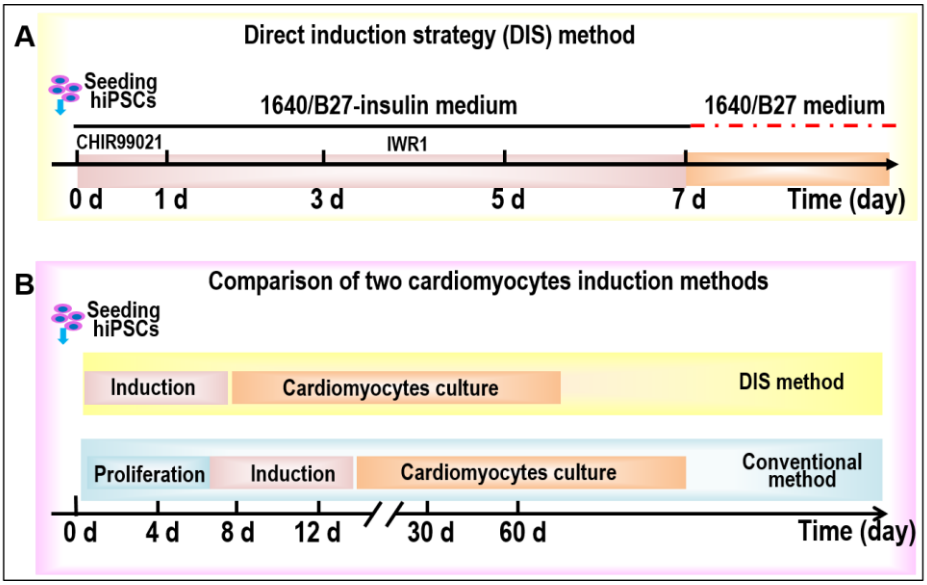

Figure 5: Direct Induction Strategy (DIS) in differentiating human iPSCs into cardiomyocytes on paper. A. The detailed process of cardiomyocytes differentiated from human iPSCs using DIS. B. Comparison of DIS and conventional approach in the differentiation of cardiomyocytes from hiPSCs.

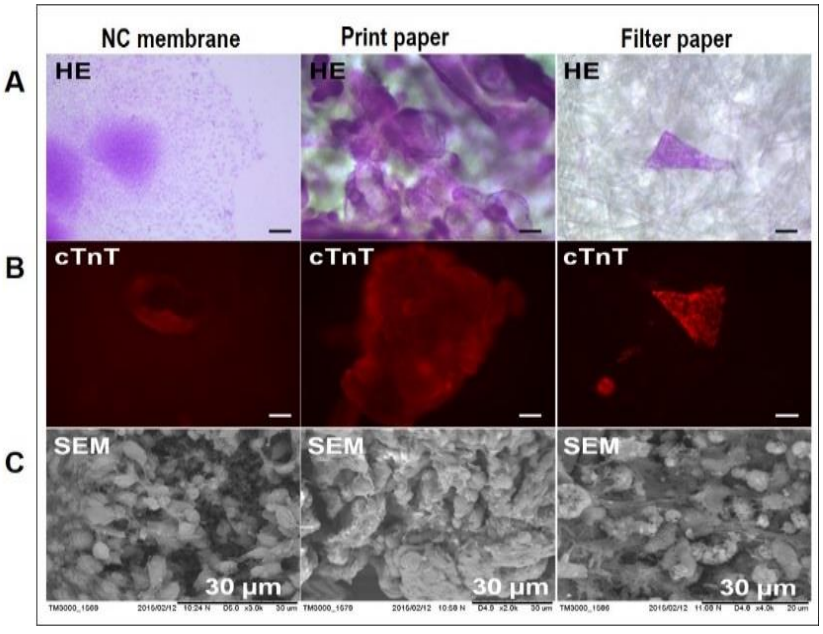

Figure 6. Direct human iPSCs differentiated into cardiac tissues on paper. A: The human iPSC-derived cardiac tissues on the three types of paper were stained with H&E after 3 three months. B: The cardiac tissues were stained with a cardiac-specific marker, cTnT, by immunofluorescent imaging. C: The SEM images of cardiac tissues on the three types of paper. Scale bar: 100 μm.