Comparative Analysis of Two-dimensional and Three-dimensional Stem Cell culture: Implications for Applications in the biomedical field

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Abstract

Stem cell expansion is a crucial part of regenerative medicine and modern biomedical research. Cultivating a large numbers of high-quality stem cells is essential to the development of cell therapies for conditions such as heart failure and neurodegenerative diseases, as well as applications in disease modeling and drug screening.[1] In vitro, stem cells are grown either on flat 2 dimensional surfaces (ex: petri dish) or within three-dimensional systems (ex: organoids). While 2D systems are currently the standard, 3D culture systems are gaining recognition for their ability to better mimic the cells' natural microenvironments. However, widespread adoption of 3D systems is restricted by issues in reproducibility and standardization, which must be addressed to achieve their intended benefits. Overcoming these challenges would have an incredible effect for both research and clinical adaptations, potentially improving the physiological relevance, efficiency, and scalability of stem cell-based therapies.[1 2] This research discusses and compares the characteristics, advantages, and limitations of both 2D and 3D stem cell culture systems, emphasizing their implications for regenerative medicine, disease modeling, and drug discovery.

Introduction

2D cultured stem cells are grown as monolayers on flat and rigid substrates, which are usually made of plastic. Due to that, they have limited opportunities for cell-cell and cell-extracellular matrix interactions, mainly only having contact with the artificial substrate[2]. These cells offer several advantages, they're very simple and cost efficient since their analysis is very straight forward and they have a well-established protocol to follow. Another strength of 2D systems is their high reproducibility, making it suitable for standardization and giving more consistent results across different experiments and laboratories, allowing comparison and making the results more credible, making it the best fit for standardization. As a result, 2D systems are particularly suitable for large-scale screenings, basic biology research, and specifically easy stage studies of cells [2]. However, despite their advantages, 2D cultured stem cells do have their limitations as well. Given that they're grown in a 2D environment and the complex 3D microenvironment found in living organisms can't be accurately replicated in a 2D system, they lose physiological relevance since their cell behavior can differ significantly from in vivo situations [5]. As well as the altered differentiation and signaling in 2D systems frequently result from the limited cell-cell and cell-matrix interactions causing unpredictability and inconsistencies in cell function, differentiation potential and gene expression [6 1]. Additionally stem cells in 2D cultures often experience rapid loss of stemness since the environment they're in may induce senescence which would cause the loss of stem cell properties [5]. Therefore, while 2D culture is ideal for studies and experiments where cell yield, consistency and simplicity are critical, it shouldn't be solely relied on for clinical translation or modeling complex tissues, since the system can't mimic the complexity required for that purpose.

3D cultured stem cells are grown on biomimetic scaffolds, spheroids or matrices which enables interactions from all directions[2]. This environment provides plenty of opportunities for enhanced cell-cell and cell-extracellular matrix[4]. 3D stem cell culture systems offer significant advantages in

comparison to traditional 2D approaches, primarily in terms of physiological relevance. The 3D microenvironment more closely simulates the complex cell-cell and cell-extracellular matrix interactions that occur naturally within living tissues. By more accurately mimicking biochemical cues present in vivo, 3D cultures are better able to replicate actual and accurate cellular behaviors, leading to results that are more representative of biological contexts.[6] Another notable advantage of 3D systems is their ability to better maintain the natural properties of stem cells, resulting in improved stemness, differentiation capacity, and proliferation rates. A study was conducted and has found that stem cells cultured within 3D systems have been shown to proliferate more actively, this finding was supported by the expression of Ki67, a protein found in the nucleus of actively dividing cells which is why Ki67 is a common marker used to asses proliferation rates. When comparing 3D and 2D cultures as shown in table 1, 69.4% of cells in the 3D environment were Ki67-positive, compared to only 57.4% in 2D culture. This difference is statistically significant ($p = 0.0022$), indicating that 3D cultures provide an environment that supports greater stem cell proliferation and viability.[7]

Another experiment was carried out to test the differentiation capacity in both 2D and 3D cultured stem cells showed that 3D culture techniques greatly enhance the differentiation potential of stem cells. As neural stem cells grown in 3D cellulose scaffolds showed significantly improved differentiation into both neuronal and glial lineages when compared to their 2D counterparts. This was measured using immunostaining, If a cell is GFAP-positive, it means the stem cell is turning into an astrocyte, a type of helper cell in the brain, for lineage specific markers, the proportion of GFAP-positive cells, marking astrocytic differentiation, was significantly higher in 3D cultures as illustrated in figure 1 (18.45% ± 2.8) than in 2D cultures (3.50% ± 2.7), with the difference being highly significant ($p < 0.01$). Similarly, the percentage of βIII-tubulin-positive cells, an indicator of neuronal differentiation, was also markedly higher in the 3D system (16.46% ± 4.5) compared to the 2D condition (0.79% ± 0.7), the difference can be observed in figure 2, once again reaching statistical significance ($p < 0.01$). For a comprehensive quantitative overview of these results, see the tables provided in the screenshot. These data make it clear that 3D culturing environments greatly facilitate the differentiation of stem cells into specific desired lineages, adding to their utility in both basic research and application-driven studies.[8]

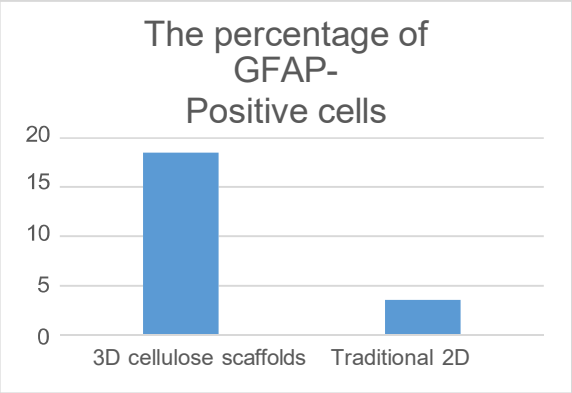

Figure 1. GFAP comparison chart

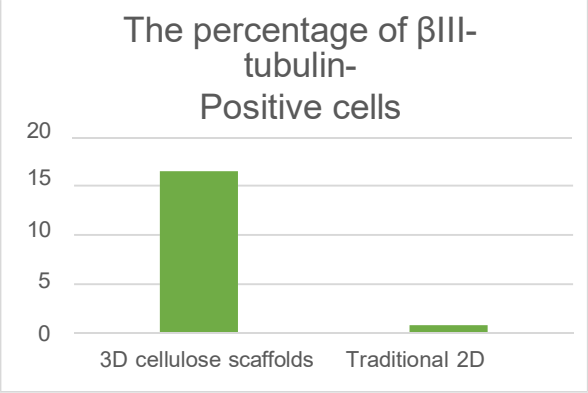

Figure 2. βIII-tubulin comparison chart

Lastly, 3D cultures provide more physiologically relevant drug responses, as cells grown in three dimensions tend to react to drugs and external signals in a manner much closer to what is observed within the body. The mimicking of a natural environment improves the predictive value of clinical studies, drug development and toxicity screening[2 3].

However, the use of 3D cultures does present several disadvantages. The main disadvantage is the difficulty in achieving standardization and reproducibility. The variability in scaffold materials, matrix composition, and protocols can introduce inconsistencies, leading up to 30-40% variation in cell behavior[10], making it harder to reproduce experiments or compare data across different laboratories and studies. This variability can limit the wider usage of 3D cultures in research and clinical fields[1]. Another limitation is the increased technical complexity. Setting up and maintaining 3D cell cultures demands more advanced techniques, greater expertise, and more complex equipment compared to traditional 2D systems1, 3D culture is almost 3 times more costly1 and takes 20-50% more time due to its complexity. Analyzing cellular behavior in a 3D environment often requires modern imaging and quantification methods to accurately observe cell activity, like confocal microscopy which are used to view cells inside thick 3D cultures because regular microscopes can't focus clearly at different depths. These methods detailed images that allow us to observe how cells behave in the 3D environment.[11] Additionally, 3D culture methods are often not ideal for high-throughput testing or large-scale production, as some 3D systems are hard to use in larger scales, for example, spheroid culture throughput is often limited to hundreds of spheroids per experiment, unlike 2D cultures which can easily handle thousands, making 3D systems less useful in situations that require large quantities or compatibility with automated equipment[4]. In summary, while 3D stem cell culture systems offer great abilities and improved physiological relevance, they also present new technical, logistical, and standardization challenges that must be addressed for standardization and consistent use in both basic and clinical research.

A key issue facing the standardization of 3D culture system is scalability and reproducibility as many 3D culture approaches are not readily applicable for large-scale or automated production, limiting their broader application[4]. That is caused by many other complications faced by 3D systems. Including protocol inconsistencies[1] in methods used for spheroid or organoid formation, such as hanging drop, spinner flask, or bioreactor techniques, often result in variable sizes and shapes of the cultured structures, reducing reproducibility. Alongside material since differences in scaffold composition and source, whether natural or synthetic, can significantly impact cell behavior and experimental outcomes[2 4]. As well as difficulties in the measurement and analysis of 3D cultures which introduces further difficulties due to optical limitations and the inherent heterogeneity of 3D tissues, complicating accurate evaluation of cell growth, differentiation, and other aspects[4]. To address these challenges, researchers are developing defined synthetic scaffolds[2 4], such as PEG-based hydrogels, which offer a uniform composition designed to minimize the unpredictability associated with animal-derived materials like Matrigel. Scaffold free techniques, including spheroid formation, microfluidics, or bioreactors, eliminate the need for physical scaffolds altogether, enhancing reproducibility and reducing batch-to batch variation[9]. The implementation of automated systems and bioreactors allows for spheroid and organoid growth under tightly controlled, scalable conditions, therefore improving yield and ensuring consistency across cultures[9]. Furthermore, advancements in imaging and analysis, such as computer assisted quantification, confocal microscopy, and light-sheet microscopy which all

provide more precise and comprehensive evaluation of 3D cultures, facilitating better quality control and process monitoring[4]. Together, these strategies represent promising approaches to overcoming the current limitations and advancing the standardization of 3D stem cell culture systems yet the problem is not yet fully resolved, as there are still difficulties in scaling these methods, achieving consistent results across different systems, and using them in everyday lab and clinical processes. The complexity and variability of 3D culture models remain as a major obstacles to their wider use in both research and industrial fields, emphasizing the ongoing need for further research and improvement in this field

Comparison point	Research status	Main references
Proliferation	Very well researched	3D cultures show higher proliferation (Ki67+) than 2D; supported by Cuesta-Gomez et al., 2023
Differentiation capacity	Very well researched	Enhanced lineage-specific differentiation in 3D vs. 2D shown by Basmahan et al., 2020; Couvrette et al., 2023
Maintenance of stemness	Fairly well researched	3D better preserves pluripotency and reduces senescence Yin et al., 2020; Cuesta-Gomez et al., 2023
Drug response	Fairly well researched	3D models show more clinically relevant drug responses (Kumar et al., 2024)
Scalability and yield	Well researched	2D easier to scale and yields more cells; 3D scalability improving but limited (Liu et al., 2024)
Cellular morphology	Very well researched	3D cultures maintain native morphology and polarity (Park et al., 2025).

Table 1. Findings across different researches

Methodology

This study conducted a systematic review through secondary data of published research comparing 2D and 3D stem cell cultures, focusing on proliferation, differentiation, stemness maintenance, drug response, scalability, technical complexity, and reproducibility. With selected quantitative data including comparative percentages and stem cell markers. The research was conducted across two main databases: PubMed and Google Scholar. Keywords including but not limited to “regenerative medicine” “3D/2D stem cell culture” “stem cell expansion” and “3D standardization challenges” were used combined with Boolean operators to maximize relevancy. The targets were comparisons, experimental studies, and articles published during the last 10 years. The study selection process is summarized using a PRISMA flow diagram which can be seen in figure 3 to ensure methodological transparency.

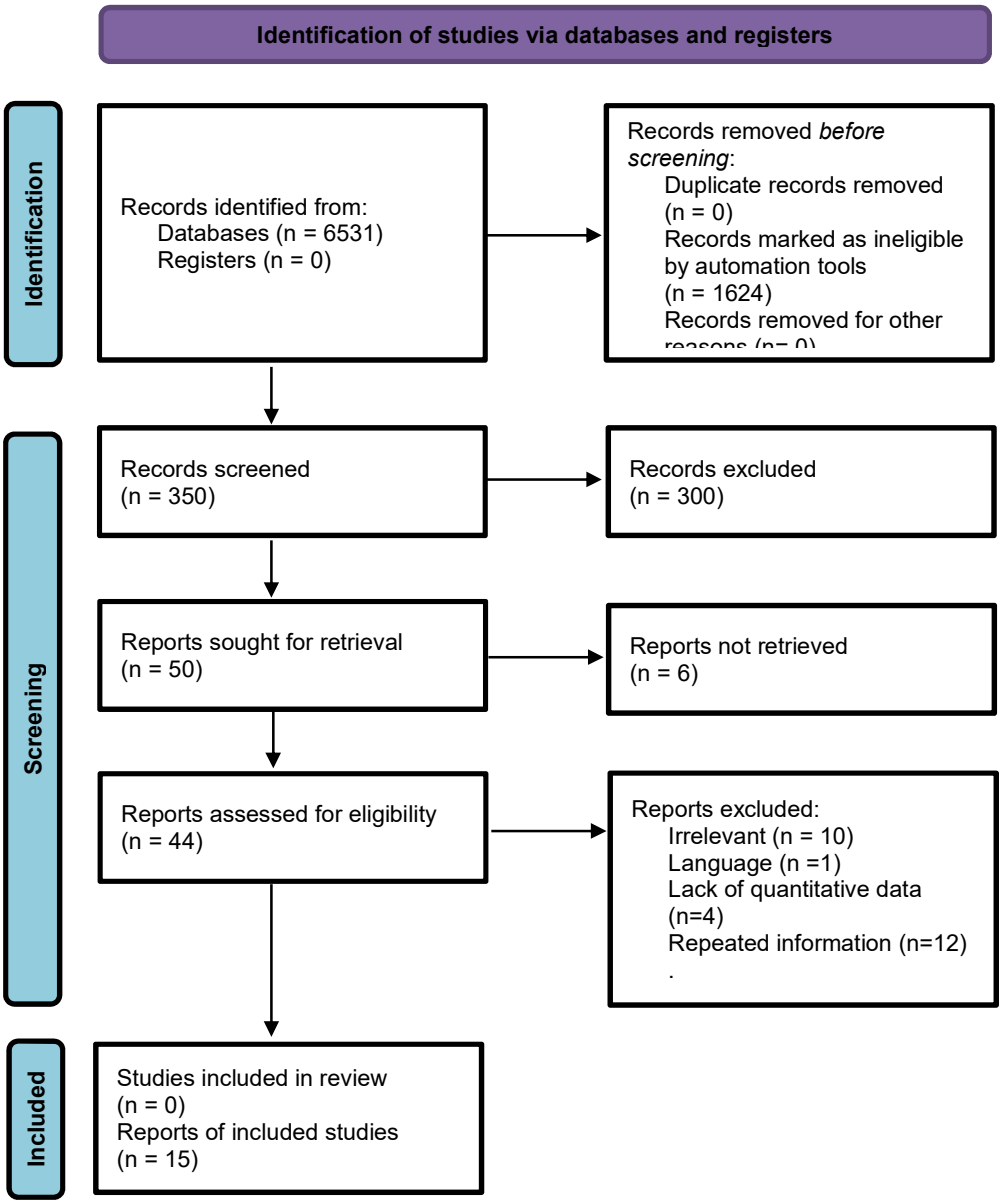

Figure 3. PRISMA diagram representing the reviewing process

Results

The results of this study highlight significant differences between 2D and 3D stem cell culture systems in terms of proliferation and differentiation capacities. Analysis of cell proliferation, measured by Ki67 expression, demonstrated that stem cells grown in 3D cultures exhibited a higher proliferation rate more than those cultured in 2D. As seen in Figure 1, 69.4% of cells in the 3D environment were Ki67-positive compared to 57.4% in 2D cultures, a difference that is statistically significant ($p = 0.0022$). This finding indicates that 3D culture conditions provide a more favorable environment for stem cell expansion, likely due to enhanced cell-cell and cell-extracellular matrix interactions within a biomimetic scaffold.

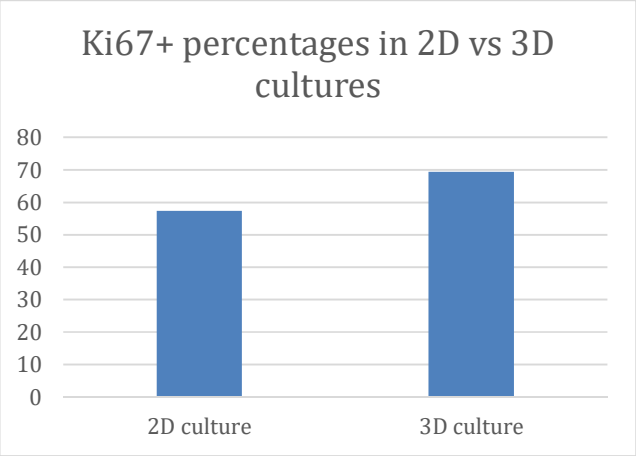

Figure 4. Ki67+ percentages comparison

Differentiation efficiency tests highlighted the enhancement of 3D cultured stem cells' abilities. Neural stem cells cultured within 3D cellulose scaffolds showed an increase in differentiation into both astrocytic and neuronal lineages compared to 2D cultures. The proportion of GFAP-positive cells, representing astrocyte differentiation, was significantly greater in 3D cultures (18.45% ± 2.8) than in 2D systems (3.50% ± 2.7) with $p < 0.01$. Similarly, βIII-tubulin-positive cells, indicative of neuronal differentiation, were considerably more abundant in the 3D environment (16.46% ± 4.5) compared to the 2D condition (0.79% ± 0.7), also reaching statistical significance ($p < 0.01$). These results demonstrate that 3D culture systems not only promote higher proliferation rates but also significantly enhance stem cell differentiation toward specific neural lineages, reinforcing their physiological relevance.

Additional to the proliferation and differentiation differences, the comparative analysis of 2D and 3D cultures revealed fundamental 3D culture systems better replicate the complex microenvironment of living tissues by providing multidirectional cell-cell and cell-matrix interactions, which supports the maintenance of stemness and reduces senescence compared to traditional 2D systems. As well as, stem cells grown in 3D environments have shown to exhibit drug responses more accurate to in vivo behavior, improving the predictive accuracy of clinical studies. However, 3D cultures currently face challenges in standardization and reproducibility; variability in scaffold composition, matrix properties, and culture protocols can introduce inconsistencies, complicating comparative studies and broader adoption. These challenges are compounded by increased technical complexity, requiring advanced imaging and analytical methodologies to accurately evaluate cell behavior within three-dimensional matrices.

In contrast, 2D cultures offer advantages in simplicity, cost-effectiveness, and easy scalability, with well-established protocols ensuring high reproducibility across experiments. These qualities make 2D systems especially suitable for large-scale screenings and basic biological studies where consistency and yield are critical. However, the inability of 2D cultures to accurately capture the in vivo microenvironment limits their value and use in clinical translation and complex tissue modeling. Collectively, these data underline that while 3D culture systems provide superior physiological relevance and improved cellular function, addressing current technical and logistical challenges is essential to achieve their full potential for regenerative medicine and biomedical research.

These findings are further supported by a broader comparison between 2D and 3D cultured stem cells across key functional parameters such as proliferation, differentiation, stemness, drug response, scalability, technical complexity, and reproducibility as seen in table 2. The table highlights how 3D systems consistently outperform 2D in mimicking physiological conditions and supporting functional maturity, while 2D systems maintain advantages in simplicity, scalability, and reproducibility.

Comparison point	2D cultured stem cells	3D cultured stem cells
Cell shape	Cells have a flat and stretched shape with an average height of ~1–3 μm because they can only grow in two directions on the surface14	Cells maintain their natural shape and grow into 3D clusters or spheroids ~100 300 μm diameter, which are made up of multiple layers14
Proliferation	Moderate proliferation (~57.4% Ki67+). The flat surface limits cell cell interaction and alters signaling	Higher proliferation (~69.4% Ki67+). The 3D microenvironment supports multidirectional interactions making the cells more physiologically relevant
Differentiation Capacity	Limited differentiation with low marker expression (e.g., GFAP 3.5%, βIII-tubulin 0.79%)	Significantly enhanced differentiation (GFAP 18.45%, βIII-tubulin 16.46%). The 3D environment mimics in vivo signaling, improving specificity
Maintenance of Stemness	Rapid loss of stemness due to the artificial microenvironment. Promoting senescence and spontaneous differentiation	Better stemness preservation due to constant cell-matrix interactions. Supporting controlled differentiation for regenerative applications. With as higher cell survival (~77%) after 14 days compared to 2D cultures[12]
Drug response	Inaccurate and unpredictable results due to limited cell-matrix interactions and altered signaling as the cells often have little resistance to drugs making it appear as though drugs	Closely matches in vivo conditions making 3D systems more predictive and valuable due to their environment and improved resistance
Cost	For large-scale studies, it is much cheaper than using 3D culture	3D cell culture is generally more expensive and takes longer than 2D methods, but it offers more reliable drug screening by better mimicking how cells behave in vivo
Scalability and yield	Highly scalable and standardized. Easy to	Limited scalability due to inconsistent protocols and material

	automate and ideal for large-scale expansion in research and industry	variability. Bioreactor systems are improving yield, but standardization is still evolving
Technical complexity	Simple setup with well-established protocols. Imaging and analysis are straightforward, making it accessible and cost-effective and only taking a few hours	Technically demanding, requiring advanced scaffolds, imaging, and analysis tools. High complexity can limit widespread use without specialized training and equipment while being more time consuming up to 12 hours[13]
Reproducibility	High reproducibility with defined protocols and materials	Variable reproducibility due to scaffold diversity leading up to 30-40% variation
Best uses	Ideal for studies and experiments where cell yield, consistency and simplicity are critical. Ex: Large scale production which 2D cultures represent ~ 90% of the field13	Ideal for studies requiring physiological relevance. Ex: Regenerative medicine and drug modeling with growth rates >30%13

Table 2. Overview of key points in 2D vs 3D cultures

Discussion

This study investigates how two-dimensional and three-dimensional stem cell culture systems influence stem cell proliferation and differentiation, with particular attention given to their suitability for regenerative medicine and biomedical research. The primary question addressed is whether 3D culture environments offer greater physiological relevance and functionality compared to traditional 2D cultures. The key findings observed in this research were that stem cells cultured in 3D microenvironments proved to have higher proliferation rates and enhanced differentiation capacity compared to 2D systems. These findings were supported by two experiments conducted to test and compare each ability. The outcome of these experiments suggests that while 2D systems are more cost-efficient and simple, 3D microenvironment proved to provide more effective cues for both cell expansion and specialization. The consistency of these findings with current literature, such as studies by Cuesta-Gomez et al., 2023 and Basmahan et al., 2020 mentioned in table 1, reinforces their validity and relevance. Previous research has also reported that 3D systems has improved maintenance of stemness, reduced senescence, and drug responses that better mimic in vivo behavior. The enhanced performance of 3D systems was expected as it's a well-established fact in the cell culture field due to the more in vivo accurate microenvironment the cells are cultivated in. Making the study's findings in strong agreement with prior research highlighting the importance of microenvironmental cues in regulating stem cells. However, this study draws largely on specific scaffold and cell types, which may not fully represent other materials or cells, and variability in culture conditions across laboratories could limit generalizability. This study uses quantitative markers (Ki67, GFAP, βIII-tubulin) with statistical data to ensure objectivity and fair comparison, As well as proliferation and differentiation capacity results are based on experiments conducted in controlled laboratory environments to have a clear observation on the cell behavior. However there are several limitations and factors that may have influenced the outcomes such as material variability and protocol inconsistencies which contribute

to differences across experiments. As well as, the advanced tools required for 3D systems which may introduce measurement limitations, affecting accuracy. By gathering and analyzing existing research, this review highlights the benefits and limitations of both systems, helping researchers make more informed decisions when selecting the most suitable culture method for their work. These findings confirm that 3D culture systems enhance the physiological relevance of in vitro models, making them valuable for accurate tissue modeling and drug screening, while 2D systems remain preferable for high-throughput screenings due to their simplicity and consistency; therefore, it's incredibly important that future research should focus on overcoming 3D standardization challenges by implementing well established protocols to unify the materials and conditions, developing synthetic scaffolds to improve reproducibility and scalability, adopting automated bioreactor systems for large scale 3D stem cell production, and exploring scaffold-free 3D techniques to reduce variability. Improving these methods will not only make research results more reliable, but also have a significant impact on future medical treatments and drug development. This study highlights the need to shift toward more complex but clinically accurate 3D models in biomedical applications while providing a helpful starting point for researchers in designing studies that are both scientifically and practically relevant, helping to advance research that is more consistent and applicable to the real world.

Conclusion

This study demonstrates that three-dimensional stem cell culture systems significantly enhance proliferation and differentiation capacities compared to traditional two-dimensional cultures, therefore offering a more physiologically relevant result for regenerative medicine and biomedical research. While 2D systems maintain advantages in simplicity, reproducibility, and scalability, their inability to mimic the complex in vivo microenvironment limits their potential for clinical applications and complex modeling. In the contrast, 3D cultures better replicate natural cell interactions, improving abilities including relevance, differentiation capacity, and stemness maintenance with predictive and accurate drug responses. However, challenges in standardization, reproducibility, and technical complexity currently limit the standardization of 3D methods. Addressing these issues through standardized protocols, synthetic scaffold development, automated bioreactor integration, and scaffold-free approaches is essential. Overcoming these challenges will allow more reliable, scalable, and clinically accurate stem cell models, ultimately advancing both biomedical research and the medical field. That's why it's so important to keep researching and improving 3D stem cell culture, so we can fully unlock its potential and move closer to better treatments and real progress.

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