Optimization of extracellular matrix production from human induced pluripotent stem cell-derived fibroblasts for scaffold fabrication for application in wound healing

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Abstract
Extracellular matrix is a key component of all tissues, including skin and it plays a crucial role in the complex events of wound healing. These events are impaired in chronic wounds, with chronic inflammation and infection often present in these non-healing wounds. Many tissue engineering approaches for wound healing provide a scaffold to mimic the native matrix. Fibroblasts derived from iPS cells (iPSF) represent a novel source of matrix rich in pro-regenerative components, which can be used for scaffold fabrication to improve wound healing. However, in vitro production of matrix by cells for scaffold fabrication requires long cell culturing times which increases cost. The aim of this work is to optimize the iPSF matrix production by boosting matrix deposition, without affecting its composition. A good candidate technique to achieve this goal is macromolecular crowding, which is known to promote conversion of procollagen into mature collagen and its accumulation. We tested two molecular crowders, Ficoll and Carrageenan—in combination with ascorbic acid—over a prolonged period of time. Ficoll in combination with ascorbic acid notably increased collagen deposition and matrix dry weight compared to ascorbic acid alone, and did not affect matrix composition as measured by RT-PCR. Interestingly, Carrageenan did not affect collagen quantity, but it significantly increased glycosaminoglycan deposition. Finally, we successfully fabricated scaffolds from harvested matrix and confirmed their ability for cell growth and viability. This work lays the foundation for development of a time and cost effective protocol for novel iPSF ECM production for tissue engineering scaffolds.

Keywords
ECM, fibroblasts, iPSC, macromolecular crowding, scaffolds

1 | INTRODUCTION

Extracellular matrix (ECM) is one of the key components of skin, playing a critical role in maintaining its structure and integrity.1 Housing the cells within skin, it mostly consists of macromolecules, including collagen, glycosaminoglycans (GAGs), proteoglycans, laminins, and elastin, that provide mechanical and biochemical function and cues.2 ECM deposition is essential for proper wound healing, which is a complex, dynamic and finely orchestrated process that involves a series of overlapping events that lead to skin repair, or—
larger defects—scar formation. In chronic wounds the healing process is impaired resulting in chronic inflammation and formation of non-healing wounds. These non-healing wounds cause severe health challenges, which results in them also being a significant economic burden. For instance, diabetic foot ulcers are chronic wounds that are susceptible to infection, possibly leading to the development of gangrene, and results in limb amputations in some cases. Therefore, there is a persistent unmet need for developing more efficient wound healing treatments, and tissue engineering offers a lot of promise.

Integra’s Omniprgraft®, a collagen-glycosaminoglycan (CG) scaffold developed for skin regeneration, has been approved by FDA for diabetic foot ulcers treatment, however it has 50% success rate in wound closure. In addition to Omniprgraft, other bioengineered skin substitutes such as Apligraft® and Dermagraft® have been developed and implemented in clinics, although with similar success rates. Recently we functionalized collagen-based scaffolds with ECM grown by induced pluripotent stem (iPS) derived fibroblasts (iPSF) and tested its potential in wound healing. The motivation for the inclusion of iPS-derived fibroblast ECM was due to its previous demonstration of increased ECM production compared to source fibroblasts. This matrix is enriched with pro-regenerative components (e.g., collagen III, collagen IV, fibronectin) similar to those produced by embryonic fibroblasts and, notably, embryonic wounds heal completely without forming a scar. Our work showed that scaffolds made of iPSFs ECM when seeded with healthy or diabetic foot ulcers fibroblast, enhanced the production of key ECM components and VEGF release in both types of fibroblasts and induced robust immune response to cues. In addition, iPSF scaffolds induced higher GAG, VEGF and higher cell content in diabetic foot ulcers fibroblasts when compared to scaffolds made of source fibroblasts.

One of the limitations of using cell-derived matrix is achieving sufficient in vitro production of ECM for scaffold fabrication within an acceptable timeframe. In order to scale-up ECM production, we have tested several cell culture conditions and identified optimal cell density and media composition that increased matrix deposition twofold without adversely affecting the ratio of different matrix components. Nonetheless, in traditional 2D cell culturing methods, cells are seeded on a plastic surface with specific cell media that is quite different from physiological conditions. In vivo, cells are surrounded by dense and complex environment made of neighbouring cells and ECM. Synthetic macromolecular crowders can be used to mimic these physiological conditions in vitro, by creating macromolecular crowding effect or an excluded volume effect. Namely, in an uncrowded environment molecules freely diffuse, however, when macromolecules are present they occupy a certain volume and restrict the other molecules to the remaining space preventing them from diffusing freely. As a consequence, they enhance matrix deposition by accelerating enzymatic processes that facilitate pro-collagen conversion into mature collagen, leading to increased collagen deposition. Moreover, macromolecular crowders create cell specific microenvironments eliminating the need for feeder cell layers or protein coating of plastic surface, which overall allows for generation of improved cell culturing systems. Macromolecular crowders can be used for both scientific (e.g., investigation of cellular biological functions) and clinical/industrial purposes (e.g., improved cell expansion and development of cost effective protocols). Ideal crowders have the following characteristics: molecular weight between 50 and 200 kDa, high water solubility; and they are not prone to self-aggregation. Commonly used synthetic crowders include ficolls, carrageenan, dextrans, polyethylene glycol and polyvinyl alcohol. Most studies, to our knowledge, that implemented macromolecular crowders to increase collagen deposition were done in a short time period of 2–7 days and did not exceed 14 days. However, our prior observations suggest that in order to produce reasonable amounts of matrix for scaffold fabrication, a much longer cell culturing time of at least 3–5 weeks is necessary.

The aim of this work is to optimize and scale-up in vitro ECM production from iPSFs, by using macromolecular crowders to increase the rate of matrix deposition without affecting its constituents. This matrix can then be used for scaffold fabrication with applications in wound healing. It should be noted that this matrix could also be used for other disorders requiring collagen treatment for example, articular cartilage disorders, however, iPSF matrix was investigated so far only in context of skin. hence, further research should be conducted to investigate the beneficial effect of iPSF matrix in other tissues. In order to scale-up in vitro ECM production, we tested two types of macromolecular crowders, Ficoll and Carrageenan, for their efficiency in increasing matrix deposition in vitro from iPSFs, over an extended culture period. Finally, we confirm that we can fabricate scaffolds made of harvested ECM and evaluated their capacity to support cell growth in vitro.

2 | MATERIALS AND METHODS

2.1 | Cell culture conditions

All cells (fibroblasts and iPSFs) were kindly provided by the Garlick lab and previously fully characterised and described. The iPS line (Post-iPSF) (gift of the lab of Dr. Konrad Hochendlinger, Boston, MA) (Maherali et al., 2008), was derived by reprogramming BJ fibroblasts using separate dox-inducible vectors Oct4, Sox2, Klf4, cMyc, and Nanog. In mM adenine (Sigma), 8 mM HEPES (Sigma), 0.5 μg/ml hydrocortisone (Sigma), 10−10 M cholera toxin (MP Biological), 10 ng/ml epidermal growth factor (Austral Biological), and 5 μg/ml insulin (Millipore) Cells were seeded at 32 k/cm2 confluency and cell media was refreshed every 72 hr supplemented with 100 μg/ml ascorbic acid. For molecular crowding media was supplemented with Ficoll (37.5 mg/ml Fc70 + 25 mg/ml Fc400) or Carrageenan (75 μg/ml), and refreshed every 72 hr.

Samples were collected after 1 week (for RT-PCR analysis), however an insufficient volume of ECM was generated for scaffold fabrication. Hence, we analysed 3 week cultures for collagen, GAG and DNA quantification, and for scaffold fabrication.

2.2 | RNA extraction and RT-PCR

For RNA extraction, cells were collected after 7 days in culture using Qiagen RNA Isolation Kit (#74106). Upon extraction, RNA concentration
2.3 | Collagen, GAG, and DNA quantification

For collagen quantification, samples were collected after 3 weeks and resuspended in 100 mM acetic acid. Total collagen was analysed with Sircol Soluble Collagen Assay (Biocolor, S1000) according to the manufacturer’s instructions. Collagen concentrations were calculated from the standard curve. As Ficoll+ ascorbic acid was brought forward for further experimentation, we had those additional samples available for analyses which gave n = 6 for this group in experiments in Figures 2b and 3b.

For visualisation of collagen in 2D culture, cells were stained with Sirius Red/Fast Green Collagen Staining Kit (Chondrex, 9046) according to manufacturer’s instructions. Images were taken using ECLIPSE 90i microscope (Nikon, Tokyo, Japan).

Samples for GAG analysis were collected after 3 weeks in Papain Extraction buffer (0.2 M sodium phosphate buffer, 8 mg/ml sodium acetate, 4 mg/ml EDTA, 0.8 mg/ml cystein HCl, and 1 mg/ml papain enzyme) according to manufacturer’s instructions, and left overnight at 67°C. GAG content was quantified using Blyscan Glycosaminoglycan assay (Biocolor, B1000) according to manufacturer’s instructions. The concentration of GAGs was calculated from the standard curve.

Samples previously prepared in buffer for GAG quantification were used in this assay. DNA content in samples was quantified using PicoGreen assay according to manufacturer’s instructions. The concentration of DNA was calculated from the standard curve.

2.4 | Scaffold fabrication

For collagen scaffolds, collagen-slurry was generated by blending 5 mg/ml tendon bovine collagen (Sigma-Aldrich, Germany) in 0.5 M acetic acid based on previous studies. For fibroblast and iPSC scaffolds a 5 mg/ml slurry was prepared by blending matrix with bovine collagen in 1:1 ratio. Prepared slurry was then degassed, freeze-dried at −10°C, (60 min ramp) and sublimated at 100 mTorr at 0°C for 17 h.

For histology analysis scaffolds were fixed in 4% PFA at 4°C over night, sectioned with Leica Cryostat, and stained with haematoxylin
and eosin. Images were taken using ECLIPSE 90i microscope (Nikon, Tokyo, Japan).

2.5 | Statistical analysis

For statistical analysis, t test or ANOVA was calculated using GraphPad Prism software to determine significant differences between mean values in all experiments. Bar graphs show mean values ± SEM. The differences were considered significant at *p < 0.05, **p < 0.01.

3 | RESULTS

3.1 | Macromolecular crowders effect on the expression of collagens in fibroblasts and iPFSs

In order to scale-up ECM production in vitro, we tested two types of macromolecular crowders, ficoll and carageenan, for their capacity to increase the deposition of collagen. In all experiments, two different cell types were used: a skin fibroblast cell line, and fibroblasts derived from iPFS that were reprogrammed from the fibroblast cell line.

Collagen III expression in iPFSs was significantly enhanced by addition of ascorbic acid when compared to fibroblasts ± ascorbic acid, or iPFSs without ascorbic acid. Similarly, collagen I expression was significantly enhanced by addition of ascorbic acid compared to fibroblasts ± ascorbic acid (Figure S1A-C).

Since the pro-regenerative properties of the iPFS are attributed to their matrix, it is important to maintain the same proportions of different types of collagen. Hence, we evaluated if the addition of macromolecular crowders alters the RNA expression of different types of collagen, which could consequently change the matrix properties.

Cell media was supplemented with Ficoll (37.5 mg/ml Fc70 + 25 mg/ml Fc400), Carageenan (75 μg/ml) or was left macromolecular crowders free (control). After 1 week cells were collected for RNA isolation and tested for the expression of collagen 1, 3 and 4 (COL1A1, COL3A1, and COL4A1). There was no significant difference in collagen expression between groups, which supports the conclusion that macromolecular crowders do not affect adversely production of different collagenous proteins (Figure 1a,b). Statistical significance was evaluated by the ANOVA test (*p < 0.05).

3.2 | Effect of macromolecular crowders on ECM deposition from fibroblasts and iPFSs

After confirming that cell matrix production is not adversely affected by macromolecular crowders next, we evaluated the ability of Ficoll and Carrageenan to increase ECM deposition. The cells were seeded as previously described. After 3 weeks of culture, matrix was collected and analysed for total collagen content using Sircol collagen assay.

Ascorbic acid improves collagen production, and as expected addition of ascorbic acid increased collagen deposition in fibroblasts and iPFSs (Figure 2a and Figure 3a). While this change was significant in fibroblasts, in iPFS even though there was a clear increase in collagen content it did not reach significance. Statistical significance was evaluated by the t test (*p < 0.05).

We then investigated whether Ficoll and Carrageenan can additionally increase collagen deposition in the matrix. Our results revealed that Ficoll in combination with ascorbic acid had the most striking effect on collagen deposition (Figures 2b and 3b), when compared to the untreated control, without affecting cell number (Figure S2). However, Ficoll+ ascorbic acid did not significantly increase collagen content in fibroblasts when compared to the group treated with ascorbic acid alone (Figure 2b), after 3 weeks of treatment. Nonetheless, in iPFS, addition of Ficoll to ascorbic acid significantly increased overall collagen content in ECM compared to ascorbic acid alone (Figure 3b). Interestingly, when cells were treated alone with macromolecular crowders without addition...
of ascorbic acid, Ficoll was sufficient to significantly increase collagen in fibroblasts, but not in iPSFs, confirming that a combination of Ficoll and ascorbic acid is an optimal condition for production and deposition of iPSF matrix (Figure S3). Statistical significance was evaluated by ANOVA test (**p < 0.05). For visual representation of the data, cells were stained with Sirius red and Fast green that specifically stain collagen and non-collagenous proteins, respectively (Figures 2c and 3c). Imaging revealed a trend that was consistent with the quantified data.

3.3 Effect of macromolecular crowders on GAG deposition in fibroblasts and iPSF

Besides collagen, GAGs represent an important component of the ECM; hence, we evaluated if macromolecular crowders could increase their deposition as well. After 3 weeks of culture, matrix was collected for GAG isolation and quantification. Our results suggest that Ficoll induced higher GAGs deposition, however, not significantly. On the contrary, Carrageenan had a striking effect and it significantly increased GAGs accumulation in both fibroblasts and iPSFs, compared to ascorbic acid and Ficoll+ ascorbic acid groups (Figure 4). In addition, strong stimulatory effect of Carrageenan in both fibroblasts and iPSFs was observed also in conditions without ascorbic acid (Figure S4). Statistical significance was evaluated by the t test and ANOVA test (**p < 0.05).

3.4 Scaffold fabrication and in vitro testing

We next asked if matrix produced with this method could be used for scaffold fabrication. We have previously identified Ficoll+AA as an optimal condition for matrix production (Figures 2 and 3), hence in following experiments we did not include Carageenan + AA condition.
In line with evidence that Ficoll and ascorbic acid combination can efficiently increase collagen and GAG deposition compared to ascorbic acid alone, we observed 78.83% ± 37.45 and 40.56% ± 5.88 increase in ECM dry weight in fibroblasts and iPFS, respectively (Figure 5a), after 3 weeks in culture. Finally, we successfully fabricated scaffolds from harvested fibroblasts and iPFS matrix by mixing it with bovine collagen (COL) in 1:1 ratio (Figure 5c), as per previous work.10 Scaffold cross sectional area revealed slightly different structure and pore size in scaffolds produced from harvested matrix compared to the control (Figure 5d), which is likely due to the presence of additional ECM components other than collagen. This is consistent with our previous observations for this material.10

Further, 300,000 cells were seeded per scaffold and grown for 10 days in cell media supplemented with ascorbic acid. Scaffolds made from harvested matrix tended to contract when seeded with cells, much more evidently compared to COL scaffold, suggesting higher cell activity (Figure 6a), or possibly a change in mechanical properties. Indeed, previously we demonstrated that iPFS scaffolds had significantly lower compressive modulus compared to CG and fibroblasts scaffolds.10

There was no difference in DNA content between the groups, indicating that cell proliferation was not affected (Figure 6b). In addition, GAG production was not altered in all three groups (Figure 6c). However, H&E staining of cross-sections clearly demonstrated better cell distribution and increased matrix production in fibroblasts and iPFS scaffolds with respect to collagen scaffold (Figure 6d). Statistical significance was evaluated by the ANOVA test (*p < 0.05).

4 | DISCUSSION

Fibroblasts are a type of mesenchymal cells that play multiple roles in wound healing.28 They produce ECM components that facilitate cell migration and adhesion, and provide growth factors and cytokines that promote various processes including angiogenesis and tissue repair.29 Those characteristics make fibroblast a focal point of skin regenerative medicine, and many approaches seek to exploit their matrix to promote tissue regeneration. One of the great examples of matrix mimicry are the CG scaffolds, that have been clinically successful for skin regeneration7 and recently have been FDA approved for diabetic foot ulcers treatment (Integra’s Omnigraft®).
However, clinical trials reported only 50% success in terms of wound closure. Moreover, in most cases wound healing leads to scar formation, and identifying the growth factors and matrix components that will promote scarless healing is of great interest.

IPSF are of particular interest for tissue engineering since they have higher expression of collagens compared to source fibroblast (Figure S1 and 10,11). However, our data showed that the accumulation of collagen in cell culture, measured with colorimetric assay after 3 weeks, was lower in iPSFs compared to fibroblasts (Figure 2 and Figure 3); our previous work showed that fibroblasts collagen was slightly, albeit insignificantly, higher. This finding at first seems contradictory to mRNA data which clearly suggest higher production of collagen in post-iPSFs (Figure S1 10,11). Nonetheless, mRNA data represent collagen expression normalized per total amount of transcript, while the colorimetric assay measures overall collagen accumulation in culture dish. Moreover, DNA content was lower in post-iPS then in pre-iPSF after 3 weeks of culture (Figure S2), which is also in line with observations from our previous work.  

The aim of this work was to test different macromolecular crowders, Ficoll and Carrageenan, for their potential to increase matrix deposition in a novel source of ECM, iPSF, as well as in the source fibroblasts, over 3 weeks of cell culture. Our results show that Ficoll and Carrageenan did not significantly alter expression of different collagen molecules (Figure 1) confirming that they solely affects their deposition. These observations are in line with previous work done on human corneal fibroblasts, where authors demonstrated that Ficoll did not affect gene expression profile and therefore maintained ECM composition, after 2, 4 and 6 days of culture.22

Moreover, Ficoll in combination with ascorbic acid increased collagen deposition in both fibroblasts and iPSF groups after 3 weeks, although this was significant only in iPSFs (Figures 2 and 3). These data suggest for the first time that for long term application, Ficoll does not have beneficial effect on matrix deposition in normal fibroblasts, however, this effect is rather observable at shorter time points.
(up to 3 weeks).\textsuperscript{22-24} Nonetheless, in IPSF cells, Ficoll in combination with ascorbic acid clearly boosted collagen deposition compared to ascorbic acid alone, suggesting that efficiency of macromolecular crowders could be cell specific and can still have an effect up to 3 weeks. Further, for regenerative medicine applications that want to exploit matrix from IPS fibroblasts—and possibly other cells—macromolecular crowders is a promising technique for enhanced matrix deposition.

Surprisingly, the increase in overall matrix dry weight in fibroblasts treated with Ficoll+ ascorbic acid seems to be much higher than the increase of collagen alone, when compared to corresponding ascorbic acid groups (Figure 2b and Figure 5a). This suggests that macromolecular crowders could influence deposition of ECM components other than collagen. Indeed, previous works show that besides collagen deposition, Ficoll was able to increase deposition of fibronectin—a glycoprotein abundant in ECM—in fibroblast and keratinocytes after 6 days of culture.\textsuperscript{31} Moreover, our results demonstrated that Carrageenan significantly increased GAGs deposition (Figure 4). In correspondence to this observation, it has been demonstrated that Carrageenan could inhibit sulphates activity in endothelial cells leading to increased GAG deposition.\textsuperscript{32} Moreover, Carrageenan in combination with 2\% oxygen tension, in addition to increased collagen deposition, induced significantly increased expression of elastin in tenocytes after 7 days in culture.\textsuperscript{33} Further investigation of the effect of different macromolecular crowders on deposition of other matrix components such as fibronectin, elastin and laminin could be beneficial for potential use in tissue engineering when specific matrix components are required.

Finally, ECM harvested from fibroblasts and IPSFs was shown to be suitable for scaffold fabrication (Figure 5). Importantly, cells seeded on such scaffolds are viable and show higher activity reflected in increased matrix formation compared to collagen scaffold, as shown with H\&E staining (Figure 6). Nonetheless, further optimization of scaffold stability with appropriate crosslinking method should be applied to avoid observed scaffold contraction when seeded with cells. For instance, in our previous work scaffolds showed greater stability in cell culture when they were physically crosslinked and sterilized using dehydrothermal and chemically cross-linked using 1-ethyl-2-(3-dimethylaminopropyl) carbodiimide.\textsuperscript{10} Moreover, when those scaffolds were tested for mechanical properties, IPSF scaffolds had significantly lower compressive modulus compared to CG and fibroblasts scaffolds, suggesting that the composition of ECM components greatly influences mechanical properties of the scaffolds.\textsuperscript{10}

In conclusion, in this study we show, for the first time, that combination of macromolecular crowders and ascorbic acid can scale up matrix production from a novel source of matrix, IPSF cells, by specifically boosting collagen and/or GAG deposition. In addition, IPSF matrix can successfully be used to produce functional porous scaffolds. Further work will focus on the optimization of scaffolds mechanical properties and assessing the capabilities of these scaffolds to promote wound healing.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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