Reprogramming of human induced pluripotent stem cells and growth performance in 3D peptide hydrogel matrix system and potential applications

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Abstract

Human-induced pluripotent stem cells (hiPSCs) represent invaluable tools in biomedical research due to their exceptional versatility. This study delves into the intricate realm of hiPSC culture maintenance and application within three-dimensional (3D) systems, aiming to unravel how distinct culture conditions intricately shape cellular behavior. The investigation into hiPSC applications uncovers three notable advantages. First, it demonstrates the superior functional enrichment released from hiPSCs cultured in a 3D environment. Second, it highlights the efficacy of employing a 3D generation system for hiPSC induction from somatic and blood cells. Thirdly, the comprehensive comparison also encompasses mTeSR1 medium and E8 medium across various peptide hydrogel concentrations and configurations, including 3D embedded and 3D suspension peptide hydrogel matrix. These findings have significant implications for advancing the field of hiPSC cell research and its diverse applications in biomedical science. In particular, when scrutinized the conventional 2D iPSC generation system against the innovative 3D PGmatrix system, revealing significantly superior performance in hiPSC reprogramming. Importantly, the findings highlight the high efficiency of hiPSC cell harvesting, alongside the reduced time and energy requirements of the 3D hiPSC generation system compared to traditional 2D approaches. Additionally, we demonstrate that culture conditions adaptable for cell growth can be accommodated within the 3D system while maintaining high cell proliferation, viability, and pluripotent gene expression. In summary, this research significantly advances our comprehension of how hiPSCs respond to the intricate milieu of 3D culture conditions, holding the potential to optimize hiPSC applications in various facets of biomedical research, ultimately propelling advancements in regenerative medicine, disease modeling, and drug development.

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Chapter 1 – Literature Review

1.1 Induced Pluripotent Stem Cell (iPSC)

1.1.1 Development of iPSC

Pluripotency, defined as the remarkable property of cells to differentiate into various cell types (Evans and Kaufman, 1981). Embryonic stem cells (ESCs), derived from embryos, naturally possess this distinctive property, and exhibit the capacity for self-renewal (Martin, 1981). Fibroblasts and T-lymphocytes can undergo epigenetic reprogramming upon fusion with ESCs, leading to the expression of pluripotency genes and conferring pluripotent stem cell (PSC) characteristics (Cowan, et al., 2005). Somatic cells have been found to exhibit functional pluripotency when they are induced with specific reprogramming factors and upon induction, these somatic cells adopt a behavior similar to embryonic stem cells (ESCs) and are referred to as induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka, 2006). Through bioinformatics screening of embryonic stem cells (ESCs), genes with specific functions related to cell proliferation and differentiation were identified. These transcripts, known as ESC-associated transcripts (ECATs), were then introduced into mouse fibroblast cells via retroviral transduction. The four transcription factors, namely OCT3/4, SOX2, KLF4, and c-MYC, proved instrumental in inducing pluripotency. These factors were subsequently recognized as critical components in the reprogramming process and named as OSKM or Yamanaka factors (Takahashi and Yamanaka, 2006).

The initial investigations into the reprogramming capabilities of the OSKM factors involved the use of murine parental cells. Subsequently, the successful reprogramming of various human cell types was confirmed, including somatic cells such as fibroblasts, peripheral blood cells (e.g., PBMC, T cells, B cells, CD34+), and hematopoietic stem cells into iPSCs (Hanna, et al., 2008, Nakagawa, et al., 2008, Loh, et al., 2009, Seki, et al., 2010). Acquisition of fibroblasts typically necessitates skin biopsies, while obtaining blood cells offers a less complex and minimally invasive approach. Moreover, human iPSCs have been established from keratinocytes isolated from hair follicles, mesenchymal stem cells from teeth and fat tissue, as well as renal epithelial cells in urine samples (Maherali, et al., 2008, Sun, et al., 2009, Zhou, et al., 2011). Reprogramming efforts have also extended to various human cell types, including but not limited

to cord blood cells, amnion cells, organ cells from the digestive system (such as stomach and liver cells), neural stem cells, progenitor cells, and melanocytes (Aoi, et al., 2008, Sarah, et al., 2008, Utikal, et al., 2009, Jinglei, et al., 2010, Okita, et al., 2013). Furthermore, reprogramming has been achieved in other mammalian cell lines, such as bovine, canine, caprine, equine, lagomorph, simian, avian, and ichthyoid species (Liu, et al., 2008, Ezashi, et al., 2009, Honda, et al., 2010, Nagy, et al., 2011, Ren, et al., 2011, Sumer, et al., 2011, Rossello, et al., 2013). This progressive expansion of the reprogramming methodology to various cell types and species, from in vitro to in vivo, has paved the way for advancements in regenerative medicine and preclinical studies. The continued advancement of these studies holds the potential to translate into tangible therapeutic applications, leveraging iPSCs for in vivo tissue regeneration and disease modeling.

1.1.2 Methods for reprogramming

The reprogramming of somatic cells or blood cells into iPSCs encompasses two primary categories: viral system transduction and non-viral based transfection methods. Viral vectors, recently well-established such as lentivirus, adenovirus, and Sendai virus, are commonly employed in the former. The initial study conducted by Yamanaka and Takahashi (2006) employed retrovirus vectors for the introduction of exogenous DNA into cells. The efficiency of retroviral transduction is contingent upon a high titer; however, the overall efficiency has been reported to range between 0.01% and 0.5% (Okita, et al., 2007). To enhance reprogramming efficiency and minimize heterogeneous cell aggregation, lentivirus-based systems were developed (Papapetrou, et al., 2009). The reprogramming efficiency of lentivirus lies between 0.1–1.5% (Somers, et al., 2010). Viral transduction is limited to non-clinical research due to the potential reactivation of transgenes, particularly c-myc, which can lead to tumor formation (Okita, et al., 2007). While Sendai virus (SeV), an RNA vector, represents an alternative that leaves no genetic footprint and facilitates transgene-free induction of human pluripotent stem cells. Reprogramming efficiency for Sev ranges in 0.1-1% (Fusaki, et al., 2009). Non-viral approaches commonly employ episomal DNA vectors, such as transposons PiggyBac and CRISPR activators (Woltjen, et al., 2009, Warren, et al., 2010), and the reprogramming efficiency for PiggyBac is less than 0.02% (Mali, et al., 2010). These non-viral strategies offer alternative avenues for iPSC reprogramming, circumventing the limitations associated with viral methods. Additionally, reprogramming of human iPSCs has been

achieved through the modification of synthetic mRNA, bypassing innate antiviral responses via the interferon and NF-κB-dependent pathway (Warren, et al., 2010). The transfection of mRNA resulted in the reprogramming of 0.002% of the total cell population (Miyoshi, et al., 2011). Small molecule compounds have been identified to increase the efficiency of cellular reprogramming. Notable examples include the DNA methyltransferase inhibitor, histone deacetylase inhibitors (such as valproic acid), and the ROCK pathway inhibitor (Kishigami, et al., 2006, Huangfu, et al., 2008, Noggle, et al., 2011). Histone deacetylase inhibitors have been observed to impede the TGFβ and MEK signaling pathways (Lin, et al., 2009). Combining small molecule compounds with reprogramming methods has shown promising enhancement of efficiency and colony yield in reprogrammed cells. For instance, the utilization of a doxycycline (dox)-inducible lentiviral engineered system has enabled highly reproducible kinetics and efficiency in reprogramming human and mouse fibroblast cells (Wernig, et al., 2008). The overall reprogramming efficiency for the dox-induced transgenic system can be approximately around 2-4%.

1.1.3 Sendai virus

Sendai virus (SeV) is an enveloped respiratory virus that belongs to the Paramyxoviridae family and classified as mouse parainfluenza virus type I. SeV is primarily transmitted through aerosolized particles and contact with respiratory secretions. The virus exhibits a high degree of contagiousness, facilitating its rapid spread among susceptible hosts. The animal species have been identified as susceptible hosts for SeV infection are mouse, rat, hamster, and guinea pigs. The genome of Sendai virus (SeV) is a nonsegmented single-stranded RNA molecule, encompassing 15,384 nucleotides. The six major genes of the virus code for nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN) and large protein (L). The nucleocapsid protein of Sendai virus (SeV) is responsible for forming the core nucleocapsid complex via binding to the viral genome RNA. Concurrently, the matrix protein functions in supporting the envelope structure of the virus from the interior. The fusion protein facilitates the fusion of the viral envelope with the host cell membrane during viral entry. The hemagglutinin-neuraminidase protein recognizes the cell surface receptor, specifically sialic acid. Lastly, the large protein serves as the large subunit of the RNA polymerase within the viral

structure. The virulence component of the virus is the fusion protein, which fuses to the surface of various cell types after activation (Lamb and Kolakofsky, 1996).

Upon removal of the gene encoding the fusion protein, the SeV loses its ability to generate progeny viral particles, rendering it non-virulent. This distinctive variant for research was first identified through the isolation of a mutant capable of persistently infecting cells even under nonpermissive conditions, particularly at an elevated temperature of 38 °C (Yoshida, et al., 1979). Consequently, the F-depleted SeV represents a safe tool for research and an efficient vector for delivering foreign genes into various cell types. Throughout the transfection process, the replicating virus remains confined to the cytoplasm, preventing the integration of the vector's genetic information into the host chromosome (Li, et al., 2000). Leveraging its advantages and notable expression efficiency, the modified SeV has emerged as a novel system for establishing pluripotency in differentiated tissue cells. This system facilitates the delivery and sustainable expression of reprogramming factors, allowing for the subsequent replacement of exogenous reprogramming factors with their endogenous counterparts to establish autoregulated pluripotency (Nishimura, et al., 2011). This transition from exogenous to endogenous factor expression promotes a more natural and enduring pluripotent state in the reprogrammed cells. The non-integrative nature of transgenes and the lack of infectivity in humans render SeV a promising vector safer for clinical research. SeV is known as a potentially valuable tool for therapeutic application.

1.1.4 Reprogramming factors and pluripotency gene markers

The generation of iPSC is a sophisticated process that surpasses initial predictions, involving a comprehensive interplay of genetic and epigenetic factors. Genetic factors encompass the selection and delivery of specific reprogramming factors, whereas epigenetic factors govern gene expression patterns and chromatin modification during the reprogramming process. iPSCs possess the unique ability to undergo self-renewal in culture while maintaining their capacity to differentiate into various cell lineages encompassing the full range of cell types (Takahashi and Yamanaka, 2006). The epigenetic mechanisms are chromatin modification and remodeling, which involves DNA methylation, histone modification including acetylation, phosphorylation, methylation and ubiquitylation. Dynamic changes in DNA methylation occur during the early

stages of embryonic development, whereby the genome undergoes a process of genome-wide demethylation following fertilization, followed by subsequent remethylation after the blastocyst implants. Demethylation and remethylation play an indispensable role in the reprogramming of the genome, facilitating proper developmental processes (Li, 2002). The intricate interplay between these genetic and epigenetic factors is pivotal in achieving successful iPSC generation, highlighting the complex and multifaceted nature of this transformative technology.

Oct3/4, Sox2, and KLF4, the three positive regulators, were identified as critical factors in the generation of iPSCs and the maintenance of pluripotency (Takahashi and Yamanaka, 2006). The transcription factor GLIS1 can serve as a substitute for c-MYC in the reprogramming process (Maekawa, et al., 2011), and NR5A2 could replace Oct3/4 during reprogramming (Heng, et al., 2010). SALL4 is the transcription factor that has been identified as a significant enhancer of reprogramming efficiency. (Tsumaki, et al., 2015). In conjunction with the OSKM factors, the inclusion of NANOG and LIN28 has been shown to significantly enhance the successful reprogramming of human somatic cells into iPSCs (Yu, et al., 2007). In the case of LIN28, the inhibition of the p53/p21 signaling pathway, coupled with the overexpression of Lin28, has been found to promote the reprogramming process (Hanna, et al.). The activation of Nanog expression achieved by inhibiting the transforming growth factor (TGF)-β type 1 receptor, ALK5, has been demonstrated to facilitate iPSC reprogramming, serving as an alternative to Sox2 (Ichida, et al., 2009). Notably, c-MYC was found to enhance reprogramming efficiency (Nakagawa, et al., 2014), but previous research indicated that c-MYC was not essential for cell reprogramming (Nakagawa, et al., 2008). The presence of c-MYC gene, however, posed a risk of promoting oncogenic properties and potentially leading to tumorigenicity (Okita, et al., 2007). It was further recognized that c-MYC primarily regulates cell proliferation rather than pluripotency (Scognamiglio, et al., 2016). Cyclin D1 plays a crucial role in regulating cell proliferation and apoptosis, making it a promising alternative in enhancing reprogramming efficiency, which has substituted c-MYC during the reprogramming (Edel, et al., 2010). Suppression of the TP53 has been shown to be an alternative approach for substituting c-MYC in the reprogramming process, enhancing the reprogram efficiency (Hong, et al., 2009).

Among the well-studied pluripotent genes that have captured the interest of researchers are Sox2, Oct3/4, Rex1, Nanog, Lin28, Utf1, hTERT, DNMT3B, Dppa4, GDF3, and others. The transcription factor Sox2 holds significant importance in the maintenance of pluripotency, as it

exerts an influential impact on activating the expression of pluripotent genes while concurrently repressing the expression of genes associated with cellular differentiation (Zhang and Cui, 2014). Oct3/4 is a widely acknowledged and prominent regulator of pluripotency found within the inner cell mass of the mammalian blastocyst and embryonic stem cells. The role extends to the maintenance of pluripotency and self-renewal in somatic stem cells (Baek, et al., 2020). Rex1, also known as zfp42, is a zinc finger protein that exhibits primary expression in undifferentiated stem cells and teratocarcinoma cells. This gene has been identified as a marker for pluripotency and regulated by Nanog and Sox2 (Shi, et al., 2006). NANOG, an essential transcription factor, regulates cell differentiation by activating the repressors and suppressing the activators of differentiation (Gawlik-Rzemieniewska and Bednarek, 2016). Lin28, a transcription factor involved in pluripotency, exerts regulatory control over cell differentiation by facilitating cell division and promoting the formation of iPSCs (Hanna, et al., 2009). Embryonic cell transcription factor 1 (Utf1) serves as a prominent hallmark for pluripotency, which expression is robust in undifferentiated cells, but rapidly extinguishes as cells initiate the process of differentiation. This dynamic gene expression pattern of Utf1 highlights its crucial role in maintaining the pluripotent state and its subsequent downregulation during cellular differentiation (Okuda, et al., 1998). Human telomerase reverse transcriptase (hTERT) has been identified as a crucial factor impacting telomerase activity, cell lifespan extension, and telomere length elongation (Vaziri and Benchimol, 1998). Oncogene research on the function of hTERT has revealed its impact on longevity, immortalization, and rejuvenation both in vitro and in vivo (Leao, et al., 2018). DNA methyltransferase 3 Beta (DNMT3B) encodes de novo DNA methyltransferases and has high expression in undifferentiated ES cells and no expression in differentiated cells (Okano, et al., 1998). In the epigenetic regulation of genomic imprinting, X inactivation, and genome reprogramming, DNA methylation and histone modification interact synergistically while DNMT3B activates (Li, 2002). Developmental pluripotency associated 4 (Dppa4) is one of the specific factors for embryonic/stem cells, which functions in regulating epigenome by suppressing the somatic genes and activating the ESC enhancer to promote the transition to pluripotency (Hernandez, et al., 2018). Growth Differentiation Factor 3 (GDF3) inhibits the BMP-GDF (bone morphogenetic protein) subfamily of TGFβ ligands and maintains the pluripotency in stem cells (Levine and Brivanlou, 2006).

1.1.5 Application in life science and clinical trail

The emergence of iPSCs has ushered in a transformative era in disease research and clinical investigations, offering a versatile toolkit encompassing disease modeling, gene therapy, drug screening, and toxicity assessment (Inoue, et al., 2014). Notably, iPSCs have found particular relevance in the realm of neurodegenerative diseases such as Alzheimer's, Parkinson's, and frontotemporal lobar degeneration due to their remarkable differentiative potential, notably giving rise to neurons and astrocytes. This pluripotent capacity constitutes a cornerstone for the exploration of disease mechanisms and the development of potential therapeutic interventions. The generation of iPSCs entails the reprogramming of adult somatic cells, like dermal fibroblasts, into a pluripotent state akin to embryonic stem cells, thereby providing researchers with an inexhaustible source of patient-specific cells, thereby facilitating the detailed dissection of disease mechanisms, drug screening, and personalized medicine applications (Leng, et al., 2022).

Crucially, the versatility of iPSC applications spans the spectrum from controlled in vitro studies to potential in vivo implementations. iPSCs offer a biologically safe and immune-compatible platform, derived from the patient's own cells, effectively mitigating concerns related to host immune rejection. By harnessing iPSCs originating from individuals afflicted with specific maladies, researchers are empowered to establish meticulously controlled disease models within the laboratory milieu, yielding invaluable insights into disease progression, drug response profiles, and tailored therapeutic strategies. iPSCs can be directed towards differentiating into specific cell types afflicted by the disease under investigation, a salient example being the generation of neurons to study neurodegenerative disorders such as Alzheimer's. This capacity stands as a pivotal asset for exploring disease-associated genetic mutations and distinctive phenotypic traits, thereby advancing our comprehension of disease pathophysiology and forming the basis for precision-targeted therapeutic approaches. The application of iPSC in 3D printing represents a cutting-edge intersection of regenerative medicine and advanced manufacturing techniques, and iPSCs can also be used to generate functional tissues and organs for transplantation, such as liver organoids or skin (Li, et al., 2022). In a recent illustrative study (Drager, et al., 2022), iPSC-derived microglia were leveraged to scrutinize the functional consequences of genetic perturbations in microglia, pivotal players in the landscape of neurological diseases.

Nonetheless, the application of iPSC is encumbered by several inherent challenges. Differentiation efficiency and maturation pose prominent concerns within this context. Notably, there exist notable variations in the efficacy of differentiation and the degree of maturation among distinct iPSC clones. These disparities can be attributed to multifarious factors, encompassing incomplete reprogramming processes, inherent genetic background disparities among iPSC lines, the persistence of epigenetic memory, and variations in X-chromosome inactivation patterns (Nishimura, et al., 2013). Encouragingly, various differentiation protocols and culture conditions hold the potential to ameliorate the influence of epigenetic memory associated with distinct parental cell sources, rendering it a versatile and adaptable choice within contemporary stem cell research endeavors (Kim, et al., 2011). Furthermore, achieving robust differentiation or the purification/enrichment of target cells remains an ongoing challenge. While strategies employing cell-specific promoters or cell-surface antigens have enabled the isolation of target cells with a certain level of maturation, attaining absolute cellular purity remains an elusive goal (Nishimura, et al., 2013). Additionally, temporal and financial constraints cast a shadow on the application of iPSCs. The current methods for iPSC generation necessitate a temporal investment exceeding three months, rendering them suboptimal for the expeditious treatment of specific disorders (Qian, et al., 2012). Moreover, the utilization of autologous iPSCs sourced from individual patients would inevitably result in substantial medical expenditures, further underscoring the cost-related challenges associated with iPSC-based therapies. These multifaceted impediments underscore the need for continued research and innovation to overcome the existing limitations and unlock the full potential of iPSCs in clinical and research domains. Modified protocols and culture conditions, in conjunction with pioneering methodologies, are poised to chart a path towards enhanced iPSC applications.

1.1.6 Methods for detection of iPSCs

Despite overcoming immunogenicity and ethical challenges, the application of hiPSCs into a clinical setting necessitates ensuring that the final product does not give rise to tumors after implantation (Ben-David and Benvenisty, 2011). Products derived from human pluripotent stem cells may harbor residual undifferentiated cells, which can subsequently proliferate and form teratomas (Knoepfler, 2009). Hence, the development of a highly sensitive detection method to

identify remaining undifferentiated stem cells in the final product and establish their detection limit (LOD) is of utmost importance.

Flow cytometry, which employs specific antibodies recognizing antigens labeled on stem cells, can identify residual undifferentiated iPSCs with a sensitivity of 0.1%. However, it is criticized for its time-consuming nature and its limitation to cells expressing the labeled gene (Kuroda, et al., 2012). Another method for iPSC detection is the GlycoStem sandwich assay system, an enzyme-linked immunosorbent assay technology that uses rBC2LCN to detect soluble highly glycosylated podocalyxin. This protein is specifically secreted by iPSCs into the culture medium, enabling the quantification of hiPSCs by analyzing the cell culture supernatant (Tateno, et al., 2014). Alternatively, iPSCs can be identified by measuring iPSC-specific RNA using polymerase chain reaction (PCR). Compared to other methods, PCR-based approaches offer the potential advantages of high sensitivity (Sato, et al., 2019) without additional treatment or culture steps. In mice, several studies have used specific markers to detect the iPSC, such as SSEA-1, EpCAM, PECAM, and c-Kit (Polo, et al., 2012). A SOX2-transgene, linked with enhanced green fluorescent protein and an internal ribosome entry site, was utilized for the identification of iPSCs to investigate the molecular processes involved in human iPSC generation (Tanabe, et al., 2013).

Employing lin-28 homolog A (LIN28) as the target gene, qRT-PCR assays can effectively detect 0.002% undifferentiated hiPSCs in retinal pigment epithelial (RPE) cells induced by hiPSC (Kuroda, et al., 2012). However, the number of cells hiPSC derived cells needed for treatment depends on the disease. For RPE cell transplantation, approximately $5\times10^{\wedge4}$ RPE cells are necessary, and the sensitivity of qRT-PCR is adequate for quality control. Nevertheless, a patient with heart failure demands a minimum of $10^{\wedge9}$ cardiomyocytes (Laflamme and Murry, 2005), suggesting that utilizing the LIN28/qRT-PCR method for cardiomyocyte products derived from hPSCs might result in false-negative outcomes. Hence, achieving higher detection sensitivity is imperative for maintaining the quality of myocardial cells derived from hiPSCs.

Digital PCR (dPCR), as the third-generation PCR technology, provides a novel approach for nucleic acid detection and quantification. Since the introduction of the concept of dPCR by Vogelstein and Kinzler in 1999 (Vogelstein and Kinzler, 1999), the technology has rapidly advanced and demonstrated successful applications in clinical diagnosis, quantification of genetically modified components, single-cell gene expression, environmental microbial detection,

and various other fields. dPCR divides the PCR system into numerous isolated reaction units (mostly exceeding 10,000), such as microchips or droplet compartments. Ideally, each reaction chamber or droplet contains mostly one target nucleic acid molecule. In the fluorescence signal analysis stage, terminal detection is employed to gather the fluorescence signals of each reaction unit. In comparison to qPCR, dPCR offers higher accuracy, sensitivity, and repeatability. It is easily standardized, suitable for low copy number nucleic acid detection, and exhibits higher tolerance to inhibitors, thereby mitigating detection bias arising from the template matrix (Hindson, et al., 2011). With dPCR technology, the LIN28 signal can be detected when adding 0.001% hiPSC to myocardial cells, a capability not achievable with qRT-PCR (Kuroda, et al., 2015).

For analyses using RT-qPCR and RT-dPCR, the expression level of the target gene is significantly higher in iPSCs compared to iPSC-differentiated cells. LIN28, a well-known stem cell marker, has been utilized to identify residual iPSCs across various cell types (Kuroda, et al., 2012). However, the substantial expression of LIN28 during liver differentiation renders it unsuitable for detecting residual iPSCs in liver lineage cells. Therefore, embryonic stem cell-related genes (ESRG), cartilage regulatory protein (CNMD), and secreted frizzled-related protein 2 (SFRP2) are considered more appropriate markers (Sekine, et al., 2020). In summary, it is crucial to thoroughly validate any newly proposed markers, considering variations stemming from different iPSC sources (Masaki, et al., 2008). Moreover, given the previously mentioned heterogeneity, the simultaneous detection of several validated marker genes is preferable to relying on a single gene.

1.2 3D Culture System

1.2.1 Development of 3D culture system

Over the last few decades, traditional two-dimensional (2D) cell culture has been widely employed in life sciences to study various cell types in vitro and for drug screening and testing purposes (Lee, et al., 2023). However, researchers have increasingly recognized that the physiological state and activity of cells cultured in 2D do not fully recapitulate those observed in vivo (Grace N. Li, 2007). The simplicity of the 2D cell culture model limits its ability to accurately model the intricate in vivo environment and complex cellular processes. Key factors, such as cell signal transduction, chemical gradients, and spatial structural changes, cannot be faithfully

represented in the 2D model. Consequently, results obtained from 2D culture often diverge from findings in animal and clinical experiments (Wong, et al., 2007, Huang, et al., 2015). Recognizing these limitations, researchers have increasingly realized the need to simulate important characteristics of the in vivo microenvironment in cell culture, including cell-cell interactions, cell-extracellular matrix interactions, and cell-organ interactions (Sato, et al., 2009, Sato, et al., 2011). In response, significant efforts have been devoted to developing new culture methods, particularly three-dimensional (3D) culture technology. This innovative approach aims to replicate the actual microenvironment in which cells grow in vivo, allowing for a more accurate representation of cell morphology, structure, and physiological function in vitro (Sachs, et al., 2018). In contrast to the traditional 2D monolayer culture, the 3D system offers a more conducive growth environment that enables cells to proliferate more robustly, extending the exponential growth phase (Liang, et al., 2017). For successful 3D culture, appropriate materials are critical to support and maintain cells and organoids. These materials create a 3D environment that supports cell adhesion, proliferation, and differentiation (Breslin and O'Driscoll, 2013). They not only mimic the extracellular matrix but also facilitate intercellular signal transduction. Additionally, they provide binding sites for cells, triggering biological reactions and promoting normal cell growth and gene expression. Porosity is another essential characteristic, enabling the provision of living space and facilitating the infiltration, diffusion, and transportation of nutrients, oxygen, and other bioactive substances necessary for cell growth. Over time, cells proliferate and mature within the 3D culture system, forming a structure that closely resembles the tissue of origin (Haycock, 2011). Currently, hydrogels and biopolymer microfiber materials are predominantly used in 3D culture systems to replicate the complexity of the microenvironment.

1.2.2 Constituents for 3D culture system

Hydrogel, composed of water, extracellular matrix (ECM) proteins, and growth factors, exhibits excellent mechanical properties, closely resembling the supportive strength of natural tissues. As a result, hydrogel has become a widely used scaffold material in 3D cell culture. The first generation of ECM polymers used in 3D cell culture was extracted from the basement membrane of mouse sarcoma cells and marketed as Matrigel (Orkin, et al., 1977, Kleinman and Martin, 2005). However, the uncertainty in its composition affects the biochemical and genetic

characteristics of cells (Jee, et al., 2019). Inconsistencies in biochemical and mechanical properties between different batches of Matrigel lead to a lack of experimental consistency. Additionally, Matrigel may carry potential exogenous contamination, such as the lactate dehydrogenase-elevating virus (LDV), which can affect host immune microenvironment regulation and trigger tumor behavior (Plagemann and Moennig, 1992). As a result, the applicability of Matrigel in therapeutic cell manufacturing and drug discovery may be limited. Meanwhile, developed thus far are numerous 3D cell culture systems, hydrogels emerge as exceptionally promising for their potential applications in 3D cell culture (Xu, et al., 2019).

In recent advancements, a new generation of hydrogels based on polypeptide materials has emerged, meeting specific culture environment requirements, and offering optimal choices for different cell types and applications. These hydrogels serve as ECM mimics, providing a porous structure capable of storing nutrients and soluble factors like cytokines and growth factors. They also present attachment sites, such as fibronectin, collagen, and laminin, to promote cell differentiation (Tabriz, et al., 2015). Hydrogels used in 3D printing applications, for example, require adhesive properties, solidifying without deformation after extrusion within a specific time frame (Compaan, et al., 2017). The presently developed PGmatrix exhibits adjustable attributes including gel strength, viscosity, and self-healing kinetics, rendering it amenable for various applications such as injection, liquid transfer, or 3D bioprinting. Importantly, it obviates the necessity for post-printing crosslinking through ultraviolet or visible light, as well as the use of supplementary chemical agents, thus establishing its versatility and ease of use in academic and research settings (Li, et al., 2021).

Acellular matrix refers to a process that involves the removal of cellular components from human or animal tissues or organs through various techniques, such as physical, chemical, and enzymatic treatments (Bejleri and Davis, 2019, Fiordalisi, et al., 2020). This process results in the retention of the extracellular matrix and tissue structure, which can be utilized for scaffold preparation. The use of natural tissues or organs is preferred over synthetic materials due to the elimination of immunogenic substances like cells and DNA, as well as the preservation of the original morphological structure and physical properties of organs (Jung, et al., 2016). Furthermore, acellular matrix retains crucial cell-to-cell recognition binding sites that are rich in growth factors and cell adhesion signals, promoting the growth, proliferation, and differentiation of cells (Hussein, et al., 2016). The sources of acellular organs and tissues include not only discarded human organs

or marginal organs unfit for transplantation but also foreign animal organs, particularly those that do not provoke an immune response in the host (Hussein, et al., 2016). However, it is important to note that decellularized tissues may experience a decline in mechanical properties, and the removal of endothelial cells can potentially lead to thrombosis (Conklin, et al., 2002). To address these limitations, acellular matrix is often modified for cell culture scaffolds, with common modification methods involving heparinization or the addition of chitosan (Qi, et al., 2011).

In the field of 3D cell culture scaffolds, various materials are used in combination with cell encapsulation materials to replicate the form of tissues. Adding fibers or particles enhances the strength of hydrogels and serves as a structural framework for cell adhesion. Natural tissue engineering polymers like collagen, fibrin, sodium alginate, and chitosan demonstrate excellent biocompatibility without causing side effects, promoting cell adhesion, proliferation, and minimizing the risk of immune rejection (Li and Henry, 2011, Kong, et al., 2021, Xiong, et al., 2021). Synthetic polymers such as polylactic acid (PLA), polycaprolactone (PCL), and polyurethane (PU) are commonly used materials (Zhao, et al., 2021, Zhang, et al., 2022). PLA, a thermoplastic polymer, offers favorable mechanical properties and biodegradability, making it widely employed in the preparation of vascular grafts. It provides mechanical support through gradual degradation in the body, with its final degradation products being water and carbon dioxide (Enrico, et al., 2015, B, et al., 2018). To leverage the advantages of different materials, composite scaffolds are often used, incorporating two or more significantly distinct substances. Composite materials allow for the synergistic utilization of the characteristics of multiple materials, fulfilling both mechanical and physiological requirements (Jia, et al., 2020). Bio-glass or bio-ceramics are bioabsorbable materials that enhance the regenerative activity of newly formed tissues, while metals exhibit high compressive strength, particularly exceptional fatigue resistance (Velasco-Ortega, et al., 2016, Shao, et al., 2018). The combination of these materials expands the possibilities in tissue engineering and provides a broader range of options for creating scaffolds with desired properties.

1.2.3 3D Peptide hydrogel culture system

Thus far, a diverse array of 3D cell culture systems has been developed to emulate the microenvironment conducive to cellular growth. These systems encompass natural polymers,

biopolymers, synthetic polymers, and hybrid polymers. Among these, self-assembled peptide hydrogels stand out as highly promising for applications in 3D cell culture (Xu, et al., 2019). The self-assembly phenomenon in peptide molecules is a widespread occurrence in nature. Through self-assembly, peptide molecules can give rise to proteins with distinct functionalities, thereby playing pivotal roles in biological evolution and the preservation of biological diversity. By methodically engineering and controlling the molecular architecture of peptide molecules and manipulating the corresponding external conditions, peptide molecules spontaneously arrange into nanofiber mesh structures boasting a water content exceeding 95% (Zhang, et al., 1993, Xu, et al., 2021). This assembly is governed by non-covalent forces, such as hydrophobic interactions, hydrogen bonding, electrostatic interactions, and π-π stacking (Sampaziotis, et al., 2015). This intricate structure closely mimics the porosity and configuration of the in vivo extracellular matrix. Consequently, these peptide hydrogels not only furnish a suitable microenvironment for cellular growth but also have the potential to stimulate the regeneration and reconstruction of impaired tissues.

The fundamental building block of a peptide molecule is the amino acid, with nature boasting a repertoire of 20 common amino acids. Through the judicious combination of different amino acids, a diverse array of peptide hydrogel materials with distinct functionalities can be synthesized. To date, several peptide hydrogels have found utility in cancer cell culture investigations, with notable examples encompassing EAK, RADA16, Fmoc FF, and H9e. However, the hydrogels formed from the first three peptides are characterized by poor mechanical properties or poor cyto-compatibility (Zhang, et al., 1995, Truong, et al., 2015, Worthington, et al., 2015, Caliari and Burdick, 2016, Xu, et al., 2021). In contrast, H9e hydrogels exhibit appropriate mechanical strength, displaying unique deformability and recombination capabilities (Huang, et al., 2013). The commercially available product PGmatrix, derived from H9e, allows for the facile fabrication of various patterns, including simple star shapes or renal organ configurations (Li, et al., 2021). Importantly, PGmatrix demonstrates minimal cell damage and a high degree of biocompatibility under mechanical stress, consequently enhancing the biological activity of cells. This enhanced activity is evidenced by augmented extracellular matrix secretion and an increased production of immune response proteins by mesenchymal stem cells (Li, et al., 2022).

Furthermore, the H9e hydrogel system could serve as a bridge between in vitro and in vivo chemical drug screening, offering a more realistic and reliable platform for evaluating the efficacy

of potential drugs or compounds. Researchers can observe the effects of drugs on Head and Neck cellular proliferation, migration, and invasion more authentically (Kumar, et al., 2015). Liang et al (2017) found that the interaction between H9e hydrogel and hydrophobic drugs camptothecin is negeligible, allowing the drug diffused well in hydrogel and working on the cells. Moreover, this interaction does not hinder the effective diffusion of chemopreventive agents throughout the entire 3D structure, ensuring consistent compound exposure to cells. This, in turn, facilitates the precise assessment of the impact of these chemicals on HepG2 and SW480 cell growth (Xu, et al., 2019). The study also demonstrates that cryopreserved human-induced pluripotent stem cells (hiPSCs) can be cultured directly in a 3D PGmatrix after thawing, and this approach outperforms a commonly used PEG-based hydrogel (Li, et al., 2022).

1.2.4 Application of 3D cultured cells

3D cell culture simulates the growth state of cells in vivo better than 2D. The major applications are mainly divided into four categories: regenerative medicine, tumor models, drug screening and cell differentiation research. The rapid advancement of medical techniques has brought about significant changes in disease treatment. However, certain challenges persist in the treatment process, such as slow tissue and organ repair or the heightened risk of infection in wounds (Deng, et al., 2022). The application of 3D cell culture involves the artificial reconstruction of damaged tissues to enhance their morphology and function. This approach holds immense potential in vascular regeneration, organ restoration, and tissue repair. In the case of thicker tissues recovery, lacking a dense distribution of blood vessels and unable to maintain tissue activity was a common challenge. 3D printing technology allows the construction of multi-scale vascular systems, encompassing vascular channels and capillary networks (Lee, et al., 2014). By integrating blood vessel formation within the process of printing cells and matrices, this technology demonstrates promising prospects in facilitating vascular development in thicker tissues (Wang, et al., 2021).

Utilizing three-dimensional porous scaffolds to expedite tissue regeneration has long been a conventional approach in the field of tissue engineering (Kim, et al., 2021). The graphite oxide and carboxymethyl chitosan (CMC) composite scaffolds have advantageous mechanical and physicochemical properties. These scaffolds facilitate new bone regeneration within the site of rat

skull defects, creating a favorable microenvironment that promotes osteoblastic differentiation of cells (Ruan, et al., 2016). This underscores the potential of such composite scaffolds in facilitating tissue regeneration. 3D culture for cells, cell spheroids and organoids have a significant impact in regenerative medicine. These approaches offer novel solutions for overcoming challenges in tissue repair, organ restoration, and vascular regeneration, holding promise for improving patient outcomes. PGmatrix, a product originating from H9e, simplifies pattern printing, exhibits strong biocompatibility, and, when subjected to mechanical stress, minimizes cell damage while enhancing cellular activity and the production of immune response proteins (Li, et al., 2021, Li, et al., 2022).

In oncology research, the cultivation of tumor cells in 3D conditions enables self-assembly, leading to the formation of structures that closely resemble those found in vivo. In this way, researchers can observe and analyze how tumor cells interact, exchange signals, and influence within the population (Edmondson, et al., 2014, Antoni, et al., 2015). In an experimental setting involving the cultivation of Head and Neck Squamous Cell Carcinoma (HNSCC) cells within a three-dimensional PGMatrix hydrogel matrix, researchers identified that ficlatuzumab exerts inhibitory effects on the migration, invasion, and proliferation of HNSCC cells, particularly in scenarios where these cellular processes are facilitated by TAF (Kumar, et al., 2015). Notably, studies have revealed distinct behaviors of tumor cells when cultured in 3D compared to traditional 2D cultures. Mouse breast cancer cell lines exhibited resistance to various Alkylating agents when cultured in spherical 3D structures, whereas they were not resistant in 2D cultures (Kobayashi, et al., 1993). Similarly, investigations comparing 3D and 2D cultures of epithelial ovarian cancer cells found that the IC50 (half-maximal inhibitory concentration) of these cells to the anticancer drug paclitaxel increased by 100 times during 3D culture (Nicholson, et al., 1997). The characteristics displayed by 3D tumor spheroids closely resemble those observed in solid tumors in vivo. This similarity holds tremendous significance for cancer research, providing a valuable platform for studying biomarkers, investigating the efficacy of anticancer drugs, exploring invasion and metastasis processes, and studying tumor angiogenesis (Kim, 2005, Liu, et al., 2011).

In drug development, preclinical drug toxicity assessment commonly relies on animal models (Collins, et al., 2019). Before conducting animal testing, in vitro cell testing serves as the initial step in preclinical drug toxicity evaluation (Breslin and O'Driscoll, 2013). Traditional 2D cell culture models have been widely used for this purpose, but they may not always align with in

vivo experimental outcomes. Several studies have highlighted the limitations of 2D culture in accurately predicting drug toxicity and off-target effects on vital organs, such as the liver and kidneys (Maltman and Przyborski, 2010). In contrast, 3D cell culture models have shown superior capabilities in evaluating drug toxicity, offering more accurate predictions of potential adverse effects and efficacy. The research findings reveal that when H9e hydrogel is utilized in conjunction with hydrophobic drugs, it exerts minimal interference on the process of drug delivery and distribution (Liang, et al., 2017). This synergy facilitates the effective diffusion of chemopreventive agents across the entire three-dimensional structure, thereby ensuring a uniform cellular exposure to these compounds. Such precision in exposure is pivotal for the precise evaluation of the impact of these chemicals on cell growth, as substantiated by previous studies (Xu, et al., 2019). Incorporating 3D cell models in drug development holds the promise of enhancing the effectiveness of toxicity testing and providing a more comprehensive understanding of drug behavior, leading to improved drug safety and efficacy assessments (Astashkina, et al., 2012, Jee, et al., 2019). By employing 3D cell models, it becomes possible to achieve precise in vitro drug delivery, leading to reduced costs and complexities associated with animal testing, as well as mitigating ethical disputes (Pampaloni, et al., 2009). Consequently, the integration of 3D cell models into the drug development process holds significant potential in enhancing the efficiency and accuracy of toxicity testing, ultimately in the identification of drug screening while increase the alignment with animal models.

1.3 Extracellular Vesicles (EVs) and Exosomes

Extracellular vesicles (EVs) are present within cells and can be categorized into three main types based on their size: exosomes (30-150 nm), microbubbles (150 nm to 1 μm), and apoptotic bodies (1-5 μm) (Gowda, et al., 2020). Initially, the hypothesis surrounding the functions of these vesicles was primarily focused on loading and transporting cellular metabolic waste (Pan and Johnstone, 1983). Subsequently, in 1987, Johnstone introduced the term "exosomes" to describe these specific vesicles (Johnstone, et al., 1987).

1.3.1 Formation of exosomes

Exosomes exhibit distinct differences from the other two subtypes, microbubbles, and apoptotic bodies, in terms of size, function, biological origin, and various other aspects (Abhange, et al., 2021). The generation of exosome affected by diverse factors, including microorganisms, extracellular stimuli, and stress conditions (De Toro, et al., 2015). The process of exosome formation involves several stages. Initially, cells generate early endosomes through Endocytosis (Lee, et al., 2012). These early endosomes migrate within cells and gradually mature into late endosomes, known as multivesicular bodies (MVB), which consist of internal vesicles (ILVs) (Keller, et al., 2006). The regulation of this process is closely linked to the cholesterol content on MVB (Mobius, et al., 2002). When MVB fuses with lysosomes, it undergoes dissolution. However, if MVB binds to the cell membrane, its internal ILVs are released into the extracellular space, resulting in the formation of exosomes (Hessvik and Llorente, 2018). The formation of exosomes is intricately controlled by the involvement of key proteins, such as the Endosomal Sorting Complex Required for Transport (ESCRT), Alix, and other related proteins or cofactors (Colombo, et al., 2013). Additionally, specific lipids and proteins also play regulatory roles in this process (Baietti, et al., 2012, Pocsfalvi, et al., 2016). Exosomes play a crucial role in intercellular communication by transmitting signals between cells, thereby participating in various essential biological processes (Kalluri and LeBleu, 2020, Liu, et al., 2020).

Exosomes are derived from various sources, including bodily fluids such as blood and urine, with nearly all body fluids containing these microscopic vesicles. These exosomes are extensively used in clinical research. However, for standard research purposes, exosomes obtained from cell cultures or blood are more commonly utilized. One notable distinction exists between 2D and 3D cultures regarding exosome release. Cells cultured in a 3D system tend to release exosomes that are smaller and more abundant compared to those in 2D monolayer cultures (Thippabhotla, et al., 2019). The 3D culture system demonstrates a higher activity in exosome production. In 2D culture, the maximum period for conditioned media collection is typically limited to 48 hours. In contrast, the 3D culture allows for an extended collection period of up to an additional 4 days without negatively affecting cell viability. Notably, 3D cultures require a considerably lower number of cells for exosome production. The increased exosome secretion observed in the 3D culture system, coupled with other related findings, underscores the efficiency of the 3D system for investigating exosome functions in a more physiologically relevant environment. This approach provides a more accurate model for studying cellular communication within biological systems. Extracellular

vesicles (EVs), including exosomes, play a crucial role in cell-to-cell communication. Studying EVs derived from 3D cultures offers a more realistic representation of cellular-level communications compared to traditional 2D cultures.

1.3.2 Structural composition and function of exosomes

Exosomes, being derived from various cells, exhibit diverse compositions. According to the latest data from ExoCarta database, it comprises a total of 9,769 proteins, 3,408 mRNA, 2,838 miRNAs, and 1,116 lipids. Proteins are a crucial constituent of exosomes, and they can be categorized into common proteins found in almost all exosomes and special proteins specific to their cell of origin. Among the common proteins are flotillin, Alix, TSG 101, transmembrane protein superfamily members (CD9, CD63, CD81, CD82), heat shock proteins, actin, tubulin, and others (Urbanelli, et al., 2013, Rajagopal and Harikumar, 2018, Lin, et al., 2020). Detecting these common proteins can confirm the presence of exosomes (Shao, et al., 2018). On the other hand, special proteins found in exosomes are unique to their source cells. For instance, exosomes derived from malignant ascites of ovarian cancer patients may contain epithelial cell adhesion proteins (Runz, et al., 2007). Identification of specific proteins in exosomes can thus help determine their origin, aid in disease diagnosis, and assess treatment effectiveness.

The lipid components of exosomes vary based on the characteristics of the source cells and typically include cholesterol, sphingomyelin, phosphatidylserine, ceramide, and others (Record, et al., 2014, Leidal and Debnath, 2020). Lipids form the stable membrane structure of exosomes, protecting them from degradation. The structure of exosomes provides an insight for drug carriers, wherein exosomes were used to inhibit pancreatic cancer growth in mouse models (Kamerkar, et al., 2017). Apart from lipids and proteins, exosomes also contain a significant number of nucleic acids, revealing the mutational state of the source cells. With advancements in gene sequencing, the nucleic acids present in exosomes are analyzed to better understand their function (Valadi, et al., 2007, Jiang, et al., 2017). These nucleic acids in exosomes actively participate in processes like transcription, translation, and modification, thereby regulating gene expression and function in target cells (Jiang, et al., 2017). Exosomes possess a complex composition comprising proteins, lipids, and nucleic acids, which are reflective of their source cells. This diversity contributes to

their role as essential mediators of intercellular communication and highlights their potential utility in disease diagnostics and therapeutic applications.

1.3.3 Separation and purification methods of EVs and exosomes

Exosomes play a crucial role in early disease detection and treatment. However, due to their small size (30-150 nm), low density (1.13-1.19 g/ml), and coexistence with similar components in body fluids (such as cell fragments and proteins), isolating exosomes presents significant challenges. Moreover, the choice of separation techniques can influence exosome activity (Paolini, et al., 2016), necessitating stringent requirements for the separation technology of extracellular vesicles (Cui, et al., 2018). Utilizing standard separation techniques and quantitative methods is paramount to obtain exosomes effectively and efficiently.

Ultracentrifugation is currently the most used technique for separating extracellular vesicles, with approximately half of researchers opting for this approach (Zarovni, et al., 2015). The principle of ultracentrifugation relies on exploiting the density and size differences between exosomes and impurities present in the sample. This method separates a wide range of samples, and it also boasts low operating costs. However, there are certain drawbacks associated with ultracentrifugation, including its time-consuming nature (typically exceeding 4 hours), poor repeatability, and instability. Despite undergoing multiple rounds of centrifugation, the precipitation may still contain numerous impurities, such as co-purified protein aggregates, virus particles, and subcellular organelles (Tauro, et al., 2012). Additionally, the high-speed centrifugation involved in this method may lead to potential damage to the exosomes, resulting in a reduction of their biological activity (Jeppesen, et al., 2014).

An improved technique, known as density gradient centrifugation, utilizes the principles of ultracentrifugation. This method differs in its utilization of two or more separation media with varying densities, such as sucrose and iodixanol (Tauro, et al., 2012). The process involves initially removing larger impurities through low-speed centrifugation, followed by the addition of the sample to the top of the separation medium for overspeed centrifugation. One of the primary advantages of this approach is its ability to achieve higher separation purity. However, density gradient centrifugation demands preliminary preparation, involves tedious operations, and requires longer centrifugation times (often exceeding 16 hours) (Gupta, et al., 2018, Liu, et al., 2021). Both

ultracentrifugation and density gradient centrifugation techniques have their specific merits and drawbacks. The desired level of purity and efficiency need to be considered when selecting the most appropriate method for separating extracellular vesicles.

The ultrafiltration method is a straightforward and effective technique based on molecular size differences, commonly employed for exosome separation. This method relies on the use of one or more ultrafiltration membranes with varying pore sizes or molecular weight cutoff values (MWCO) to obtain exosome while removing impurities (Cappione, et al., 2014). The advantages of the ultrafiltration method are its simplicity, as it does not necessitate expensive specialized equipment or the use of harmful chemical reagents. This makes it a convenient and cost-effective option for researchers. However, the risk of ultrafiltration membrane is the blockage caused by exosomes, leading to reduced recovery rates and efficiency (Xu, et al., 2018).

Chromatography is another separation method that resembles ultrafiltration, as it is also based on molecular size differences. The technique exploits the differential migration rates of particles in a gel matrix. Larger particles are unable to enter the pores of the gel, leading to their faster elution, while smaller particles enter the pores and elute slower (Yang, et al., 2020). This size-dependent separation allows for the isolation of exosomes from the sample based on their relative size compared to the pore diameter of the gel. Chromatography offers a simple and economical approach, particularly suitable for processing large-scale samples.

The immunoaffinity method is a powerful approach based on the specific binding between antibodies and the membrane proteins of exosomes for exosome isolation (Pin, et al., 2017). This unique method allows for the identification and isolation of exosomes from specific cell sources, making it invaluable in targeted exosome research. The target proteins commonly employed in this technique include transmembrane proteins such as CD63, CD9, CD81, annexin, and epithelial cell adhesion molecule (EpCAM), which are frequently present on the surface of exosomes (Tauro, et al., 2012, Hongyun, et al., 2016). The immunoaffinity method is known for the high affinity and specificity for capturing extracellular vesicles, and the lower requirement of specialized equipment. The challenge is to consider the antigen-antibody binding necessitates sufficient incubation time, leading to potential challenges of time-consuming operations and lower extraction efficiency. The immunoaffinity method is hard to apply in large-scale use and analysis. Additionally, once the antibody binds to the extracellular vesicle, separation under mild elution conditions is difficult.

The submicron-sized magnetic beads were introduced to the immunoaffinity capture process, resulting in a significant increase in the production of extracellular vesicles (10-15 times higher than ultracentrifugation) (Zarovni, et al., 2015). Furthermore, the discovery of the Tim4 protein, which specifically binds to phosphatidylserine on the surface of extracellular vesicles, has provided a promising solution. This interaction requires the participation of Ca2+. By forming a complex with exosomes, the addition of a Ca2+ chelating agent facilitates the detachment of intact exosomes from the complex, effectively resolving the issue of difficult separation after specific binding between antibodies and extracellular vesicles (Nakai, et al., 2016).

The precipitation method is based on the physical and chemical properties of compounds and exosomes, using co-precipitation or reverse screening to separate and purify exosomes. Mainly including polymer precipitation method and organic solvent precipitation method (PROSPR). In recent years, Polyethylene glycol (PEG) is the most commonly used reagent for polymer precipitation method (Weng, et al., 2016). When PEG is introduced, the solubility of extracellular vesicles decreases, leading to their precipitation, which can then be separated through low-speed centrifugation (Yu., et al., 2018). The PEG precipitation method has demonstrated a higher yield of extracellular vesicles compared to traditional ultracentrifugation (Rider, et al., 2016). However, this improved yield comes with a drawback: in addition to extracellular vesicles, the extracted products also contain non-extracellular vesicles, immunoglobulins, viral particles, immune complexes, and other contaminants (Yu., et al., 2018). As a result, the purity of the isolated vesicles is compromised, which challenges the removal of the impurities during the process. This impurity issue impacts on subsequent analysis and downstream applications (Zarovni, et al., 2015). PROSPR is a method that utilizes organic solvents, such as methanol, ethanol, acetone, and others, which are miscible with water. The primary objective of this method is to significantly reduce protein solubility, leading to the precipitation and subsequent reverse separation of extracellular vesicles (Gallart-Palau, et al., 2016). The PROSPR method offers several advantages, including its simplicity, speed, and cost-effectiveness. Moreover, the use of acetone as an organic solvent maintains the structural integrity of exosomes, which is well-suited for biomarker detection in various patients during clinical practice. However, PROSPR shows a lower protein recovery in PAGE than differential ultracentrifugation (Dash, et al., 2021).

1.3.4 Quantification and verification of EVs and exosomes

According to a proposal by the International Society of Extracellular Vesicles (ISEV) in 2018, there are three main methods for identifying extracellular vesicles: transmission electron microscopy (TEM), nanoparticle tracking analysis (NTA), and Western blot (WB). These three quantification and verification methods are essential tools in the characterization and study of extracellular vesicles and exosomes for their morphology, size, concentration, and protein composition.

TEM is a powerful imaging technique with a high resolution of 0.1-0.2 nm, making it suitable for observing the ultrastructure of exosomes' bilayer capsule membranes and accurately measuring their size. Exosomes visualized using TEM typically exhibit a cup-shaped shape with a double-layer membrane structure or a hemispherical shape with a depression on one side (Yellon and Davidson, 2014). NTA is a dynamic light scattering (DLS) technology that allows for rapid and dynamic detection of nanoparticles in the range of 10-2000 nm. By recording the motion trajectory of individual nanoparticles and conducting mathematical calculations, NTA provides measurements of the concentration and size distribution of nanoparticles (Shao, et al., 2018). One of the advantages of NTA is its simple and fast sample processing, which preserves the original state of exosomes and enables the recovery of exosomes in their natural form after testing (Rafal, et al., 2017). Western blot (WB) is a widely used method for protein identification, enabling qualitative and quantitative analysis of specific proteins on the surface of extracellular vesicles. As recommended by MISEV2018, the identification of exosomes involves the simultaneous detection of several types of proteins, such as transmembrane proteins (CD63, CD81, CD9), membrane-binding proteins, and common contaminants (albumin, soluble acetylcholinesterase) (Thery, et al., 2018).

1.4 Summary

The development of iPSCs has profoundly transformed life science and clinical research. These iPSCs, derived from somatic cells, possess remarkable pluripotency and self-renewal capabilities akin to embryonic stem cells. This reprogramming process involves introducing crucial transcription factors (OCT3/4, SOX2, KLF4, and c-MYC), collectively known as OSKM or Yamanaka factors, into somatic cells, enabling them to regain pluripotent characteristics. iPSC

technology has transcended various human cell types and species, finding applications in regenerative medicine, tissue regeneration, disease modeling, and drug screening. Notwithstanding challenges, such as differentiation efficiency and cost, iPSCs offer an encouraging platform for personalized medicine and cutting-edge technologies like 3D printing and tissue organoid generation.

In parallel, 3D cell culture systems have emerged as a significant advancement over traditional 2D culture methods, better replicating the in vivo microenvironment. These 3D systems consider critical factors like cell signal transduction, chemical gradients, and spatial structural changes, creating an environment conducive to robust cell growth. Utilizing common materials such as hydrogels and biopolymer microfibers that mimic the extracellular matrix and support intercellular signaling, 3D cultures facilitate cell proliferation and maturation, closely resembling native tissues. Particularly promising are self-assembled peptide hydrogels for cancer cell culture and drug screening due to their realistic representation of in vivo behaviors. With diverse conditions and compositions, 3D systems hold promise as culture methods for iPSCs, spanning their generation, culture, differentiation, and drug screening applications, among others.

EVs including exosomes, microbubbles, and apoptotic bodies, serve pivotal roles in intercellular communication and diverse biological processes. Exosomes, typically sized between 30-150 nm, originate from early endosomes and undergo regulation involving proteins, lipids, and cofactors. These exosomes are derived from various sources, making them valuable for clinical research. Their structural composition, encompassing proteins, lipids, and nucleic acids, mirrors that of their source cells, emphasizing their potential for applications in diagnostics and therapeutics. Effective research in this field necessitates methods for isolating, quantifying, and verifying EVs and exosomes. These encompass techniques such as ultracentrifugation, density gradient centrifugation, ultrafiltration, chromatography, immunoaffinity capture, and precipitation methods. Quantification and verification methods, including TEM, NTA, and WB, are essential components of this research. The amalgamation of 3D cell culture techniques and the study of exosomes has paved new paths toward comprehending intercellular communication and the multifaceted roles exosomes play in health and disease. These innovations amplify our capacity to harness the potential of exosomes in various research and clinical applications, ranging from diagnostics to therapeutics.

In summary, iPSCs, 3D cell culture systems, and EVs, particularly exosomes, represent significant advancements in cellular research with promising applications in regenerative medicine, disease modeling, drug screening, and diagnostics. Addressing challenges like differentiation efficiency, cost considerations, and isolation methods is imperative to fully unlock their potential for transformative impacts in the field.

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Chapter 2 Material and Methods

2.1 3D cell culture

2.1.1 hiPSC 3D embedded culture within PGmatrix

hiPSC cell suspension were first mixed with PGworks, then add PGmatrix to the mixture and mix thoroughly, to reach a final concentration of 0.2%, 0.5% and 1%. The mixing ratio of cell suspension, PGworks and PGmatrix (v/v/v) following the PGmatrix-hiPSC using guide (PepGel LLC). The mixture with cell density of 2×105 cell/mL was plated in a 24-well plate at 500uL/well and incubated at 37°C for 30 min to form hydrogel. After gelation, mTeSR medium or E8 medium supplemented with PGgrow at ratio of 1000:1 (v/v, medium: PGgrow) was added gently on the top of hydrogel to feed cells, and the medium was changed on day 3 and day 4.

2.1.2 hiPSC 3D suspension culture within PGmatrix

hiPSC cell suspension was mixed thoroughly with PG-3DSUSP solution and PGworks at ratios of 2:1:0.03 (cell suspension: PG-3DSUSP: PGworks), to achieve a cell density of 1×105 cells/mL. The mixture was transferred to a 6-well plate (3.94mL/well) and incubated at 37°C for cell culture. To feed hiPSC, 2-3 mL of mTeSR supplemented with PGgrow were added to the 6-well plate at day 1, 3 and 4, respectively.

2.1.3 hiPSC 3D culture medium collection

3D embedded culture: Gently aspirate 1.5 mL sample culture medium above the cell-gel matrix without disturbing the hydrogel at day 3, 4 and 5. Centrifuge the collected medium at 3,000g for 10 minutes at 4°C to remove any dead cells and debris.

3D suspension culture: Mechanically disrupt the 3D suspension culture system by pipetting at day 5. Then transfer the mixture to a 15 mL conical centrifuge tube, and centrifuge at 700 g for 5 min by swing bucket centrifuge. Collect the supernatant and filter through 0.2 μm filter to remove gel. The collected media samples from different culture conditions were used for Mass Spectrum analysis and exosome analysis.

2.1.4 hiPSC 3D culture cell harvesting

3D embedded culture: The hydrogel was disrupted by pipetting the gel and medium on the top thoroughly. The mixture was then transferred to a conical tube and added DPBS to further dilute the cell and gel mixture by more than 20 folds. After centrifugation at 300g for 5 min, discard the supernatant and collect the spheroids pellets from the bottom of tube. The spheroids were dissociated with TrypLE™ Express Enzyme (1X) (1mL/well for 24 well plate) at 37°C for 15 min to obtain single hiPSC cells.

3D suspension culture: Mechanically disrupted the 3D culture system by pipetting and transferring the mixture to a 50 mL conical tube. Then centrifuge the mixture at 700 g for 5 min by using swing bucket centrifuge. The supernatant was removed (medium collection) and the spheroids pallets were collected for further TrypLE treatment. To dissociate the hiPSC spheroids into single cells, one well of 6-well plate needs about 7 mL of 1x TrypLE and incubate at 37 °C for 15-20 min.

2.2 hiPSC 2D cell culture

A 2D cell culture of hiPSC-F cells was employed as a control. To coat the 6-well plate, 0.5 mg of vitronectin (ThermoFisher) was diluted 100-fold to achieve a final concentration of 0.5 µg/cm2. The coated plate was either incubated in the incubator for 1 hour or sealed with parafilm and stored at 4°C for use within 7 days. A total of 1×10^5 hiPSC-F cells were seeded on the plate using mTeSR as the culture medium. The medium was replaced every day, and once the cell confluence reached 80-90% as observed under the microscope, the cells were harvested and passaged.

To harvest the cells, the culture medium was removed, and the cells were washed with DPBS. 1x TrypLE was used to detach the cells from the plate. After adding 1mL TrypLE, the cells were incubated at 37°C for 3-5 minutes. Once the cells observed under microscope had round shape and detached from the plate, add 1mL culture medium to stop the trypsinization. The cell mixture was transferred to a 15 ml conical tube and centrifuged at 200g for 5 minutes. The supernatant was removed to obtain the cell pellet for further cell counting and passage.

2.3 Cell count and viability measurement

The Auto2000 cellometer (Nexcelom Bioscience LLC, Lawrence, MA) was used to measure cell number and viability using acridine orange/propidium iodide (AO/PI) assay from Nexcelom Bioscience. The live cells were stained as green fluorescence and apoptotic cells with early membrane damage were stained as fluorescent orange.

2.4 Real time-quantitative PCR

Total RNA was extracted from the cells of 14 conditions using the Direct-zol RNA MiniPrep kit (Zymo Research Corp), and 100µl of $1x10^6$/mL cells were used for each RNA extraction. RNA concentration was determined by NanoDrop, and then normalized to 5ng/µl for quantitative reverse transcription PCR (RT-qPCR). RT-qPCR was performed using the iTaq™ Universal SYBR Green One-Step kit (Bio-Rad) on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad) with the following program: initial reverse transcription at 50°C for 10 min, denaturation at 95°C for 1 minutes, followed by 40 cycles of amplification at 95°C for 10 s, annealing at 50°C or 60°C depending on primer annealing temperatures, for 30 s, and extension at 72°C for 20 s (Table 2.2). Six primer sets, targeting SOX2, OCT4, REX1, NANOG, UTF1, hTERT, were used in triplicate for each target in each run. The housekeeping gene used is hEID2. (Table 2.3).

2.5 Extracellular vesicle extraction

After collecting the culture supernatants, they were centrifuged at 3,000g for 15 minutes at 4°C to remove dead cells and debris. The resulting supernatant was then subjected to further centrifugation at 10,000g for 20 minutes at 4°C to eliminate any remaining cell debris and microvesicles. The supernatant was subsequently transferred to an Amicon Ultra 5 mL centrifugal filter with a 3 kDa membrane (Merck Millipore), which had been prewashed with DPBS and centrifuged at 14,000g for 10 minutes. The Amicon filtration system was then centrifuged at 14,000g for 30 minutes to concentrate the EVs and remove the peptides in the solution. Finally,

the filter was inverted in a clean microcentrifuge tube and centrifuged at 4,000g for 10 minutes to collect the purified EVs.

2.6 EVs detection via NTA

To visualize and evaluate the size and concentration of EVs, dynamic light scattering (DLS) method was applied by the instrument NanoSight NS500 system. Prior to injection into the compartment, the EV solution underwent filtration using 0.22 μm syringe filters (VWR). Simultaneously, a non-conditioned control medium underwent the same process. For each measurement, we captured five 30-second videos of nanoparticles exhibiting Brownian motion. These videos were subsequently analyzed utilizing the Nanoparticle Tracking Analysis (NTA) software 2.3. The obtained results were presented in the form of a frequency-sized distribution graph, which depicted the number of particles and their corresponding diameters as computed from the video data. Subsequently, the concentration of released EVs was calculated to determine the average number of EVs, while the diameter data enabled to specifically narrow down the nanoparticle population to specific EVs, such as exosomes.

2.7 Alkaline Phosphatase Live Stain

Prepare a 1X AP Live Stain working solution, dilute the 500X stock solution in DMEM, and remove the growth medium from the target cultures. Wash the cultures with pre-warmed DMEM for 2-3 minutes, aspirate, and repeat. Apply the 1X AP working solution directly to the adherent cell culture. Incubate for 20-30 minutes, being cautious of media containing interfering components. Afterward, wash the culture twice with DMEM for 5 minutes each to remove excess stain. Add fresh DMEM before visualizing fluorescent-labeled colonies under FITC-filtered fluorescent microscopy. Capture images within 30-90 minutes, mark robust fluorescent colonies for selection, and replace DMEM with fresh growth medium.

2.8 CytoTune-Reprograming for h-Fibroblast

In the transduction procedure using Sendai CytoTune (ThermoFisher) for adherent cells, specifically BJ cells, which are human fibroblasts (BJ cell) obtained from normal neonatal male foreskin, several steps are followed. Initially, the cells are revived and expanded, involving the thawing of a stock vial containing 1e6 cells, suspending them in pre-warmed f-medium, and subsequently transferring them to a T25 flask for incubation. The cell culture is maintained at 37°C with 5% CO2, with medium changes every 2 days until reaching 80-90% confluency. Upon reaching the desired confluency, the cells are harvested by removing the culture medium, rinsing with DPBS, and adding 0.05% trypsin/EDTA. After observing cell separation under a microscope, the TrypLE™ Express Enzyme (1X) is neutralized, and the cells are centrifuged, resuspended, and counted for viability.

Moving to the transduction phase on Day 0, the cells are prepared by plating a specific quantity onto a 6-well plate using BJt-medium. The remaining cells are cryopreserved. Following an incubation period of one to two days to ensure cell adhesion, transduction is performed. This involves warming BJt-medium, adding TrypLE™ Express Enzyme (1X) to detach cells from the plate, calculating the virus volume needed, and thawing the required Sendai reprogramming viruses. The virus is then added to the BJt-medium, which is used to replace the existing medium in the wells containing the cells. The cells are incubated for 24 hours.

From Day 1 to Day 8, the medium is changed regularly, and GFP expression is observed under a fluorescence microscope. Vitronectin-coated plates are prepared on Day 5 or 6. On Day 7, the cells are harvested, counted, and seeded onto the vitronectin-coated plates. At the same time, cells are collected for RNA extraction and qPCR detection of vector genes. On Day 8, the medium is changed from BJt to E8.

From Day 9 to Day 28, colony formation and isolation are carried out. The plates are observed daily for colony growth, with colonies typically appearing around Day 12. The cells are maintained by changing the medium every other day. Around 21-28 days after transduction, the colonies are ready for transfer. Live staining using AP is performed for selecting reprogrammed colonies. The undifferentiated iPSC colonies are manually picked and transferred to vitronectin-coated culture plates for further expansion or analysis. This involves cutting the colonies into pieces and transferring them to a new plate, followed by incubation and regular medium changes.

Once the colonies reach the desired confluence, they are ready for passaging and continued culture in complete Essential 8™ Medium until they exceed two 60-mm plates in quantity.

2.9 CytoTune-Reprograming for hPBMC

In the transduction procedure using Sendai CytoTune (ThermoFisher) for hPBMC cells (human peripheral blood mononuclear cells), which are vital components of the immune system, several steps are followed over a multi-day process. Initially, the cells are revived from frozen stock and maintained in suspension, with regular medium changes and monitoring for confluency. On Day 0, transduction is performed, involving the calculation and addition of reprogramming viruses, followed by a 24-hour incubation.

From Day 1 to Day 8, medium changes and GFP observation are carried out to detect successful transduction. Vitronectin-coated plates are prepared, and cells are eventually transferred onto these plates for further culture. On Day 7, the culture medium is changed to E8 medium.

From Day 9 to Day 28, colony formation and isolation are conducted. The plates are observed daily for colony growth. After 21-28 days, colonies are ready for transfer, and live staining is performed for selecting reprogrammed colonies. Undifferentiated iPSC colonies are manually picked and transferred to vitronectin-coated culture plates, where they continue to be cultured and expanded in complete Essential 8™ Medium.

Table 2.1 Grouped culture conditions

hiPSC-hPBMC-mTeSR-0.5%PG concentration
hiPSC-fibroblast-mTeSR-0.5%PG concentration
hiPSC-hPBMC-E8-0.3%PG concentration
hiPSC-fibroblast-E8-0.3%PG concentration
hiPSC-hPBMC-mTeSR-0.5%PG concentration-Day4
hiPSC-hPBMC-mTeSR-0.5%PG concentration-Day6
hiPSC-hPBMC-mTeSR-0.2%PG concentration
hiPSC-hPBMC-mTeSR-1%PG concentration
hiPSC-hPBMC-mTeSR-Suspension
hiPSC-hPBMC-mTeSR-modified-Suspension
hiPSC-fibroblast-mTeSR-2D

Table 2.2 Real time quantitative PCR protocol design

	qPCR protocol 52°C	
Primer Tm	50°C	10 min
	95°C	1 min
	45 cycles of:	
	95°C	10 sec
	52	30 sec
	72°C	20 sec

	qPCR protocol 60 °C	
Primer Tm	50°C	10 min
	95°C	1 min
	45 cycles of:	
	95°C	10 sec
	60°C	40 sec
	72°C	/

Table 2.3 Real time quantitative PCR primer design

Gene	Primer ID	Primer sequence	Tm(°C)	Citation
SOX2	SOX2-F1	CAACCAGAAAAACAGCCC	52	(Li, et al., 2021)
	SOX2-R1	TCTCCGACAAAAGTTTCC		
REX1	REX1-F	GTTTCGTGTGTCCCTTTC	52	(Li, et al., 2021)
	REX1-R	CTTTCCCTCTTGTTCATTC		
OCT4	OCT4-F	AAAGAGAAAGCGAACCAG	52	(Li, et al., 2021)
	OCT4-R	CCACATCCTTCTCGAGCC		
NANOG	NANOG-F1	TGTGATTTGTGGGCCTGA	52	(Li, et al., 2021)
	NANOG-R1	GTGGGTTGTTTGCCTTTG		
UTF1	UTF1-F	CTCCCAGCGAACCAG	52	(Li, et al., 2021)
	UTF1-R	GCGTCCGCAGACTTC		
hTERT	hTERT-F	GGAGCAAGTTGCAAAGCATTG	60	(Li, et al., 2021)
	hTERT-R	TCCCACGACGTAGTCCATGTT		
hEID2	hEID2-F	GAAGCCTGCAGAGCAAGG	60	(Li, et al., 2021)
	hEID2-R	ATATCGAGGTCCACCCTGTG		

Reference

Li, Q., G. Y. Qi, X. M. Liu, J. F. Bai, J. K. Zhao, G. S. Tang, Y. S. Zhang, R. Chen-Tsai, M. Zhang, D. H. Wang, Y. Y. Zhang, A. Atala, J. Q. He and X. Z. S. S. Sun (2021). "Universal Peptide Hydrogel for Scalable Physiological Formation and Bioprinting of 3D Spheroids from Human Induced Pluripotent Stem Cells." Advanced Functional Materials 31(41).

Chapter 3 – Results and Discussion

3.1 Comparative Analysis of hiPSC Reprogrammed from Human Fibroblasts: Traditional 2D vs. 3D PGmatrix Generation System

In the comparative analysis of hiPSC-F generation between the traditional 2D culture system and the 3D PGmatrix system, several discernible differences come to light, primarily in the context of cellular morphology, as depicted in Figure 3.1G and H. In the conventional 2D system, regardless of whether it relies on feeder cells or operates independently, hiPSC colonies are required to originate from a monolayer of reprogrammed BJ cells (Figure 3.1G). However, it's important to note that not all colonies emerging from this process exhibit the characteristic features of hiPSCs. In two distinct experimental trials, the ratio of colony formation (Figure 3.1G) to hiPSC colonies identified through Alkaline Phosphatase (AP) live staining (Figure 3.1E) was observed to be 29:11 and 112:19, underscoring the variability in the quality of formed colonies.

In stark contrast, the hiPSC colonies generated within the 3D PGmatrix system surpass those produced by the conventional 2D approach (Figure 3.1A). Based on colony formation efficiency, the 3D system exhibits greater efficacy and expedites the reprogramming process considerably when compared to traditional 2D methodologies. Following multiple passages and the cultivation of parental hiPSC-F cells, differences in terms of viability and proliferation patterns between the two systems become apparent (Figure 3.1B, 1C, S4, S5). The utilization of the 3D PGmatrix embedded system offers the advantage of enabling hiPSCs to be diluted into individual wells of a 96-well plate, where they can spontaneously form single spheroids within each well (Figure S2). This capability streamlines experimental processes, providing a convenient and efficient means of studying hiPSC behavior and responses in a controlled and organized manner. Importantly, it is noted that the 2D system, especially concerning cell survival, displays lower stability compared to the 3D system. A pivotal aspect of this comparative study lies in the assessment of pluripotency gene expressions via real-time PCR for hiPSCs generated from both the 2D and 3D systems (Figure 3.1D). Notably, hiPSC-F cells cultured in the 2D system exhibit a challenge in maintaining OCT4 gene expression when juxtaposed with the control hiPSC-F cells. Conversely, hiPSC-F cells generated and cultured within the 3D system demonstrate consistent gene expressions.

In the realm of experimental complexity, the 2D system necessitates the meticulous selection of colonies during each passage to procure pure hiPSC colonies while concurrently eliminating differentiated cells or BJ cells (Figure 3.1E, G). In contrast, the 3D PGmatrix hiPSC generation system (for both embedded and suspension) presents a distinctive advantage. Successfully induced hiPSCs from reprogrammed BJ cells form discernible spheroids (Figure 3.1H, S1). During the harvesting process, un-reprogrammed BJ cells are enzymatically digested, and the hiPSC-F spheroids can be disassembled into smaller cell clusters or spheroids, facilitating subsequent passaging or other experimental procedures (Figure 3.1F). This streamlined approach inherent to the 3D system simplifies the isolation and maintenance of hiPSC colonies, thereby enhancing both efficiency and the purity of hiPSC-F culture.

3.2 Comparative Analysis of hiPSC Generation from Human PBMC: Traditional 2D Culture vs. 3D PGmatrix System

When comparing the induction of hiPSC-P derived from hPBMC using both the traditional 2D culture system and the 3D PGmatrix system, a striking divergence in cellular morphology becomes evident (Figure 3.2G, H). This discrepancy arises primarily from the unique characteristics of the starting material, hPBMC (human peripheral blood mononuclear cells), which are predominantly suspension cells. The conventional 2D system encounters challenges in the attachment process for reprogrammed hPBMC, which may not be ideal for their transformation into hiPSCs. In contrast, the 3D PGmatrix generation and culture system effectively address this issue.

Notably, the hiPSC-P colonies generated within the 3D PGmatrix system surpass those produced by the traditional 2D approach (Figure 3.2A). After multiple passages and the cultivation of parental hiPSC-P cells, the 3D system demonstrates superior performance in terms of both viability and proliferation compared to the 2D system (Figure 3.2B, C). This discrepancy suggests that the 3D system is better equipped to maintain the hiPSC-P cells, considering the potential epigenetic influence stemming from the suspension parental cells, hPBMC. Moreover, when assessing pluripotent gene expressions among different groups of hiPSC-P cells, the 3D system exhibits an advantage in preserving the pluripotent gene properties of the cells (Figure 3.2D). The process of passaging hiPSC-P cells mirrors that of hiPSC-F cells, entailing the selection of pure

hiPSC colonies while eliminating differentiated cells from a 6-well plate (Figure 3.2E, G). Consequently, hiPSCs successfully induced from reprogrammed hPBMC cells form spheroids within the 3D system (Figure 3.2H), rendering them easier to distinguish and handle. During the harvesting process, un-reprogrammed hPBMC cells are unable to proliferate, resulting in higher purity of iPSC-P cells within the 3D systems (Figure 3.2F, H). This streamlined approach inherent to the 3D system simplifies the isolation and maintenance of hiPSC colonies, thereby enhancing both efficiency and the purity of hiPSC-P culture.

3.3 hiPSC proliferation supported by PGmatrix-hiPSC 3D culture system

The PGmatrix-hiPSC for 3D culture was previously examined and compared with the 2D culture system, involving Matrigel and vitronectin, leading to the observation of reduced variance in fold expansion and cell viability across multiple passages (Li, et al., 2021). The PGmatrix (PGmatrix-hiPSC) provided a stable growth environment compared to 2D and also superior hiPSC properties than those existing advanced 3D technologies such as PEG based hydrogels (Mebiol gel) and aggregated hiPSC spheroids generated by nonadherent U-Bottom 96 well plate (Li, et al., 2021). Thus, it holds potential for further optimization or adaptation to suit various cell culture conditions. In this context, several factors were considered, including the culture medium, hydrogel concentration and culture duration, and the choice between 3D embedded and 3D suspension PGmatrix systems. Within this framework, two specific hiPSC cell lines, one derived from fibroblasts (hiPSC-F) and the other from PBMC (hiPSC-P), were investigated within the PGmatrix-hiPSC 3D culture system. Notably, the results demonstrated consistently high viability of hiPSCs, ranging from 95% to 99%, among all tested culture conditions (Figure 3.3A-D). However, the rate of cell proliferation was found to be culture condition-dependent, exhibiting significant variation in expansion fold, with values ranging from 7 to 20 (Figure 3.3A-D).

hiPSC cell lines: Both hiPSC cell lines, hiPSC-F and hiPSC-P, displayed similar growth performance within the PGmatrix culture system (Figure 3.3A). For E8 culture medium, 0.3% gel concentration was selected because at 0.5% concentration, gel became stronger and was harder to retrieve spheroids. Under the same seeding density of 1×10^5 cells/mL and cultured with mTeSR medium, both cell lines formed physiological 3D colonies (spheroids) with sizes ranging from 30-60 μm in diametercf (Figure 3.3E-J). Notably, both cell lines exhibited significantly higher

proliferation rates in mTeSR medium, with a fold expansion ranging from 14-16, compared to E8 medium, where the fold expansion was in the range of 9-11. Additionally, hiPSCs cultured in 0.3% PGmatrix with E8 showed reduced sphericity and a more polarized morphology (Figure 3.3G, H). The decreased cell growth rate and reduced sphericity within E8 medium may be attributed to the lack of growth factors in E8 medium during cell growth (Li, et al., 2022).

PGmatrix concentration: Figure 1B demonstrates that high hydrogel concentrations of 0.5%-1% provide better support for cell growth compared to the lower hydrogel concentration of 0.2%. Li's study reported that the 0.2% PGmatrix exhibits an elastic modulus of 240 Pa, while the 0.5% PGmatrix shows an elastic modulus of over 600 Pa. The weaker gel at 0.2% concentration can be easily disrupted during medium replacement on day 3 and day 4, leading to cell loss and reduced harvested cell numbers. Moreover, the polarized cells observed within the 1% PGmatrix with mTeSR (Figure 3.3J) further indicate that gel strength may exert an influence on the behavior of three-dimensional (3D) cell growth, particularly in the context of cellular morphology. This stress stimulation has the potential to stimulate induced pluripotent stem cells (iPSCs) and enhance various facets of cellular development, including derivation and maturation processes, such as for the generation of cardiomyocytes (Kolanowski, et al., 2017).

Culture duration: As depicted in Figure 3C, with a seeding density of 1×105 cells/mL, hiPSC exhibited a proliferation rate of approximately 7-8 folds at day 4, followed by a transition into the exponential growth phase with cell fold expansion reaching 14-16 at day 5. Subsequently, the cell growth rate appeared to decelerate, and the cell population proliferated further to 17-19 folds by day 6. Based on the data, the optimal culture duration for hiPSC appears to be at day 5 for the tested condition and seeding density in this study, during the exponential growth phase, which is conducive for harvesting the cells for various hiPSC-based applications. The exponential growth phase may vary as seeding density or culture conditions such as medium type and hydrogel conditions.

3D Embedded culture vs 3D suspension culture: Figure 1D demonstrates the comparison between the 3D hiPSC growth performance in the 3D embedded and 3D suspension culture (PGmatrix 3D Suspension (Susp)) conditions with mTeSR medium. The results indicated that the suspension culture exhibited similar hiPSC cell proliferation with embedded system, with 15-19 folds for P-mT-Susp. A modified PGmatrix 3D Suspension (MSusp) with higher viscosity was

used for 3D hiPSC-P with mTeSR1 (P-mT-MSusp) with the hope of increasing cell holding capacity, which may improve cell seeding capacity. Cells in MSusp presented similar viability as those in regular Susp (Figure 3.3D), however, such high viscosity resulted in higher cell loss rate in cells isolation process leading to lower proliferation folds.

3.4 Stable pluripotent gene expression in 3D PGmatrix-hiPSC system

To evaluate the genetic effects under five primary culture conditions, the expression levels of 6 genes (SOX2, OCT4, REX1, NANOG, UTF1, and hTERT) associated with pluripotency stem cell were quantitatively determined by real-time qPCR in hiPSC-P. hiPSC-F cultured in PGmatrix-hiPSC at 0.5% gel centration with mTeSR1 complete medium was used as control, which was reported to show superior or similar gene expression compared to hiPSC cultured in 2D (Li, et al., 2022). Embryonic gene hEID2 selected among hEID2, hCAPN10, and hZNF324B according to our preliminary testing was used as the housekeeping gene. Overall, all pluripotent genes remained relatively stable, with minor variation among all the culturing conditions, typically ranging from 1 to 2-fold changes in their levels of expression (Figure 3.4A-G).

Comparison of hiPSC-P and hiPSC-F cultured in PGmatrix at 0.5% gel concentration with mTeSR1 showed similar gene expression levels as hiPSC-F (Figure 3.4A). Pluripotent gene expression analysis in hiPSC-P cultured in mTeSR and E8 revealed better adaptation of cells in the mTeSR-based culture system, consistent with observed proliferation results (Figure 3.4A). In contrast, for iPSC-F, gene expression data indicated better adaptation to the E8-based culture system compared to mTeSR (Figure 3.4A), which contrasts with the cell proliferation results. This discrepancy in gene expression patterns agrees with previous findings when comparing the 2D culture system with the 3D PGmatrix system. These gene expression alterations reflect dynamic cellular responses to distinct culture environments, potentially affecting metabolic states and cell behavior, however, the overall culture performance is maintained at a similar level, revealing the potential of this 3D peptide hydrogel system being able to provide consistent growth environment with slight accommodations to the different culture conditions. Further study is necessary to elucidate the underlying mechanisms governing these differences in gene expression and cellular responses among the culture systems. Additionally, the examination of cell line adaptation in different 3D PGmatrix culture systems was performed with both mTeSR and E8. hiPSC-F showed

better adaptability to E8-based PGmatrix (Figure 3.4A), which might be attributed to their epigenetic memory as adhesive cells. Despite being induced into iPSCs from parental cells, not all epigenomic features may be fully cleared even after extensive passaging, potentially influencing cellular behavior and responses to the culture environment. Conversely, hiPSC-P performed better in the mTeSR system over E8 (Figure 3.4A) and in the Susp system over the embedded system (Figure 3.4D), with increased expression in SOX2 and REX1, and decreased expression in hTERT. As PBMCs mainly consist of suspension cells, their superior performance in suspension culture aligns with their physiological characteristics. Regarding culture duration, as depicted in Figure 2B, pluripotency gene expression of hiPSC-P aligns with the proliferation results, demonstrating a similar trend with minor increases observed on day 6. Figure 3.4C illustrates that higher hydrogel concentrations (0.5%-1%) afford superior support for cell growth compared to the lower concentration of 0.2%, which is consistent with the cell proliferation results.

3.5 Functional enrichments released by hiPSCs in varied culture conditions

Release of EVs: Human-iPSC has been harnessed as a tool to explore extracellular vesicles (EVs) that carry therapeutic peptides or nucleic sequences within their membrane (Wiklander, et al., 2019). The NTA analysis revealed the presence of extracellular exosomes related components and other nanoparticles (Figure S3). Investigating EV secretion by hiPSC across various culture systems unveiled distinct patterns (Figure S6). Notably, a comparative analysis of extracellular vesicles (EVs) secreted by different culture systems highlighted substantial disparities in EV secretion between 3D ($2x10^8$) and 2D ($1x10^8$) systems (Figure S6). Specifically, the 3D culture system demonstrated a significantly higher rate of EV secretion compared to the 2D system, implying that the 3D environment exerts a crucial influence on promoting the release of EVs. Within the E8-based culture systems, hiPSC-F ($1x10^8$) exhibited notably greater EV secretion than hiPSC-P ($9x10^7$) (Figure S6). Conversely, in the context of hiPSC-P cultured in 3D PGmatrix systems, 0.5% PG ($1.67x10^8$) demonstrated heightened EV secretion in comparison to E8 ($9x10^7$).

3.6 Discussion

The 3D PGmatrix system has demonstrated significant advantages over traditional 2D culture methodologies, notably in fostering sustained cell proliferation across multiple passages, as evidenced by recent studies (Li, et al., 2022). This robust cell proliferation is complemented by the unwavering and stable expression of pluripotency-associated genes, as depicted in Figure 2 A-D. These findings firmly establish the PGmatrix 3D culture system as a dependable platform for maintaining human induced pluripotent stem cells (hiPSCs) in a pluripotent state. Notably, the hydrogel matrix exhibits the requisite mechanical strength, featuring unique deformability and recombination capabilities (Huang, et al., 2013), meeting the criteria for providing cells with a stable culture system. This adaptability extends to encompass a diverse range of hydrogel concentrations and culture conditions, underscoring its versatility for accommodating various cell types and diverse research applications.

Beyond its pronounced cell proliferation benefits, the PGmatrix system offers substantial advantages in terms of functionally enrichment release (EVs). The 3D culture system serves as a critical bridge between in vitro and in vivo chemical drug screening, furnishing a more realistic and reliable platform for evaluating the efficacy of potential drugs or compounds. Since the slight modifications made to the culture conditions such as hydrogel concentration, culture medium, culture duration, and cell lines, caused nonsignificant difference for the cell growth or on gene markers, conditions developed for particular experiments such as drug delivery or hiPSC maturation could be effectively affect the cells without compromising the regular cell growing performance. Notably, researchers can observe the effects of drugs on head and neck cellular proliferation, migration, and invasion more authentically (Kumar, et al., 2015). Furthermore, previous research has shown that the interaction between hydrogel and hydrophobic drugs is negligible, facilitating effective drug diffusion within the hydrogel and their action on the cells (Liang, et al., 2017). The tunable pore dimensions within the 3D matrix hydrogel scaffold significantly enhance nutrient or growth factor transport, thereby enabling precise control over drug delivery and facilitating directed cell differentiation protocols. Additionally, 3D cell culture promotes the secretion of extracellular vesicles (EVs), both in vivo and in vitro (Thippabhotla, et al., 2019). The distinct hydrogel structure uniquely positions it for EVs research, providing a

precisely regulated microenvironment conducive to the study of EV secretion dynamics and their intricate interactions with encapsulated cells.

The 3D PGmatrix system also outperforms the traditional 2D culture approach in inducing the hiPSC from somatic cells. The 3D system's ability to address the attachment challenges posed by hPBMC, primarily suspension cells, results in the generation of a greater number of hiPSC-P colonies. Moreover, this system maintains superior viability and proliferation of hiPSC after multiple passages, suggesting its suitability for preserving these cells despite potential epigenetic influences from the suspension parental cells. Pluripotent gene expression analysis underscores the 3D system's advantage in preserving pluripotency in both hiPSC-F and hiPSC-P cells. The 3D system streamlines the isolation and maintenance of hiPSC colonies through the formation of easily distinguishable spheroids during the induction process, leading to higher purity and efficiency in hiPSC culture. These findings highlight the remarkable advantages of the 3D PGmatrix system over traditional 2D culture systems for hiPSC generation, particularly when dealing with distinct cell sources and their associated challenges.

These compelling findings collectively underscore the practicality and efficacy of the 3D PGmatrix culture system, positioning it as an instrumental asset for preserving hiPSC pluripotency and facilitating a wide range of research and therapeutic applications. Although concerns regarding the erasure of epigenetic memory through prolonged culture persist (Li, 2002, Li, et al., 2022), the 3D matrix culture system exhibits remarkable adaptability to diverse cell types and specific research paradigms, promising sophisticated applications including organoid generation and cell differentiation. Encouragingly, various differentiation protocols and culture conditions hold the potential to ameliorate the influence of epigenetic memory associated with distinct parental cell sources, rendering it a versatile and adaptable choice within contemporary stem cell research endeavors (Kim, et al., 2011).

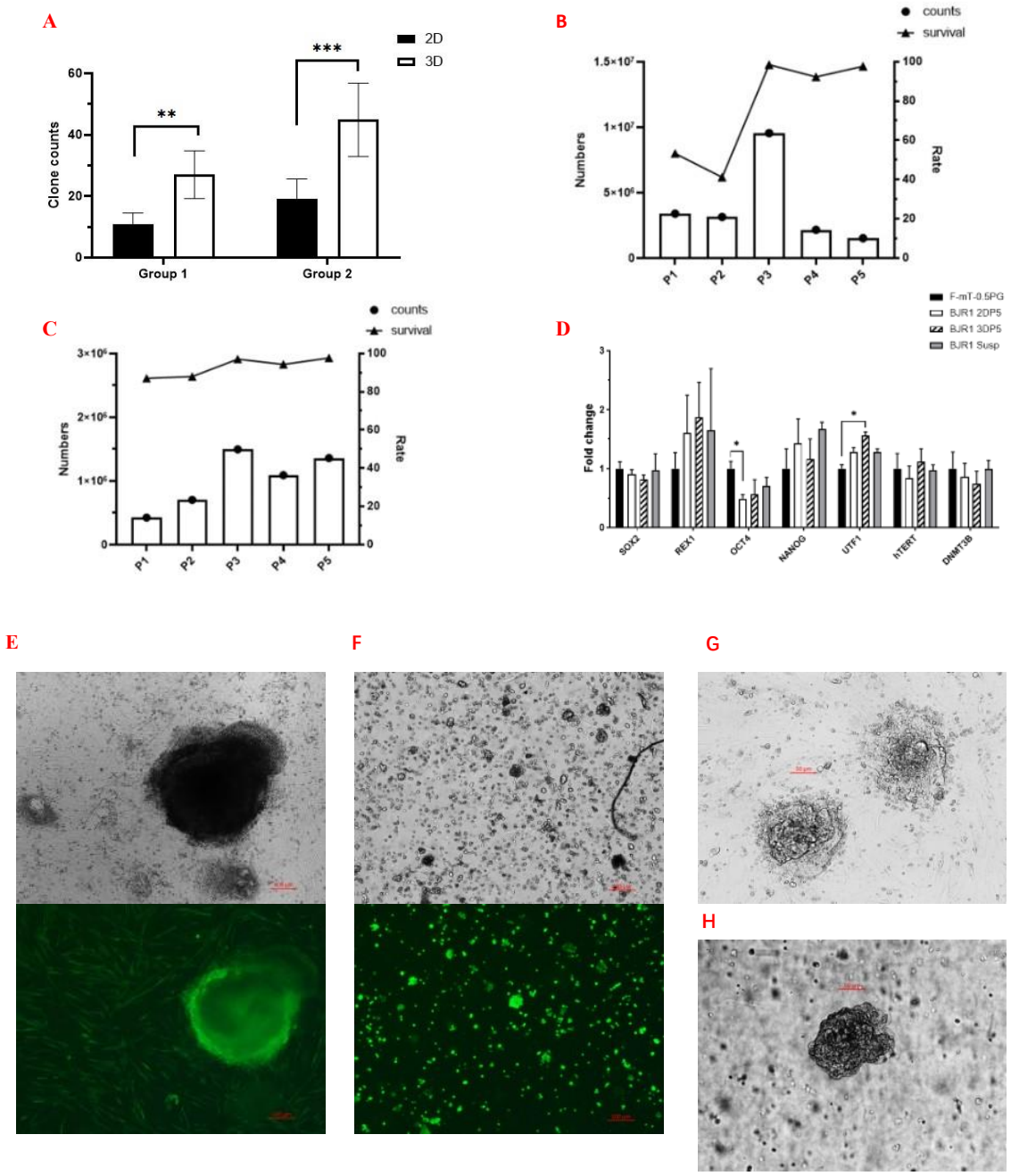


Figure 3.1 Comparison of 2D and 3D iPSC generation system for hiPSC-F

A. hiPSC-F colony formation counts between 2D and 3D generation system; B. hiPSC-F proliferation and viability of 2D generation system over 5 passages; C. hiPSC-F proliferation and viability of 3D generation system over 5 passages; D. Pluripotency gene expressed in hiPSC-F generated from 2D, 3D embedded and suspension system; E. AP stain image of hiPSC-F generated in 2D; F. AP stain image of hiPSC-F generated in 3D; G. Cell morphology of hiPSC-F generated in 2D, colonies formed from the reprogrammed BJ cells, which have the potential to be hiPSC-F or not; H. Cell morphology of hiPSC-F generated in 3D.

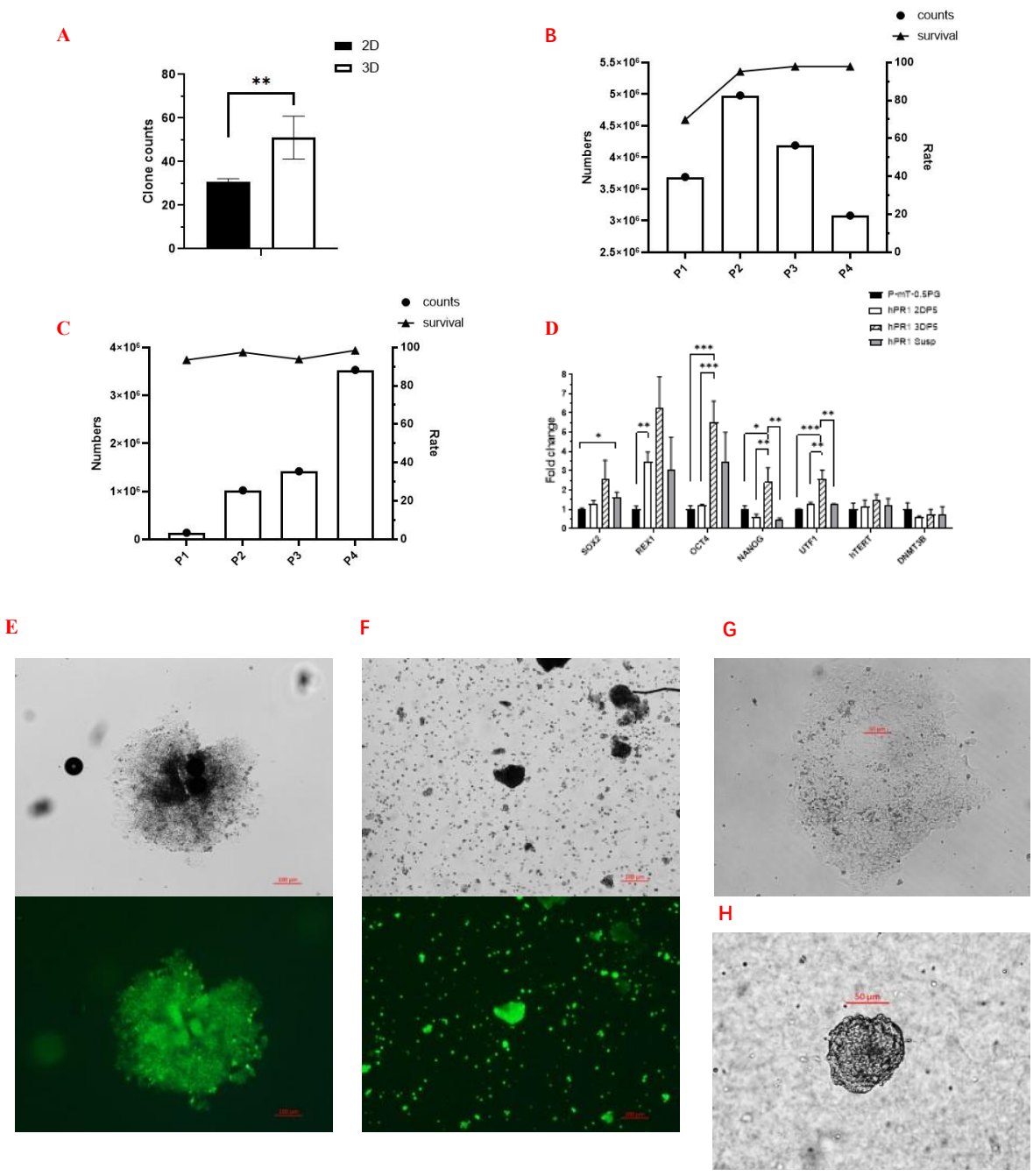


Figure 3.2 Comparison of 2D and 3D iPSC generation system for hiPSC-P

A. hiPSC-P colony formation counts between 2D and 3D generation system; B. hiPSC-P proliferation and viability of 2D generation system over 4 passages; C. hiPSC-P proliferation and viability of 3D generation system over 4 passages; D. Pluripotency gene expressed in hiPSC-P generated from 2D, 3D embedded and suspension system; E. AP stain image of hiPSC-P generated in 2D; F. AP stain image of hiPSC-P generated in 3D; G. Cell morphology of hiPSC-P generated in 2D colonies formed from the reprogrammed hPBMC cells, which have the potential to be hiPSC-P or not; H. Cell morphology of hiPSC-P generated in 3D.

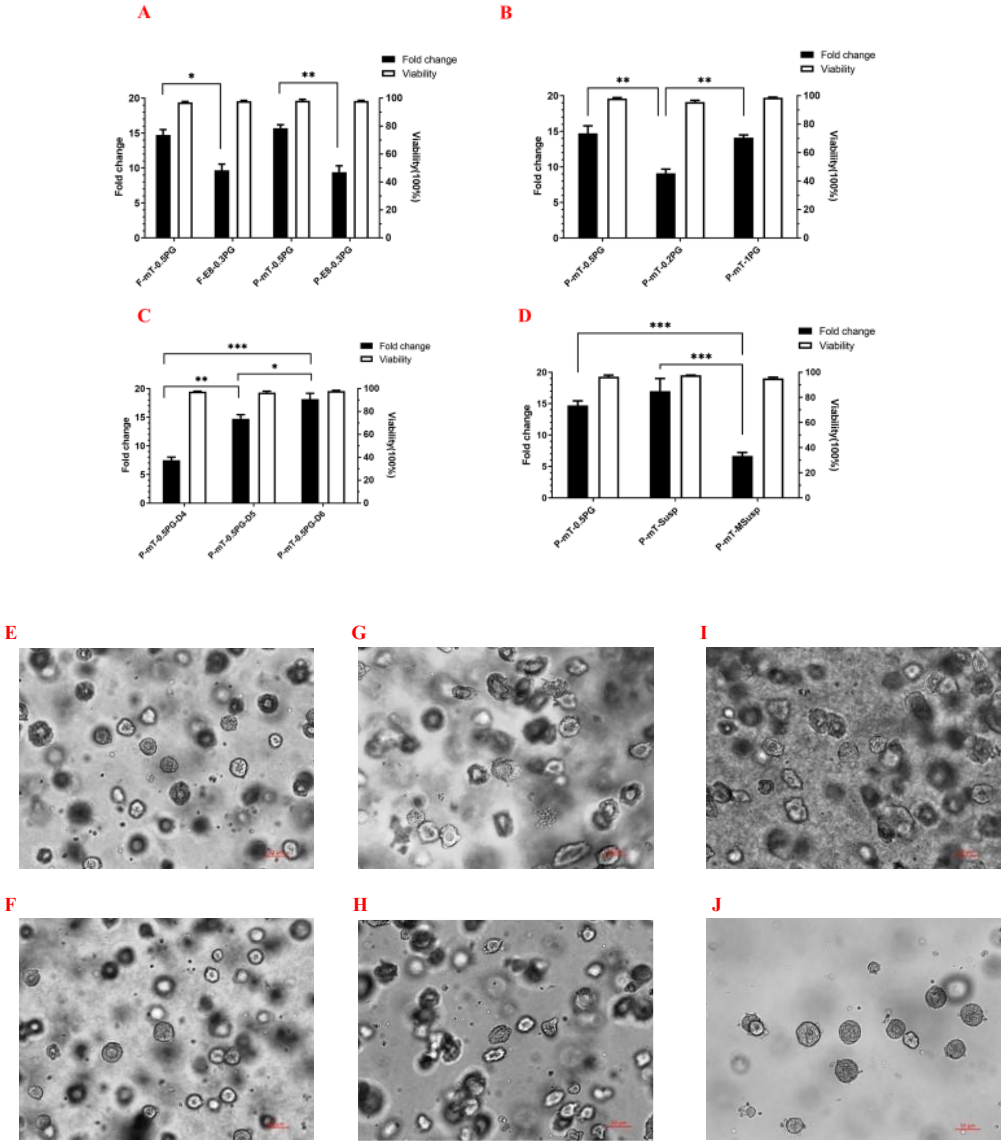


Figure 3.3 Effects of 3D culture conditions and cell lines on hiPSC growth viability and proliferation.

A. Comparison of cell line and culture medium: hiPSC derived from fibroblast cells (Applied Stemcell) vs PBMC (Applied Stemcell); Culture medium mTeSR (Stemcell Technologies) vs Essential 8™ Medium (ThermoFisher); B. Comparison of hydrogel composition and concentration: 0.2% vs 0.5% vs 1% PG concentration; C. Comparison of culture duration: 4 days' culture vs 5 days' vs 6 days'; D. Comparison of 3D culture system: 3D embedded system vs 3D suspension system and modified suspension system; E. Cell morphology of hiPSC-F cultured in 3D 0.5%PG mTeSR system; F. Cell morphology of hiPSC-P cultured in 3D 0.5%PG mTeSR system; G. Cell morphology of hiPSC-F cultured in 3D 0.3%PG E8 system; H. Cell morphology of hiPSC-P cultured in 3D 0.3%PG E8 system; I. Cell morphology of hiPSC-P cultured in 3D 1%PG mTeSR system; J. Cell morphology of hiPSC-P cultured in 3D suspension mTeSR system.

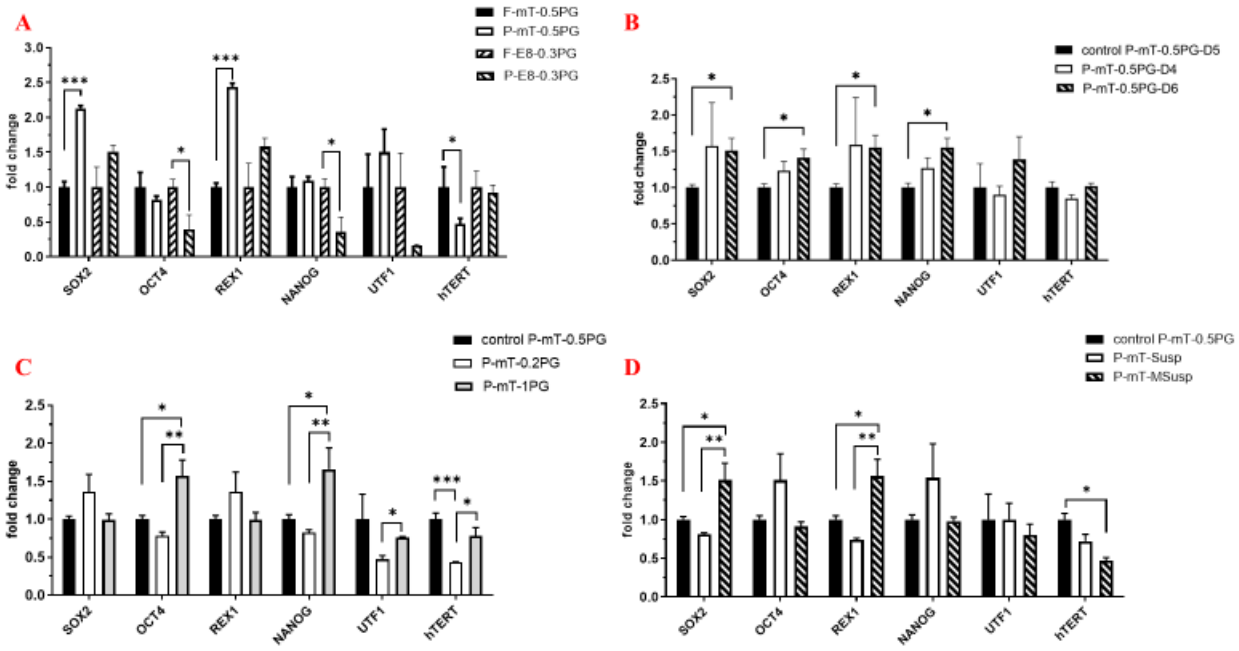


Figure 3.4 Effects of 3D culture conditions and cell lines on hiPSC pluripotent gene expressions.

A. Pluripotent gene expression of hiPSC-P vs hiPSC-F cultured in 3D 0.5%PG mTeSR system, and hiPSC-P vs hiPSC-F cultured in 3D 0.3%PG E8 system; B. Pluripotent gene expression of hiPSC-P cultured in 3D 0.5%PG mTeSR system for 4 days vs 5 days vs 6 days; C. Pluripotent gene expression of hiPSC-P cultured in 3D 0.2% vs 0.5% vs 1% PG mTeSR system; D. Pluripotent gene expression of hiPSC-P cultured in 3D embedded system vs 3D suspension system and modified-suspension system.

Figure S1

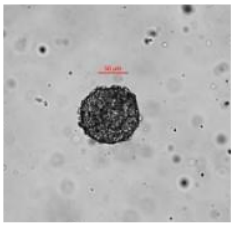


Figure S2

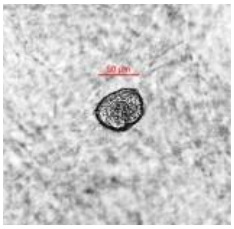


Figure S3

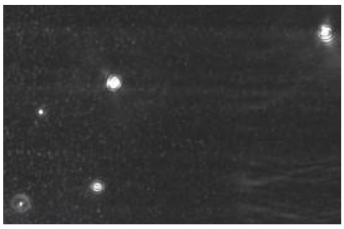


Figure S4

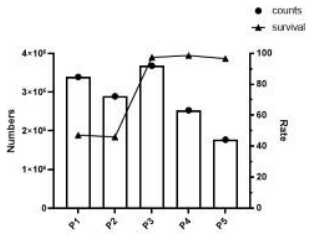


Figure S5

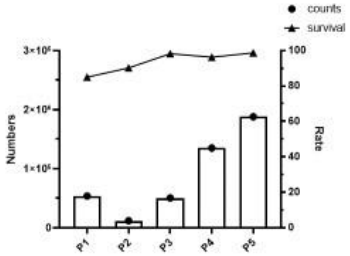
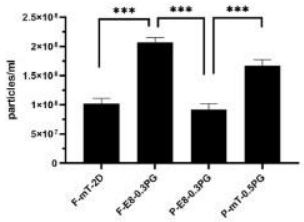


Figure S6



Supplemental Figure 1. hiPSC-F generated in 3D suspension system.

Supplemental Figure 2. hiPSC-F spheroids generated from 96-w cell dilution.

Supplemental Figure 3. Detection of EVs by NTA. The capture of the nanoparticles in the medium collected from conditional culture.

Supplemental Figure 4. hiPSC-F proliferation and viability of 2D generation system over 5 passages (the other trail).

Supplemental Figure 5. hiPSC-F proliferation and viability of 3D generation system over 5 passages (the other trail).

Supplemental Figure 6. The release of extracellular vesicles (EVs) release from hiPSC cultured in different conditions.

Table 3.1 NTA data

	Sample	particles/ml	Mean Diameter
1	hiPSc-Fibroblast-2D-mTeSR	1.02e+08 +/- 9.04e+06	100.00 nm
2	hiPSc-Fibroblast-0.3%PG-E8	2.07e+08 +/- 8.35e+06	88.00 nm
3	hiPSc-hPBMC-0.3%PG-E8	9.21e+07 +/- 1.02e+07	101.1 nm
4	hiPSc-hPBMC-0.5%PG-mTeSR	1.67e+08 +/- 1.02e+07	176.4 nm

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Chapter 4 – Conclusion and Discussion

4.1 Conclusion

In conclusion, this comprehensive investigation has unveiled the intricate interplay between culture conditions and the behavior of hiPSCs. Through systematic exploration of various culture conditions, this study has shed light on how the 3D PGmatrix systems impact hiPSC growth, gene expression, and hiPSC applications as hiPSC inducing and functional enrichment release. Notably, the 3D PGmatrix system has demonstrated remarkable advantages over traditional 2D culture methodologies in hiPSC reprogramming. It excels in maintaining hiPSC pluripotency, fostering sustained cell proliferation, and providing a versatile platform adaptable to different cell types and research needs, and meanwhile the culture condition could be modified to accommodate different cell lines or experimental supplements, such as for the application of drug delivery and the differentiation of hiPSC.

Another significant finding is the profound influence of EV secretion in 3D culture systems. The 3D culture system has emerged as a superior environment for promoting EV release compared to traditional 2D systems. This suggests that the 3D matrix plays a pivotal role in modulating the secretion dynamics of functional enrichments, which are of growing interest in various research fields. Moreover, the 3D PGmatrix system's superiority extends to hiPSC generation, especially when faced with challenging cell sources like suspension cells (hPBMC). It outperforms 2D methods by generating more hiPSC colonies with enhanced viability, proliferation, consistent maintenance, and pluripotency stable expression. This streamlined approach simplifies colony isolation and maintenance, improving overall efficiency and purity. In essence, this study underscores the practicality and efficacy of the 3D PGmatrix culture system, positioning it as a promising tool of hiPSC research, regenerative medicine, disease modeling, and drug discovery. While challenges in hiPSC remained as epigenetic memory, the system's adaptability and versatility hold promise for future stem cell research endeavors, including organoid generation and cell differentiation studies.