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ABOUT COVER

Editorial Board member of *World Journal of Stem Cells*, Dr. Perez-Campo is currently an Associate Professor in the Department of Molecular Biology at the University of Cantabria (Spain). She obtained her degree in Biological Sciences from the University of Salamanca (Spain), where she then went on to complete her PhD in 1999. Dr. Perez-Campo undertook her postdoctoral research at the Paterson Institute for Cancer Research (United Kingdom; currently known as Cancer Research UK Manchester Institute) under the supervision of Prof. Lacaud, where she remained for more than 10 years working in the field of stem cell biology. Upon returning to Spain, she joined the University of Cantabria and focused her research efforts on the molecular mechanisms that control mesenchymal stem cell (MSC) differentiation towards the osteoblastic and adipogenic lineages, and how those mechanisms are altered in osteoporosis. (L-Editor: Filipodia)

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Practical choice for robust and efficient differentiation of human pluripotent stem cells

Mei Fang, Li-Ping Liu, Hang Zhou, Yu-Mei Li, Yun-Wen Zheng

ORCID number: Mei Fang 0000-0002-9435-3119; Li-Ping Liu 0000-0003-4445-8403; Hang Zhou 0000-0001-8659-5030; Yu-Mei Li 0000-0002-9501-5314; Yun-Wen Zheng 0000-0001-9002-3190.

Author contributions: Fang M, Liu LP, and Zheng YW designed the study; Fang M drafted the manuscript; Zheng YW, Li YM, Liu LP, and Zhou H contributed to reviewing and revising the manuscript; all authors approved the final manuscript; Fang M and Liu LP contributed equally to this work; Li YM and Zheng YW are senior authors and co-correspondents of this work.

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Mei Fang, Li-Ping Liu, Hang Zhou, Yu-Mei Li, Yun-Wen Zheng, Institute of Regenerative Medicine, Affiliated Hospital of Jiangsu University, Jiangsu University, Zhenjiang 212001, Jiangsu Province, China

Yun-Wen Zheng, School of Biotechnology and Health Sciences, Wuyi University, Jiangmen 529020, Guangdong Province, China

Yun-Wen Zheng, Department of Gastrointestinal and Hepato-Biliary-Pancreatic Surgery, University of Tsukuba Faculty of Medicine, Tsukuba, Ibaraki 305-8575, Japan

Yun-Wen Zheng, Yokohama City University School of Medicine, Yokohama, Kanagawa 234-0006, Japan

Yun-Wen Zheng, Division of Regenerative Medicine, Center for Stem Cell Biology and Regenerative Medicine, The Institute of Medical Science, the University of Tokyo, Tokyo 108-8639, Japan

Corresponding author: Yun-Wen Zheng, PhD, Associate Professor, Department of Gastrointestinal and Hepato-Biliary-Pancreatic Surgery, University of Tsukuba Faculty of Medicine, Tennodai 1-1-1, Tsukuba, Ibaraki 305-8575, Japan. ywzheng@md.tsukuba.ac.jp

Abstract

Human pluripotent stem cells (hPSCs) have the distinct advantage of being able to differentiate into cells of all three germ layers. Target cells or tissues derived from hPSCs have many uses such as drug screening, disease modeling, and transplantation therapy. There are currently a wide variety of differentiation methods available. However, most of the existing differentiation methods are unreliable, with uneven differentiation efficiency and poor reproducibility. At the same time, it is difficult to choose the optimal method when faced with so many differentiation schemes, and it is time-consuming and costly to explore a new differentiation approach. Thus, it is critical to design a robust and efficient method of differentiation. In this review article, we summarize a comprehensive approach in which hPSCs are differentiated into target cells or organoids including brain, liver, blood, melanocytes, and mesenchymal cells. This was accomplished by employing an embryoid body-based three-dimensional (3D) suspension culture system with multiple cells co-cultured. The method has high stable differentiation efficiency compared to the conventional 2D culture and can meet the requirements of clinical application. Additionally, *ex vivo* co-culture models might be able to constitute organoids that are highly similar or mimic human organs for

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potential organ transplantation in the future.

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Core tip: Identifying a practical way to efficiently differentiate pluripotent stem cells is essential in regenerative medicine. After considering the advantages and limitations of current approaches, we summarize the ideal conditions and systems. We also provide potential choices for efficiently and robustly differentiating human pluripotent stem cells into target cells and tissues in different germ layers.

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INTRODUCTION

The first five lines of human embryonic stem cells (hESCs) were obtained in 1998 from the inner cell mass of a 3- to 5-day-old fertilized embryo^[1]. Subsequently, induced pluripotent stem cells (iPSCs) were created by reprogramming fibroblasts^[2]. Human pluripotent stem cells (hPSCs), including hESCs and human (h)iPSCs, have the ability to self-renew and differentiate into any cell type from all germ layers^[3], driving the development of regenerative medicine. The cells and organoids derived from hPSCs have various potential applications including complex diseases studies, cell-based drug screening, and limitless transplantation treatments^[4,5]. With the rapid development of regenerative medicine technology, many differentiation approaches based on hPSCs have been explored. However, some challenges remain. To meet the needs of clinical application and basic research, high efficiency and stability are the key objectives during hPSC differentiation into high-quality target cells. Thus, it is important to identify an efficient and robust differentiation approach that can increase the differentiation ratio of target cells, produce stronger functions in cells, generate more complete structural organoids, or be reproduced in different cell lines or in other laboratories. Currently, there are great differences in these experimental schemes. Differentiation efficiency^[6] and stability are impacted by whether the method is based on an embryoid body (EB) or a two-dimensional (2D) or 3D system, or if single or multiple cell co-cultures are used.

In this review article, we combine the experiences of our laboratory with a summary of existing mainstream approaches involving hPSC differentiation, with the goal of providing a reference and time-saving guide for future experimental design.

DIFFERENTIATION INDUCTION FROM HPSCS

EB-based differentiation system

EB has been a very common model of *in vitro* hPSC differentiation for more than 50 years^[7]. The EB-based method is widely used to differentiate majority of cell lineages from the three germ layers (Table 1) and has an obvious advantage in improving the differentiation efficiency of some cells^[8-10], such as hematopoietic progenitors^[11] and melanocytes^[12]. Combined with suspension bioreactor technology, this advantage can be further amplified for large-scale production^[13]. Additionally, EB formation provides an excellent way to assess and manipulate developmental potential^[7]. Differentiation predictions can be made in the early stage of EB to predict which germ layer hPSC is likely to differentiate into, which can save on the cost for subsequent differentiation and indirectly improve differentiation efficiency. For example, Spalt like transcription factor 3 (*SALL3*) expression in EB indicates a high probability of differentiating into the ectoderm and a low chance of differentiating into the mesoderm/endoderm^[14]. Our study also confirmed these findings, and we found that iPSC lines that expressed

Table 1 Summary of current approaches for human pluripotent stem cells differential direction into targeted cells or tissues

Targeted cells or tissues	Cultural approaches				Check points of differential status			Ref.
	EB formation	2D or 3D	2D Surface or 3D system	Multiple cells co-culture	Gene expression	Protein level	Under <i>in vitro</i> or <i>in vivo</i>	
Neural progenitor	-	2D	Matrigel	-	<i>PAX6, TBR2</i>	-	<i>In vitro</i>	[21]
Brain	EB	3D	Low attachment plate, Matrigel	-	<i>PAX6, SOX2, FOXG1, TBR2, ARHGAP11B</i>	Nestin	<i>In vitro</i>	[21]
Midbrain	EB	3D	Matrigel	-	<i>SOX1, VMAT2, TH, NURR1, DAT, GIRK2, PITX3, AADC, ANLN, FAH, MBP, GLA35ST1, ACSL1, CLDN11, CHAT, MAPT, GFAP, S100B, ALDH1L1</i>	-	<i>In vitro</i>	[30]
Brain	-	3D	Microwell, bioreactor	Neuronal, astrocyte	-	-	<i>In vitro</i>	[54]
Retinae	-	3D	Matrigel	-	<i>SIX3, PAX6, RAX, OTX2, VSX2, PRKCZ, MITF</i>	TJP1, LAMB1, RHO, OPN1LW/OPN1MW, OPN1SW	<i>In vitro</i>	[31]
Retinae	EB	3D	Low attachment plate	-	<i>BRN3B, PAX6, RAX, SIX3, LHX2, CHX10, OTX2</i>	RHO, PKC α , Arl13b, OPN1SW, OPN1MW	<i>In vitro</i>	[37]
Retinae	-	3D	Matrigel	RPE, retinal organoid	<i>CRX, RCVRN, NRL, GNAT1, RHO, CHX10, OPN1LW/MW, OTX2, RLBP1, PROX1</i>	-	<i>In vitro</i>	[32]
Melanocyte	EB	3D	Microwell, low attachment plate	-	<i>MITF, PAX3, SOX10, KIT, TYR, TYRP, DCT, PEARL</i>	Melanin	<i>In vivo</i>	[12]
Hepatic stellate cells	-	2D	Matrigel	-	<i>NCAM, KDR, PDGFRα, P75NTR, ALCAM, ACTA2, COL1a1, LRAT, RELN, PCDH7, PDGFRβ, SYP, GFAP, PPARγ, NGF, α-SMA</i>	Desmin, PDGFR β , P75NTR, ALCAM, PDGFR α , CD73, KDR, NCAM	<i>In vitro</i>	[23]
Liver	EB	3D	Microwell	iPSC endoderm cell, HUVEC, BM-MSC	<i>ALB, G6PC, CYP2C9, CYP2C19, CYP3A4, CYP3A7</i>	CYP3A4, ALB, Urea, NTCP	<i>In vitro</i>	[52]
Liver	-	3D	Microwell	iPSC-tHE, iPSC-EC, iPSC-STM	<i>TBX3, ADRA1B</i>	AFP, ALB, Complement factor H, Coagulation factor VIII, Transferrin, AAT	<i>In vivo</i>	[50]
Intestinal	-	3D	Matrigel	-	<i>KLF5, ECAD, SOX9, Klf6</i>	Villin	<i>In vivo</i>	[33]
Entersphere	-	3D	Matrigel	Pan-epithelium cell, HLF, HUVEC	<i>SOX9, CK20, CDX2, NNKX2.1, LGR5, OLFM4, TACSTD2, VIL1, APOA1, FABP2</i>	E-cadherin, Cytokeratin18, α -SMA	<i>In vitro</i>	[34]
Cardiomyocyte	-	2D	Matrigel	-	-	TNNT2, ACTN2	<i>In vitro</i>	[67]
Cardiomyocyte	EB	3D	Low attachment plate	-	<i>TBX5, NKX2.5, GATA4</i>	TNNT2, TNNT3, MYH6, MYL7	<i>In vitro</i>	[47]
Cardiomyocyte	EB	3D	Suspension bioreactor	-	-	TNNT2, α -Actinin, MLC-2v, MLC-2a	<i>In vitro</i>	[13]
Heart	EB	3D	Matrigel	hESC-CPC, hESC-MSC, HUVEC	<i>KDR, MESP1, NKX2.5, TBX5, GATA4, ISL1, PDGFR-α, MEF2C, CD90, CD73, CD105, CD44, CD31, cTNT, β-MHC, MLC2v, KCNA4, KCNJ2, KCNH2</i>	-	<i>In vivo</i>	[35]
Hematopoietic cell	EB	3D	-	-	<i>RUNX1, SCL/TAL1</i>	CD34, CD43, CD45	<i>In vitro</i>	[68]

T Cell	-	2D	-	-	<i>TRA, TRB, RAG1, RAG2</i>	CD8ab, LMP2, TCR, TCRab-CD3	<i>In vivo</i>	[69]
Macrophage	EB	3D	Low attachment plate	-	<i>MAF, CSFR1, FLT3, CCR2</i>	CD14, CD45, CD11b, CD16, TNF-α	<i>In vitro</i>	[11]
Liver sinusoidal endothelial cell	EB	3D	Low-cluster plate	-	<i>CD31, CDH5, CD34, F8, STAB2, LYVE1, FLK1, FLT4, FCGR2B</i>	-	<i>In vitro</i>	[55]
Platelet	-	3D	Ultra-low attachment plate	-	<i>CD41a, CD13, CD42b, CD31, CD34, CD43, CD41b</i>	Thrombospondin4, Platelet factor 4	<i>In vivo</i>	[49]
Mesenchymal cell	-	2D	Matrigel	-	<i>CD146, CD73, CD140a, CD90, CD105, CD44, PDGFRβ, CSPG4, NES, LEPR, ADRB2, KITLG, IGFBP2, TNC, CXCL12, ADRB3</i>	-	<i>In vitro</i>	[26]

-. None; EB: Embryoid body; 2D: Two-dimensional; 3D: Three-dimensional; RPE: Retinal pigment epithelium; iPSCs: Induced pluripotent stem cells; HUVECs: Human umbilical cord vein endothelial cells; BM-MSC: Bone marrow mesenchymal stem cell.

higher levels of *SALL3* on day 7 of EB formation showed greater potential for melanocyte differentiation^[15]. Additionally, miR-371-3 plays both a predictive and functional role in neurogenic differentiation^[16], and the low expression of fibroblast growth factor 1 (commonly known as *FGF-1*), ras homolog family member U (commonly known as *RHOU*), and thymidine phosphorylase (commonly known as *TYMP*) genes are associated with low hepatic differentiation^[17], which can be used to predict the differentiation potential in early EB. Therefore, EB-based differentiation systems not only help to increase the percentage of target cells, but also contribute to the prediction of differentiation potentials in the early stage, which improves efficiency directly and indirectly, respectively.

Matrigel-mediated system

Matrigel, a natural extracellular matrix, is widely used in hPSC maintenance and can also be used in 2D and 3D hPSC differentiation (Table 1). During 2D differentiation, the culture vessels are first coated with Matrigel, followed by single cell or cell cluster inoculation. The role of Matrigel in 2D is adherence of cells or cell clumps to a culture vessel. Furthermore, the major component of Matrigel is laminin, which promotes the formation of a rigid neuroepithelial structure^[18]. Laminin-positive basement membranes are crucial for continuous epithelial integrity^[19]. A massive volume increase of the human neocortex results from expansion of the cortical area and the related emergence of extensive cortical folding^[20], which is thought to be due to the increase of the proliferative potential of neural progenitors (NPs)^[21]. As this study shows, two human ESC lines were differentiated into NPs in Matrigel-coated 2D adherent culture. Jaenisch and his colleagues constituted human cerebral organoids in an EB-based 3D system, which displayed markedly increased outgrowth of neuroepithelial tissue surrounding ventricle-like structures^[21]. Other desired cells can also be differentiated in a Matrigel-coated 2D culture system such as hepatocytes^[22], hepatic stellate cells^[23], intestinal epithelium^[24,25], mesenchymal cells^[26], cardiomyocytes (CMs)^[27], monocytes, and macrophages^[28]. Thus, the Matrigel-based 2D culture approach is a basic method for the directed induced differentiation of hPSCs.

Matrigel can also be used for 3D differentiation of hPSCs. In addition to coating the substrate, the Matrigel-based 3D construct is formed by adding mixed Matrigel and special differentiation medium^[29] in the hPSC differentiation process, resulting in differentiation in the solution of a suspended system. A 3D differentiation system provides enough space for establishing an organoid, and promotes cellular communication and interaction among cells compared to a 2D approach. Currently, many target cell lineages or tissues can be differentiated in this way including the brain^[30], retinae^[31,32], intestinal organoids^[33,34], and heart^[35]. Interestingly, after adding Matrigel, retinal induction cells increase by up to 30%-70% of the total cells in the low cell adhesion plate^[18]. Because this gel promotes the epithelialization of hPSCs toward retinal differentiation, researchers have tried to use 3D Matrigel methods for differentiating hPSCs. Epithelialized cysts are obtained by floating culture clumps of Matrigel/hESCs and the subsequent floating culture results in self-formation of retinal organoids^[31]. This includes patterned neuroretina, ciliary margin, and retinal pigment epithelium, which autonomously generates stratified retinal tissues, comprising photoreceptors with ultrastructure of outer segments in long-term culture. This system

has been validated in two lines of human hPSCs^[31]. Clearly, the use of Matrigel is common in 2D or 3D differentiation of hPSCs into target cells. However, the Matrigel-embedded 3D differentiation system has distinct advantages in self-organizing and generating organoids with a more complete structure when compared to a 2D culture.

3D suspension system

During hPSC differentiation, there are many decisions in creating a 3D floating state such as a non- or ultra-low attachment plate, microwell plate, and suspended bioreactors. At present, a variety of cell lineages have been generated by using 3D suspension system such as eye^[36-38], skin^[39], brain^[40-43], liver^[44,45], heart^[46,47] and blood^[11]. For example, during the 3D differentiation process, the authors generated iPSC-derived fully functional hepatocyte-like organoids in gene expression, protein secretion, and biotransformation^[48]. Likewise, iPSC-derived platelets can be harvested by using a 3D differentiation system, and it is very similar to human platelets in terms of both ultrastructural features and *in vivo* and *in vitro* functional characterizations^[49]. Thus, the 3D differentiation system can produce cells with ideal functions. The yield of differentiated cells is also important. The omni-well-array culture platform can produce massive and miniaturized iPSC-derived liver buds on a clinically relevant large scale ($> 10^8$)^[50]. Hama *et al.*^[13] designed a protocol that generated $> 90\%$ hiPSC-derived CMs that yielded on average 72 million cells per 100 mL in a 3D bioreactor. These results show that the yield from the 3D suspension system is remarkable in contrast to the 2D system. To test the reproducibility of the CM 3D differentiation protocol, a previous study compared biologically independent experiments with various passage numbers of iPSCs, and found minor inter-experimental variations^[13]. Overall, the 3D differentiation culture appears to have advantages in differentiation efficiency and stability over the 2D system. This indicates that the 3D differentiation method is optimal when hPSCs differentiation experiments are conducted.

Multiple cells co-culture system

Each organ has a variety of cell components with a certain structure and its own specific functions. Because of the communication and interaction among cells, co-culturing with different supportive and tissue-constructive cells has become attractive. The benefits of co-culturing multiple cells are that they can facilitate communication and interaction among different cells, enhance the hPSCs differentiation efficiency^[51], and better simulate the environment *in vivo*. It can bring surprises when used in a co-culture system to self-organize and generate an organoid. For example, to recapitulate hepatitis B virus-host interactions in liver organoids, Nie *et al.*^[52] co-cultured iPSC endoderm cells, human umbilical cord vein endothelial cells (HUVECs), and human bone marrow mesenchymal stem cells to form liver organoids in a 3D microwell plate, which exhibits stronger hepatic functions than iPSC-derived hepatic like cells. Furthermore, the co-culture pattern also has a higher differentiation yield^[48] and organoids with more complex functions^[53]. There are co-culture combinations in other studies such as co-culturing hPSC-derived neurons and astrocytes^[54]; co-culturing iPSC-derived hepatic parenchymal and non-parenchymal cells^[55]; co-culturing hiPSC-derived retinal pigment epithelium and retinal organoids^[32]; and co-culturing HUVECs, hESC-derived MSCs, and hESC-derived cardiac progenitor cells^[35]. Human PSC-derived organoids with multiple cell components have a complete structure and sturdy function similar to a human organ, which may provide an alternative source for organ transplantation. Therefore, the 3D culture method is a better choice for organoid generation.

Transcription factor-directed differentiation

Transcription factors (TFs) play an important role in pluripotent stem cell induction and transdifferentiation^[56]. Recently, they have been used to differentiate hPSCs into desired cells or tissues such as neural^[57], liver^[58,59], and cardiac muscle^[60,61]. A growing body of TF-directed differentiation method of hPSCs has demonstrated that efficient cell fate is reprogrammed *via* forced expression of single or multiple TFs^[62]. Sun *et al.*^[63] used the technique to design a single-step protocol for forebrain GABAergic neuron differentiation, which could generate cells similar to rodent cortical interneurons with $> 80\%$ efficiency, and the target cells showed mature functional properties within 6-8 wk. By contrast, other process takes as long as 30 wk^[64]. The TF-mediated method can differentiate hPSCs into terminal cells directly, and the experimental procedure is relatively brief.

LIMITATION AND CHOICE

Current methods for hPSC differentiation described above have various limitations. 2D differentiation culturing is performed on the surface of the culture vessel and the limited contact area limits the yield of the target cells. Furthermore, all structural components of organoids cannot be generated^[37,65]. Without 3D contact with Matrigel, Lowe *et al.*^[31] reported that most cells died and the few surviving cells formed solid cell masses on 2D culturing. Most 3D culture methods involve various intermediate stages requiring varying combinations of recombinant factors and small molecules^[63], thus rendering the method cumbersome to repeat. Although TF-mediated methods improve the differentiation efficiency of hPSCs, numerous tools for TF transfection, including plasmids and viruses, have led to the integration of exogenous genes^[66] into the target cells, thus presenting a remote prospects for their clinical application^[56]. In this situation, EB-based 3D culture systems allow for large-scale directional differentiation of hPSCs, and the co-culture method seems to constitute highly functional organoids *in vitro* to compensate for organ transplantation insufficiency.

CONCLUSION

Although only a few articles have compared the differences between 2D and 3D differentiation, it can be concluded that 3D system with EB has obvious advantages for hPSC differentiation compared to 2D culture. The details of the differentiation approaches are shown in the “cultural approaches” of Table 1. Regarding future studies, there are some key recommendations. First, the ability of EB not only can scale up culture systems and differentiation, but also predict the fate of hPSCs differentiation for reducing unnecessary waste. Second, the 3D differentiation system also has significant improvement in differentiation efficiency, and 3D space is necessary for organoid formation. Finally, it is a promising and challenging task that co-cultures multiple kinds of cells, supportive, structured, vascularized and further neurovascularized for organoid organization in 3D suspension system. Simply put, an EB-based 3D differentiation culture system is an efficient and powerful choice for hPSCs to meet the demand in clinical applications and basic research.

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