Macromolecular crowding facilitates rapid fabrication of intact, robust cell sheets

Shuwen Guan
Guangdong Academy of Sciences
Shipeng Wu
Qingdao Eighth People's Hospital
Gang Li
Guangdong Delun Medical Group
Jiangwei Xiao
Guangdong Academy of Sciences
Botao Gao (gaobotao1984@outlook.com)
Guangdong Academy of Sciences https://orcid.org/0000-0002-4411-0258

Research Article

Keywords: Cell sheet technology, extracellular matrices, human bone marrow mesenchymal stem cell, macromolecular crowding, 3T3 fibroblasts

Posted Date: September 9th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1989359/v1

Abstract

Objectives To develop a rapid and simple method to fabricate intact, robust cell sheets from common cell culture dishes by combination of a macromolecular crowding (MMC) reagent and vitamin C.

Results It was found that 3T3 fibroblasts or human bone marrow mesenchymal stem cells (hBMSCs) and their secreted cell derived extracellular matrices could be easily detached as intact cell sheets under gently pipetting after treated by MMC and vitamin C for 4 days. This method also allowed fabrication of functional multi-layered hepatic cell sheets by culturing 10×104 cells/cm2 HepG2 cells on top of confluent 3T3 fibroblast layers. What's more, MMC induced hBMSC cell sheets demonstrated 1.4 times larger area and 1.6 times greater cell number than that of cell sheets harvested from temperature-responsive cell culture dishes.

Conclusion MMC based method make it possible to fabricate various types of cell sheets more conveniently, economically, and thus may facilitate wide application of cell sheet technology.

Introduction

Cell sheet technology (CST), which is traditionally based on temperature responsive cell culture dishes (TRCD), is developed by Okano et al (Matsuda et al. 2007). This technology allows us to harvest confluent cells in the form of an intact sheet-like structure by lowering the temperature. Cell sheets retain the cell membrane proteins and extracellular matrices (ECM), thus they maximize transplanted cell survival and retention rates *in vivo* (Sekine et al. 2011). So far, CST has been clinically applied for therapy of a vast variety of diseases, such as corneal disease, heart failure, esophageal ulceration, periodontitis and so on (Takahashi and Okano 2019). Besides, CST has also been applied in building *in vitro* heart and liver models for drug screening and study of human disease (Sasaki et al. 2018; Gao et al. 2020; Gao et al. 2021). Recently, CST has been tried for production of cultured meat (Park et al. 2021). Although UpCell® TRCDs are commercially available, they are much more expensive than normal cell culture dishes and thus limit their application in common labs. There are also some advanced technologies have been developed for cell sheet fabrication, such as electro-responsive surface (Enomoto et al. 2016), photo-responsive surface (Park et al. 2021), magnetic nanoparticles (Gonçalves et al. 2017), ultrasound irradiation (Imashiro et al. 2020) and so on. However, these methods are complicated or require special equipment, thus difficult to be reproduced.

It has been reported that vitamin C (Vc) is an effective method to harvest cell sheets (Nakamura et al. 2010; Wei et al. 2012). Vc treatment promotes mesenchymal stem cell (MSC) sheet formation via stimulating ECM production and MSC sheets with sufficient ECM can be easily detached from cell culture dishes with a cell scraper. However, it usually takes about two weeks for enough ECM formation. To solve this issue, we plan to combine Vc with macromolecular crowding (MMC), a biophysical approach, to accelerate ECM formation. The principle of MMC is a bio-inspired opinion that *in vivo* highly

crowded/dense extracellular space promote conversion of the *de novo* synthesized procollagen type to collagen type (Chen et al. 2011).

In vivo, cells are encapsulated in the dense ECM environment, where the proteinase-dependent conversion of procollagen type to collagen type is rapid. In contrast, cells are normally cultured in liquid media *in vitro*, where the conversion of water-soluble procollagen type to water insoluble collagen type is very slow. Therefore, the addition of inert macromolecules in the liquid media, by mimicking the dense extracellular environment, will enable the accelerated deposition of ECM.

Therefore, we propose that the addition of inert polydispersed macromolecules and Vc in the culture media will facilitate amplified ECM production in cell sheets and thus develop a simple and rapid technique to generate ECM-rich cell sheets without usage of TRCDs. To prove our hypothesis, 3T3 fibroblasts and human bone marrow MSCs (hBMSCs) were cultured in the media containing Ficoll (MMC reagent) and Vc for rapid cell sheet fabrication. Since our method may not be suitable for harvesting ECM less cells (such as hepatocytes) as intact cell sheets, we also investigate the possibility and optimal conditions of using 3T3 fibroblasts as a feeder layer to fabricate functional hepatic tissues to further demonstrate the utility of the MMC technique in building heterotypic tissues. Finally, MMC induced cell sheets were compared with TRCD induced cell sheets in area and cell number. As far as we know, this is the first report of rapidly harvesting intact cell sheets from common cell culture dishes through MMC treatment and gently pipetting.

Materials And Methods

Cell culture

3T3 fibroblasts and HepG2 cells (ATCC, Manassas, Virginia, USA) were cultured in Dulbecco's modifed Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (PS). Cells were maintained in a 5% CO_2 humidifed atmosphere incubator at 37°C. hBMSC were kindly provided by Dr. Xiao et al. (2017) and expanded until passage 4–8. Cells were cultured in typical basal MSC culture media, comprising MEM, plus with 10% FBS and 1% PS. The media were changed every 2 days. Cell passaging was performed by trypsinization in 0.05% trypsin-EDTA.

Cell sheet detachment

Two methods were used to detach cell sheets. For TRCD method, 1×10^6 3T3 fibroblasts or 0.6×10^6 hBMSCs were seeded into 35 mm UpCell® cell cuture dish (CellSeed, Tokyo, Japan) with base media (high glucose DMEM/10%FBS/1%PS). For MMC method, 1×10^6 3T3 fibroblasts or 0.6×10^6 hBMSCs were seeded into 35-mm common cell culture dishes (Corning, NY, USA), cultured with base medium supplemented with 20 μg mL^{-1} Vc or 25 mg mL^{-1} of 400 kDa Ficoll (Ficoll400; GE Healthcare, Uppsala, Sweden) and 20 μg mL^{-1} Vc or 37.5 mg mL^{-1} of 70 kDa Ficoll (Ficoll70; GE Healthcare) and 20 μg mL^{-1} Vc or 25 mg mL^{-1} Ficoll400, 37.5 mg mL^{-1} Ficoll70 and 20 μg mL^{-1} Vc. Experimental groups were

shown in Table 1. Cells were maintained in 5% CO_2 at 37°C. The media were changed every 2 days until day 5.

Table 1
Experimental groups

Groups	Control	Vitamin C	Vitamin C/ Ficoll400	Vitamin C/ Ficoll70	Vitamin C/ Ficoll70/ Ficoll400	Temperature responsive cell culture dish
abbreviation	C	Vc	VF400	VF70	VF70/400	TRCD
Ficoll400	/	/	25 mg mL^{-1}	/	25 mg mL^{-1}	/
Ficoll70	/	/	/	37.5mg mL^{-1}	37.5mg mL^{-1}	/
Vitamin C	/	20 μg mL^{-1}	20 μg mL^{-1}	20 μg mL^{-1}	20 μg mL^{-1}	/

After 4 days culture, cells cultured on common dishes were detached as cell sheets by gently pipetting, while cells cultured on TRCDs were incubated at 20°C for 30 min to harvest cell sheets.

Fabrication for HepG2/3T3 co-culture cell sheets

2×10^5 3T3 fibroblasts were cultured in each well of 24 well plates for 4 days by using base medium containing MMC reagent and Vc as mentioned above. HepG2 cells (2.5×10^4, 5×10^4, 10×10^4, 20×10^4, 30×10^4, 40×10^4 cells/cm^2) were seeded on 3T3 fibroblast monolayer on day 5 and detached as HepG2/3T3 co-culture cell sheets on day 6 following the same protocol above mentioned (2.5Hep/T, 5Hep/T, 10Hep/T, 20Hep/T, 30Hep/T, 40Hep/T, respectively). The detached co-culture cell sheets were transferred into collagen type coated-35 mm cell culture dishes (IWAKI, Tokyo, Japan). Then, cell culture media was drained and the cell sheets were incubated at 37°C without media to attach on cell culture surfaces. Around 10 min later, 2 mL base media was added into dishes. The co-cultured cell sheets were then cultured for 1 week in base media.

RNA isolation and real-time quantitative PCR (RT-qPCR)

Total RNA was extracted by Trizol reagent (Thermo Fisher, MA, USA), and then 1 μg total RNA was reverse transcribed into cDNA by Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher). Real-time PCR was performed using the 2×RealStar Green Power Mixture (GenStar, Beijing, China) in ABI Step One Sequence

Detection System (Thermo Fisher). The PCR reaction procedures were: 95℃ for 10 min, 40 cycles at 95℃ for 15 s, 60℃ for 15 s, and 72℃ for 1 min. Primer sequences for collagen (, , ,), MMP2, fibronectin, and GAPDH are listed in Table S1 (Supplementary material). All experiments were repeated three times and were normalized to housekeeping gene GAPDH.

Morphological Examination and Histology

The cell sheets were fixed in 4% paraformaldehyde (PFA) for paraffin-embedded histological analysis. Sections were cut perpendicular to the cell sheet to a thickness of 5 μm, rehydrated, and stained with hematoxylin and eosin (H&E; Sigma-Aldrich, St. Louis, Missouri, USA).

Evaluation of liver-specific functions

Cell culture supernatants of HepG2/3T3 co-cultured cell sheets over a 24 h period were collected on day 6 after cell sheet detachment. Albumin secretion was detected by human albumin enzyme-linked immunosorbent assay (ELISA) quantitation kit (Solarbio Science & Technology, Beijing, China). Urea synthesis was determined using a colorimetric assay kit (Jiancheng Bioengineering Insititute, Nanjing, China). Measurements were performed in three independent experiments. In each experiment, replicate samples were more than three.

Statistical analysis

The results were expressed as mean ± standard deviation (SD) values. Significant differences between two groups were tested using Student's *t* test. ANOVA was used to analyze differences between three or more groups. Values of $*P < 0.05$ were considered statistically significant.

Results And Discussion

MMC reagents promotes cell sheet formation

As shown in Fig. 1A, Vc enabled detachment of some cell fragments, not intact cell sheets after 4 days culture. In contrast, combination of MMC (including VF400, VF70, VF70/400) and Vc induced cohesive cell sheets formation (Fig. 1B-D). The MMC based cell sheets were robust and easy to be detached by pipetting (Fig. 1E,F). Although all of VF400, VF70, VF70/400 allow fabrication of intact cell sheets, VF400 was decided as an optimal condition given its lower working concentration and cost than other groups.

Immunofluorescence staining showed that fibronectin and cells were completely detached from the cell culture dish and remained in the cell sheet (Fig. 1G,H). This result suggests this method dose not destroy the ECM layer in the cell sheet, which is the same as cell sheets harvested from TRCDs. Consistent with other studies (Kumar et al. 2015; Marinkovic et al. 2021), MMC did not affect gene expression for collagens (I, III, IV, V, VI); fibronectin; MMP-2 (Fig. 1I-N), confirming that MMC is a biophysical phenomenon and does not affect cell genotype, which is quite important in culturing stem cells.

Furthermore, MMC based cell sheets were tried to be stacked to observe whether they could form multilayered structure as TRCD based cell sheets. As shown in Fig. S1 A-C (supplementary materials), MMC based cell sheets stably stuck together generating double-layered and triple-layered tissue constructs. Addition of culture media did not decouple multilayered tissues, indicating the presence of ECM “glue” in the cell sheets (Fig. S1 G). HE staining of cross-sections of cell sheets also confirmed integrated multiple layered structure (Fig. S1 D-F).

Until now, although some cutting-edge techniques, such as magnetic force (Gonçalves et al. 2017) and ultrasonic vibration (Imashiro et al. 2020), have been reported to rapidly harvest cell sheets in several days, they require specific devices which are not easily available in biological labs. In this study, we proposed a simple, rapid and economical method to detach cell sheets. The materials we used, such as Ficoll, Vc, cell culture dish and pipette, are commonly available and affordable in biological labs. Therefore, we believe this method can enable more biomedical scientists to carry out CST research.

MMC treated 3T3 fibroblast layer facilitate rapid hepatic cell sheet formation

CST is a scaffold-free technology which recapitulates *in vivo* stratified structure, increases cell-cell, cell-ECM interaction and thus provide a competitive way to build functional hepatic tissue, which have a potential to be used as drug testing models or cell-based therapy for metabolic liver disease (Sakai et al. 2018; Kim et al. 2012; Kim et al. 2017; Sekine et al. 2011). However, all of these studies took advantage of cell sheets from TRCDs, MMC based CST has not been reported for fabrication of hepatic cell sheet tissues. However, hepatocytes including HepG2 cells produce small amount of ECM and not easy to form intact cell sheets. Therefore, HepG2 cells were tried to be cultured on MMC treated 3T3 fibroblast layer for co-cultured cell sheet formation. Since co-culture ratio of hepatocytes and fibroblasts has been demonstrated to be an important factor affecting hepatocellular functions (Sakai et al. 2018), various density of HepG2 cells have been seeded on 3T3 fibroblast layer.

As shown in Fig. 2A, HepG2/3T3 co-culture cell sheets containing various HepG2 cells from 2.5×10^4 cells/cm^2 to 40×10^4 cells/cm^2 were successfully fabricated, demonstrating the feasibility to obtain heterotypic cell sheet tissues by using the MMC method. HE staining confirmed stable attachment of HepG2 cells on 3T3 layers after 6 days culture (Fig. 2B). Besides, although HepG2 cell numbers did not disturb the detachment of co-cultured cell sheets, it affected area of cell sheets. It seemed that area of HepG2/3T3 co-cultured cell sheets increased with rise of HepG2 seeding cell numbers (Fig. S2).

As for liver specific functions, urea synthesis was significantly enhanced as HepG2 seeding density increased from 2.5×10^4 to 10×10^4 on day 6 (Fig. 2C). However, when the seeding density was more than 10×10^4, urea synthesis did not increased further. Similar to this trend, CCK8 assay result showed that viable cell numbers reached a plateau from 20Hep/T to 40Hep/T, which indicated that there is a cell number limitation to culture cell-dense tissues *in vitro* (Fig. 2E). This is probably because that poor nutrient, oxygen and waste transport result in cell necrosis in the central cell-layers (Miyamoto et al. 2021). What’s more, another liver function marker, albumin secretion revealed an increase from 2.5Hep/T

to 10Hep/T, but declined from 10Hep/T to 40Hep/T. By analyzing the above mentioned results, we found that 10×10^4 cells/cm^2 of HepG2 cells was the best condition for fabrication of functional hepatic cell sheet tissues.

MMC induced hBMSC cell sheets demonstrated larger area and greater cell number than that of TRCD induced cell sheets

To further explore the application of MMC based CST, hBMSC cell sheets, which have great clinical significances in regenerative medicine, were fabricated and compared with cell sheets harvested from TRCDs. As shown in Fig. 3A,B, both of TRCDs and MMC allowed fabrication of intact hBMSC cell sheets after 5 days culture, whereas area of MMC induced cell sheets was 24 mm^2, 1.4 times larger than that of cell sheets harvested from TRCDs (Fig. 3C). HE staining of cross-sections of cell sheets showed that there was no obvious difference in thickness between two types of cell sheets (Fig. 3D,E). Phase contrast microscopy exhibited that MMC treated cells were more crowded than cells on TRCDs on day 5 (Fig. 3F,G). Further, CCK8 assay showed that MMC accelerated cell proliferation since day 3, in comparison to TRCD counterparts (Fig. 3H). Possibly, MMC intensifies the efficacy of autocrine cell signals, and growth factors present in serum, and thus facilitated cell proliferation. In particular, MMC group exhibited 1.6 times greater cell number than that of TRCD group on day 5, which is consistent with the findings of HepG2/3T3 co-cultured cell sheets and confirmed that cell numbers is a major factor in regulating area of cell sheets.

Although this MMC method allows rapid fabrication of 3T3 and hBMSC cell sheets, it also has limitations. As shown in Fig. S3A, MMC treated NHDFs could not be detached as intact cell sheets with some cell fragments still strongly bind with culture surface. In contrast, NHDFs were completely detached from TRCDs by lowering temperature (Fig. S3B). CCK8 assay result demonstrated that cell number of MMC group was 1.6 times greater than that of TRCD group on day 5 (Fig. S3C), which is similar to hBMSCs. Therefore, cell number was not the culprit of partially detachment of NHDF cell sheets.

NHDFs are major cells in skin tissue for ECM production with the most prevalent ECM is collagen, which comprises 77% of the fat-free dry weight of human skin (Tracy et al. 2016; Weinstein and Boucek 1960). Since collagen type which constitute 90% of collagen (Miyamoto et al. 2021), is strongly adhesive protein, thus may inhibit NHDF cell sheet detachment. This hypothesis was consistent with Satyam et al' study. They have reported that Ficoll treated primary human corneal fibroblast sheets were not easy to be detached from commercially TRCDs due to the abundant deposited ECM (Satyam et al. 2014). Finally, we thought MMC based CST was cell-specific and seemed to be not suitable for cells have strong ability to produce collagens. We are now trying to figure out possible mechanism and develop cell culture media 2.0, which will be reported in the future.

Conclusion

Cell sheet-based tissue engineering is a powerful technology in regenerative medicine. Currently, harvesting cell sheets mainly relies on TRCD. By combination of a MMC reagent and Vc, we developed a method to harvest intact cell sheets from common cell culture dishes, including 3T3 fibroblasts and hBMSCs. It was found that MMC and vitamin C promoted robust cell sheet formation which could be easily detached under gently pipetting. This method also allowed fabrication of functional multi-layered hepatic cell sheets by culturing HepG2 cells on top of 3T3 fibroblast layers. What's more, MMC induced hBMSC cell sheets demonstrated larger area and greater cell number than that of TRCD induced cell sheets. Finally, we expect MMC based method facilitates wide application of CST, because the novel technique make it possible to fabricate various types of cell sheets more conveniently, economically and have a potential to increase efficacy of cell sheets by increasing cell numbers and ECM.

Declarations

Acknowledgments

This research was financially supported by Guangdong Academy of Sciences (Grant No. 2020GDASYL-20200102005; Grant No. 2021GDASYL-20210102004; Grant No. 2022GDASZH-2022010110), Science and Technology Planning Project of Guangdong Province (Grant No. 2020B1111560001) and Project funded by China Postdoctoral Science Foundation (Grant No. 2021M690746).

Competing interests:

The authors have declared that no conflicts of interest.

Author contributions:

Botao Gao conceived and designed the experiments and wrote the manuscript. Shuwen Guan performed the experiments and wrote the manuscript together with Botao Gao. Material preparation, data collection and analysis were performed by Shipeng Wu, Gang Li and Jiangwei Xiao. All authors read and approved the final manuscript.

Data availability:

The datasets generated during the current study are available from the corresponding author on reasonable request.

Consent to participate:

Informed consent was obtained from all individual participants included in the study.

References

1. Chen C, Loe F, Blocki A, Peng Y, Raghunath M (2011) Applying macromolecular crowding to enhance extracellular matrix deposition and its remodeling in vitro for tissue engineering and cell-based therapies. Adv Drug Deliv Rev 63:277–290. https://doi.org/10.1016/j.addr.2011.03.003
2. Enomoto J, Mochizuki N, Ebisawa K, Osaki T, Kageyama T, Myasnikova D, Nittami T, Fukuda J (2016) Engineering thick cell sheets by electrochemical desorption of oligopeptides on membrane substrates. Regen Ther 3:24–31. https://doi.org/10.1016/j.reth.2015.12.003
3. Gao B, Sakaguchi K, Matsuura K, Ogawa T, Kagawa Y, Kubo H, Shimizu T (2020) In vitro production of human ballooned hepatocytes in a cell sheet-based three-dimensional model. Tissue Eng Part A 26:93–101. https://doi.org/10.1089/ten.tea.2019.0101
4. Gao B, Sakaguchi K, Ogawa T, Kagawa Y, Kubo H, Shimizu T (2021) Functional analysis of induced human ballooned hepatocytes in a cell sheet-based three dimensional model. Tissue Eng Regen Med 18:217–224. https://doi.org/10.1007/s13770-020-00297-x
5. Gonçalves AI, Rodrigues MT, Gomes ME (2017) Tissue-engineered magnetic cell sheet patches for advanced strategies in tendon regeneration. Acta Biomater 63:110–122. https://doi.org/10.1016/j.actbio.2017.09.014
6. Imashiro C, Hirano M, Morikura T, Fukuma Y, Ohnuma K, Kurashina Y, Miyata S, Takemura K (2020) Detachment of cell sheets from clinically ubiquitous cell culture vessels by ultrasonic vibration. Sci Rep 10:9468. https://doi.org/10.1038/s41598-020-66375-1
7. Kumar P, Satyam A, Fan X, Collin E, Rochev Y, Rodriguez BJ, Gorelov A, Dillon S, Joshi L, Raghunath M, Pandit A, Zeugolis DI (2015) Macromolecularly crowded in vitro microenvironments accelerate the production of extracellular matrix-rich supramolecular assemblies. Sci Rep 5:8729. https://doi.org/10.1038/srep08729
8. Kim K, Ohashi K, Utoh R, Kano K, Okano T (2012) Preserved liver-specific functions of hepatocytes in 3D co-culture with endothelial cell sheets. Biomaterials 33:1406–1413. https://doi.org/10.1016/j.biomaterials.2011.10.084
9. Kim K, Utoh R, Ohashi K, Kikuchi T, Okano T (2017) Fabrication of functional 3D hepatic tissues with polarized hepatocytes by stacking endothelial cell sheets in vitro. J Tissue Eng Regen Med 11:2071–2080. https://doi.org/10.1002/term.2102
10. Matsuda N, Shimizu T, Yamato M, Okano T (2007) Tissue engineering based on cell sheet technology. Adv Mater 19:3089–3099. https://doi.org/10.1002/adma.200701978
11. Marinkovic M, Sridharan R, Santarella F, Smith A, Garlick JA, Kearney CJ (2021) Optimization of extracellular matrix production from human induced pluripotent stem cell-derived fibroblasts for scaffold fabrication for application in wound healing. J Biomed Mater Res A 109:1803–1811. https://doi.org/10.1002/jbm.a.37173
12. Miyamoto D, Sakai Y, Huang Y, Yamasaki C, Tateno C, Hasegawa H, Murai T, Hara T, Adachi T, Soyama A, Hidaka M, Ito S, Kanetaka K, Eguchi S (2021) Functional changes of co-cultured hepatocyte sheets subjected to continuous liver regeneration stimulation in cDNA-uPA/SCID mouse: Differences in transplantation sites. Regen Ther 18:7–11. https://doi.org/10.1016/j.reth.2021.02.004

13. Nakamura A, Akahane M, Shigematsu H, Tadokoro M, Morita Y, Ohgushi H, Dohi Y, Imamura T, Tanaka Y (2010) Cell sheet transplantation of cultured mesenchymal stem cells enhances bone formation in a rat nonunion model. Bone 46:418–424. https://doi.org/10.1016/j.bone.2009.08.048
14. Park S, Jung S, Choi M, Lee M, Choi B, Koh W, Lee S, Hong J (2021) Gelatin MAGIC powder as nutrient-delivering 3D spacer for growing cell sheets into cost-effective cultured meat. Biomaterials 278:121155. https://doi.org/10.1016/j.biomaterials.2021.121155
15. Sekine H, Shimizu T, Dobashi I, Matsuura K, Hagiwara N, Takahashi M, Kobayashi E, Yamato M, Okano T (2011) Cardiac cell sheet transplantation improves damaged heart function via superior cell survival in comparison with dissociated cell injection. Tissue Eng Part A 17:2973–2980. https://doi.org/10.1089/ten.tea.2010.0659
16. Sasaki D, Matsuura K, Seta H, Haraguchi Y, Okano T, Shimizu T (2018) Contractile force measurement of human induced pluripotent stem cell-derived cardiac cell sheet-tissue. PLoS ONE 13:e0198026. https://doi.org/10.1371/journal.pone.0198026
17. Sakai Y, Koike M, Kawahara D, Hasegawa H, Murai T, Yamanouchi K, Soyama A, Hidaka M, Takatsuki M, Fujita F, Kuroki T, Eguchi S (2018) Controlled cell morphology and liver-specific function of engineered primary hepatocytes by fibroblast layer cell densities. J Biosci Bioeng 126:249–257. https://doi.org/10.1016/j.jbiosc.2018.02.006
18. Sekine W, Haraguchi Y, Shimizu T, Umezawa A, Okano T (2011) Thickness limitation and cell viability of multi-layered cell sheets and overcoming the diffusion limit by a porous-membrane culture insert. J Biochip issue Chip 1:1–7. https://doi.org/10.4172/2153-0777.S1-007
19. Satyam A, Kumar P, Fan X, Gorelov A, Rochev Y, Joshi L, Peinado H, Lyden D, Thomas B, Rodriguez B, Raghunath M, Pandit A, Zeugolis D (2014) Macromolecular crowding meets tissue engineering by self-assembly: a paradigm shift in regenerative medicine. Adv Mater 26:3024–3034. https://doi.org/10.1002/adma.201304428
20. Takahashi H, Okano T (2019) Thermally-triggered fabrication of cell sheets for tissue engineering and regenerative medicine. Adv Drug Deliver Rev 138:276–292. https://doi.org/10.1016/j.addr.2019.01.004
21. Tracy LE, Minasian RA, Caterson EJ (2016) Extracellular matrix and dermal fibroblast function in the healing wound. Adv Wound Care 5:119–136. https://doi.org/10.1089/wound.2014.0561
22. Wei F, Qu C, Song T, Ding G, Fan Z, Liu D, Liu Y, Zhang C, Shi S, Wang S (2012) Vitamin C treatment promotes mesenchymal stem cell sheet formation and tissue regeneration by elevating telomerase activity. J Cell Physiol 227:3216–3224. https://doi.org/10.1002/jcp.24012
23. Weinstein GD, Boucek RJ (1960) Collagen and elastin of human dermis. J Invest Dermatol 35:227–229. https://doi.org/10.1038/jid.1960.109
24. Xiao E, He L, Wu Q, Li J, He Y, Zhao L, Chen S, An J, Liu Y, Chen C, Zhang Y (2017) Microbiota regulates bone marrow mesenchymal stem cell lineage differentiation and immunomodulation. Stem Cell Res Ther 8:213. https://doi.org/10.1186/s13287-017-0670-7

Figures

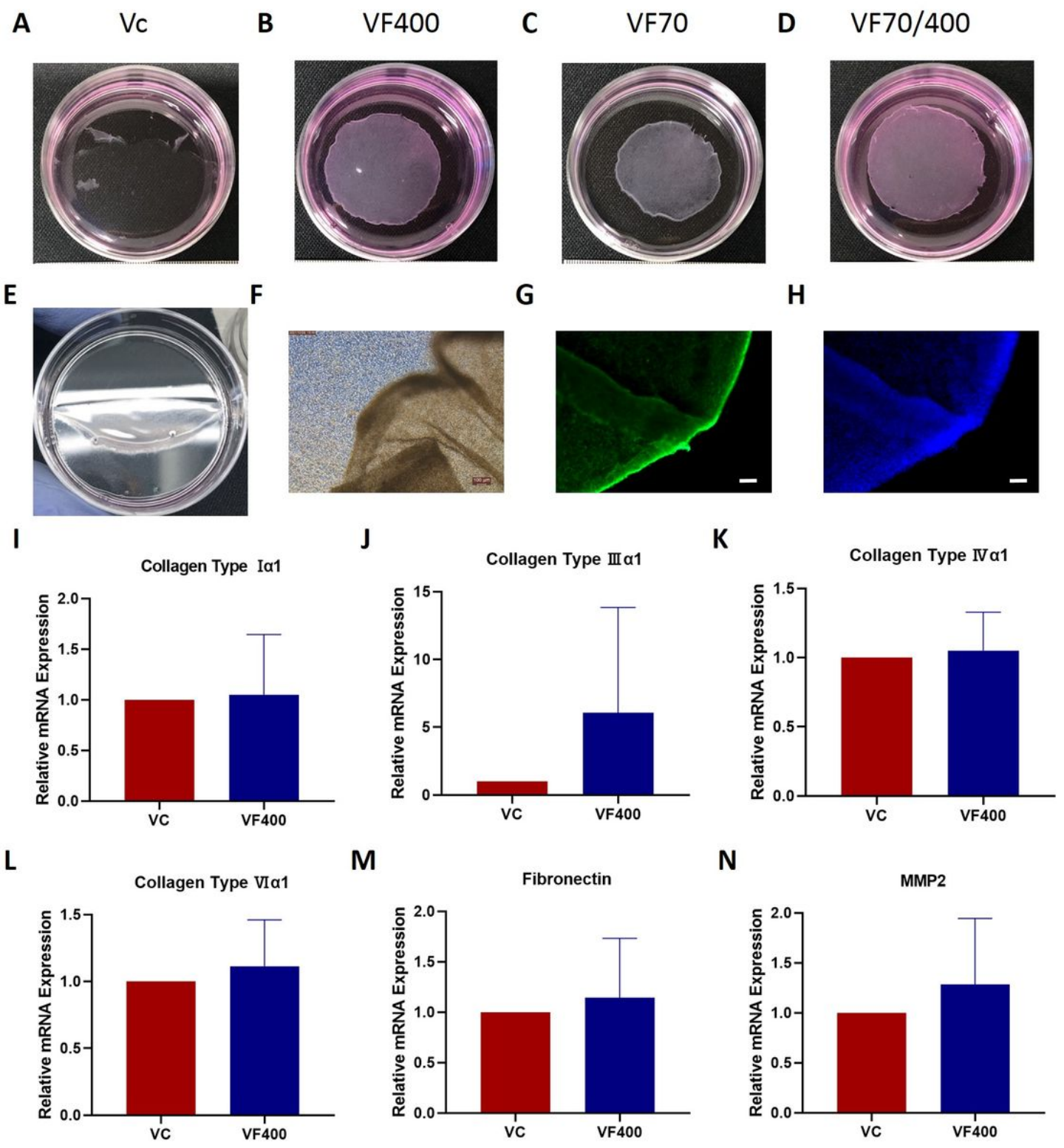

Figure 1

MMC reagents promoted cell sheet formation. Vc enabled detachment of some cell fragments, not intact cell sheets after 4 days culture (A). In contrast, VF400 (B), VF70 (C), VF70/400 (D) induced cohesive cell sheets formation. The MMC based cell sheets were robust and easy to be detached by pipetting (E,F).

Immunofluorescence staining images of fibronectin (Green) (G) and cell nucleus (DAPI, Blue) (H) showed that extracellular matrix and cells were completely detached from the cell culture dish and remained in the cell sheet (Scale bar=100 μm). There were no significant difference between VF400 and Vc in the gene expression for collagen I (I), collagen III (J), collagen IV (K), collagen VI (L), fibronectin (M), MMP-2 (N).

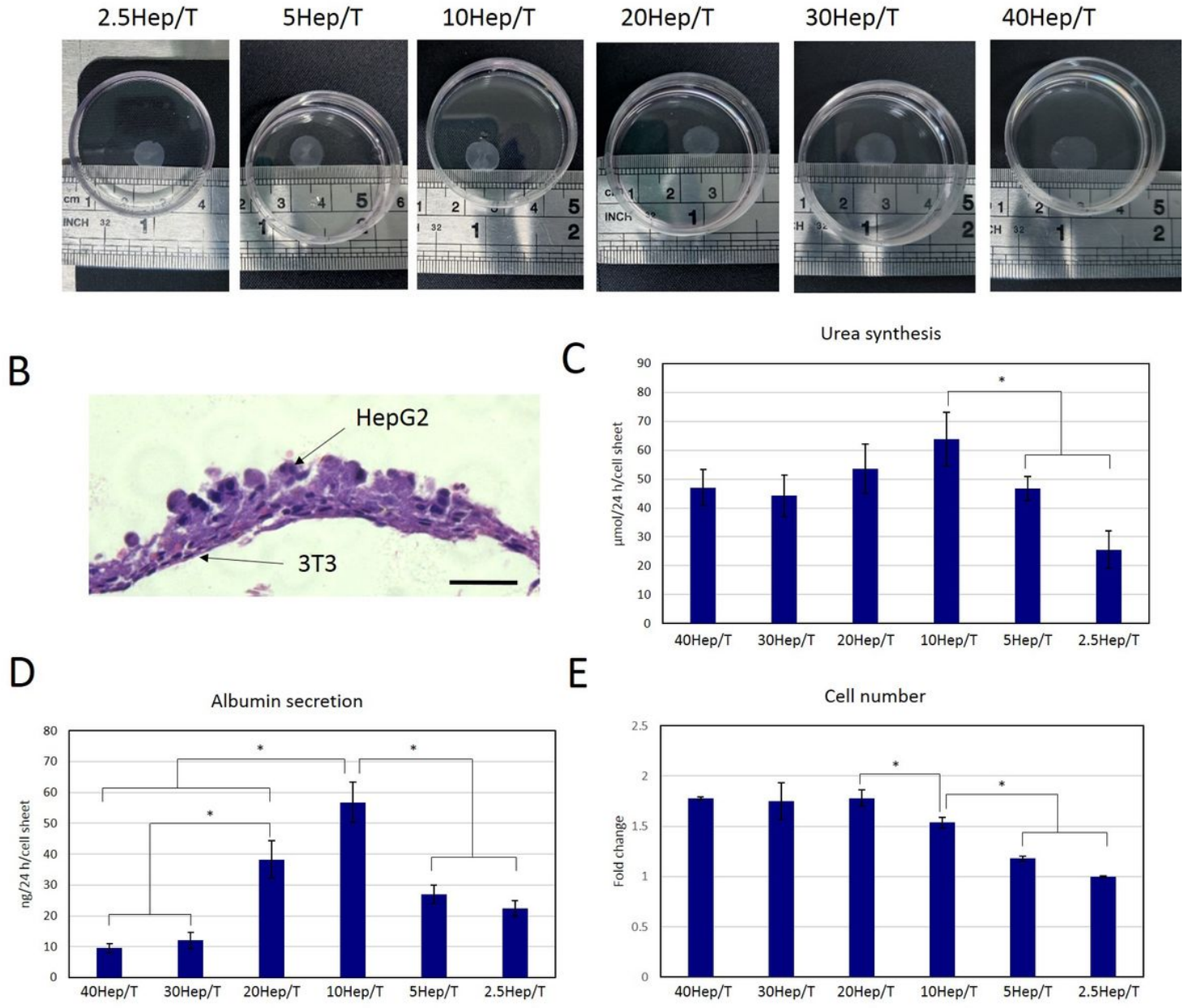

Figure 2

Functional hepatic cell sheet formation through MMC based method. (A) HepG2/3T3 co-culture cell sheets containing various HepG2 cells from 2.5×104cells/cm2 to 40×104 cells/cm2 (2.5Hep/T, 5Hep/T, 10Hep/T, 20Hep/T, 30Hep/T, 40Hep/T) were successfully fabricated. (B) HE staining images of cross-sections of HepG2/3T3 co-culture cell sheets. (C) Urea synthesis, (D) albumin secretion, and (E) cell number of various co-culture cell sheets. *$P < 0.05$.

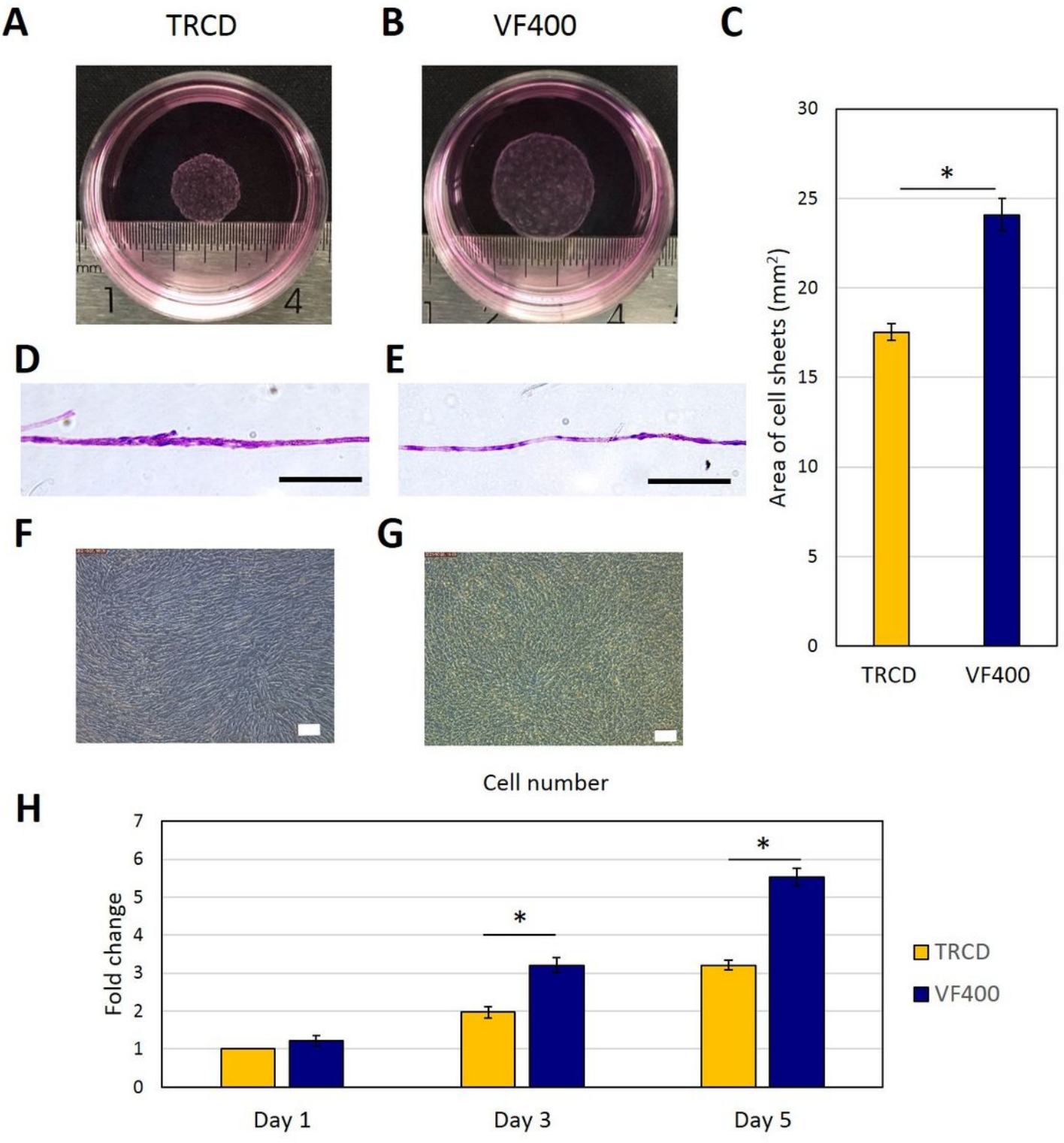

Figure 3

MMC induced hBMSC cell sheets. (A) Picture of hBMSC cell sheet harvested from TRCDs. (B) Picture of hBMSC cell sheet harvested from common cell culture dishes via MMC method. (C) Area of MMC induced cell sheets was 1.4 times larger than that of cell sheets harvested from TRCDs. (D-E) HE staining of cross-sections of hBMSC cell sheets. (F-G) Phase contrast microscopy images of hBMSC on TRCDs or MMC

treated hBMSC on common cell culture dishes. (H) CCK8 assay of hBMSC on day 1, 3, 5. *$P < 0.05$. Scale bar=100 μm.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Fig.S1.jpg
- Fig.S2.jpg
- Fig.S3.jpg
- TableS1.docx