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Concise Review

Three-Dimensional Spheroid Culture of Human Mesenchymal Stem Cells: Offering Therapeutic Advantages and In Vitro Glimpses of the In Vivo State

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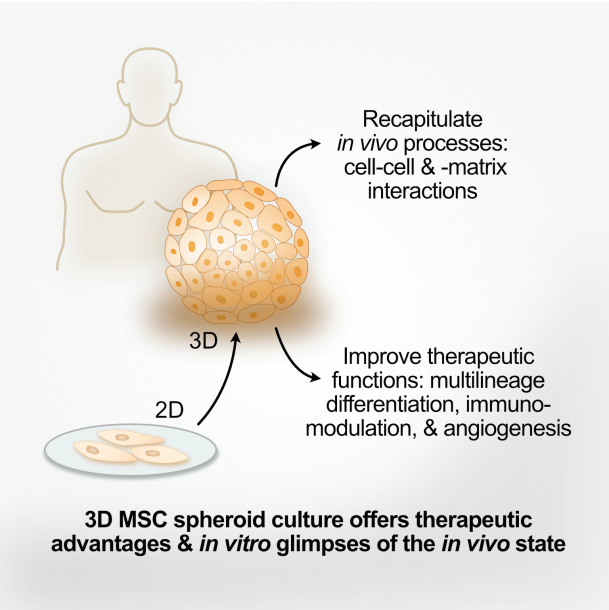
Abstract

As invaluable as the standard 2-dimensional (2D) monolayer in vitro cell culture system has been, there is increasing evidence that 3-dimensional (3D) non-adherent conditions are more relevant to the in vivo condition. While one of the criteria for human mesenchymal stem cells (MSCs) has been in vitro plastic adherence, such 2D culture conditions are not representative of in vivo cell-cell and cell-extracellular matrix (ECM) interactions, which may be especially important for this progenitor/stem cell of skeletal and connective tissues. The 3D spheroid, a multicellular aggregate formed under non-adherent 3D in vitro conditions, may be particularly suited as an in vitro method to better understand MSC physiological processes, since expression of ECM and other adhesion proteins are upregulated in such a cell culture system. First used in embryonic stem cell in vitro culture to recapitulate in vivo developmental processes, 3D spheroid culture has grown in popularity as an in vitro method to mimic the 3-dimensionality of the native niche for MSCs within tissues/organs. In this review, we discuss the relevance of the 3D spheroid culture for understanding MSC biology, summarize the biological outcomes reported in the literature based on such this culture condition, as well as contemplate limitations and future considerations in this rapidly evolving and exciting area.

Key words: 3-dimensional cell culture; multicellular spheroid; human mesenchymal stem cells; multilineage differentiation; chondrogenesis; osteogenesis; paracrine factors; immunomodulation; angiogenesis; wound-healing.

Graphical Abstract



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Significance Statement

While 2-dimensional in vitro plastic adherence is a criterion for defining human mesenchymal stem cells (MSCs), 3-dimensional (3D) non-adherent conditions may be particularly suited to understand MSC physiological processes since cell-cell and cell-extracellular matrix interactions in this system better mimic the native in vivo niche of MSCs within tissues/organs. Moreover, significant therapeutic advantages of 3D MSC spheroids have been seen, including in in vivo models. We discuss here the application of 3D spheroid culture for a better understanding of MSC in vivo biology and therapeutic uses, as well as limitations and future considerations in this rapidly evolving and exciting area.

Introduction

In vitro mammalian cell culture has clearly been indispensable for understanding normal and pathological biological processes. As invaluable as this classic 2-dimensional (2D) monolayer system has been, the inability to mimic cell-cell and cell-extracellular matrix (ECM) interactions of cells within its native organs/tissues—all 3-dimensional (3D) in nature—are known to limit the physiological relevance of monolayer in vitro culture.[1-3] Early morphologic studies demonstrate that when epithelial cells are cultured in 3D conditions, recapitulation into their in vivo native 3D structures mimicking its tissue of origin occurs.[4] In recent years, profiling technologies have increasingly documented the genetic and epigenetic alterations that 2D in vitro culture can induce in the cultured cell, which then lead to genetic/chromosomal aberrations and functional phenotypes that drift away from the original state of the isolated cell.[5-8] Alarmingly, a consistent difference reported between 2D monolayer culture and native tissue and/or uncultured cells is the upregulation of the cell cycle and proliferative pathways,[5,6] which is often a key experimental parameter in most in vitro studies. Thus, while 2D monolayer cell culture is convenient and low cost, its physiological relevance is increasingly being questioned.

Historical Background: 3D In Vitro Culture Recapitulate Physiological Outcomes With Cell-Type Specific Results

The earliest non-adherent 3D spheroids were likely spontaneous cell aggregations of differentiating pluripotent mouse teratocarcinoma cells grown in the absence of feeder cells to maintain undifferentiated conditions.[9] Termed embryoid bodies (EBs), these suspended cell aggregates resembled rounded spheroids and, more importantly, proceeded to recapitulate the tri-germ layer differentiation events which occur during early mouse embryo development. When normal pluripotent stem cells (PSCs) were first isolated from the murine blastocysts, the same spontaneous aggregation of isolated embryonic stem cells (ESCs) into non-adherent EBs could also be seen when cultured without feeder cells to allow for spontaneous differentiation.[10] 3D EB culture is now an established protocol to test the pluripotent capacity of any PSC including induced PSCs (iPSCs)[11,12] and especially human PSCs,[13,14] since the most rigorous tests of in vivo pluripotency for human cells are likely not able to be performed due to ethical concerns.[15,16]

In contrast to PSCs in which 3D suspension culture results in differentiation and developmental progression, stem cells from a number of adult organs including neural stem cells (NSCs) and mammary stem cells, are selected and identified through this same ability to survive and proliferate ex vivo from single-cells into suspended cellular spheroids in serum-free non-adherent culture.[17,18] There are key differences between 3D conditions for PSCs versus somatic stem cells, however;3D PSC-EBs arise from aggregations of cells cultured in serum conditions, whereas the somatic stem cell spheroids arise from single-cell outgrowths and are cultured in serum-free conditions. This single-cell, minimal condition used to select for normal NSCs was then adopted to select for and identify cancer stem cells from solid tumors in the brain.[19] Oddly, this same 3D NSC/brain cancer stem cell selection method has since been used to select for cancer stem cells from other solid cancers, including breast, colon, and lung.[20-22] Indeed, there is still controversy in the idea of the "cancer stem cell" in solid tumors,[23,24] and it is still of debate how reflective this stringent 3D culture method is to the actual disease state in the patient.[25] These striking differences in cellular fate and developmental outcome after the 3D culture are clearly due to both differences in the culture method as well as the inherent nature of the cultured cell itself.

3D Spheroid Culture for "2D-Defined" MSCs: Evidence for In Vivo Relevance

MSCs are multipotent somatic stem cells that can differentiate into the mesodermal skeletal/connective tissue cell types of osteoblasts, chondrocytes, and adipocytes. First found in the bone marrow (BM) as supporting stromal cells for hematopoiesis, the multilineage differentiation capacity for these stem cells was rapidly demonstrated, with similar progenitor/stem cells also found in numerous tissues and organs in quick succession.[26] An interesting defining characteristic of all MSCs is the in vitro criteria of plastic adherence in standard 2D culture.[27] This unusual criterion likely arose out of the initial need in BM aspirates to discern MSCs from hematopoietic/immune cells which can adhere (ie, monocytes, macrophages, dendritic cells, and activated lymphocytes[28,29]) prior to the advent of more sophisticated molecular selection methods. It can therefore be argued that MSCs are the ultimate adherent cell type, so a fair question to ask would be whether 3D non-adherent culture conditions are an appropriate in vitro condition for MSCs. While the answer to this question still awaits accumulation of comparative evidence of in vivo vs. in vitro MSC biological information, there has been an explosion of publications on MSCs cultured in 3D conditions as spheroids: a PubMed search for title keywords of "mesenchymal stem/stromal cells," "sphere" or "spheroid," and "3D" yields nearly 300 publications. Scaffold-based systems of 3D MSC culture have a long history of study since the most important differentiation lineages of MSCs are toward skeletal-related tissues in which non-cellular components are functionally critical. Despite large numbers of studies in this area of 3D MSC culture and tissue engineering, shortcomings still exist in each type of scaffold, including effectiveness in mimicking the native ECM microenvironment, residual harmful

solvent, uneven cell distribution, and maintenance of cell viability.[30,31] While scaffold-based culture can activate MSC-matrix interactions, scaffold-free 3D spheroid culture relies on the cultured cells themselves to modulate and/or create the microenvironment as well as interact with each other; this is likely more physiological, and since this is a more recent 3D method, studies are generating unexpected and interesting findings.[32-34] Importantly, the translational relevance of scaffold-free 3D MSC spheroids is increasingly being reported, with a very recent study evaluating detailed clinically important parameters in non-human primates.[35] It is also necessary to clarify that in vitro culture of MSCs in scaffolds to achieve tissue engineering for 3D skeletal components like bone and cartilage still mainly involves the 2D adherent culture of MSCs on the engineered surfaces, and therefore not focused upon in this review.

Despite in vitro, plastic-adherence being one of the 3 criteria for defining human MSCs, in vivo these cells are obviously found in 3D tissues/organs.[36] Indeed, the ability of MSCs to "self-assemble" into cellular aggregates and spheroids in suspension culture was reported in several studies shortly after the publication of the Minimal Criteria.[37-39] Moreover, as the stem/progenitor cells for bone and cartilage, tissues where acellular components significantly outweigh the cellular compartment, MSCs are responsible for secreting the myriad of ECM molecules specific for each lineage.[40] The multicellular 3D spheroid culture, therefore, may be particularly suited as an in vitro model to investigate MSC biology, as this method of 3D culture is known to elicit ECM secretion from the aggregated cells.[41] In fact, an early study using bone progenitor cells/osteoblasts from diverse tissues including the BM demonstrated spontaneous non-adherent spheroid formation with osteogenic lineage commitment by all these MSC-related cells, with the spontaneous 3D spheroid formation increasing protein expression of integrins and inorganic components allowing for recapitulation of osteogenesis and successful ex vivo bone formation.[42]

As with its predecessor the EB suspension culture, the culture medium used for the multicellular 3D spheroid culture is usually unchanged from its 2D culture; this may be one important reason why this multicellular aggregated 3D culture method has been found to better reflect in vivo conditions, even for cancer cells.[41,43] MSCs may be especially sensitive to its microenvironment both in vivo and in vitro, with specific ECM molecules, matrix, as well as the stiffness of its niche able to modulate differentiation and lineage commitment, as seen in the spontaneous differentiation into different lineages when cultured in vitro on plates with varying stiffness index and ECM protein coatings.[44,45] The single-cell serum-free 3D culture may, therefore, be a less suitable method to recapitulate MSC biology given its minimal and stringent conditions; it has been well documented in a standard 2D culture that such low serum, low cell density conditions strikingly alter the biological profile of the cultured cell as to call into question the physiologic relevance of such methods.[46-48] While advances in single-cell transcriptomic technology have been increasingly applied to understanding murine BM and adipose MSC in its native in vivo state,[49-51] these studies are technically and ethically challenging to conduct in the human system. For understanding human MSCs in a more physiological context, researchers have therefore increasingly turned to use multicellular 3D suspension culture to achieve this goal.

3D Culture Is Integral to MSC Chondrogenesis and May Improve Osteogenic Differentiation as well as Multilineage and Survival Capacity

From the understanding of embryonic limb development elucidated in the 1980s-1990s, the standard protocol to induce MSC chondrogenesis in vitro has required 3D suspension culture conditions to achieve high-density cell aggregation, in contrast to differentiation protocols for all other somatic lineages which are largely performed using standard 2D monolayer culture. This is likely because condensation—a process during developmental lineage commitment in which reduced intercellular spaces, increased cell-cell adhesion, and increased ECM secretion lead to 3D tissue formation—is an integral process of in vivo chondrogenesis.[52,53] Moreover, Sox9, the master transcription factor for chondrogenesis, can be induced by the process of compression and with 3D organoid culture.[54-56] While the initial protocol for MSC chondrogenic differentiation protocol is similar to the typical 3D multicellular aggregation culture, one important difference is that chondrogenic differentiation requires a serum-free environment for adequate lineage commitment,[54] but the influence of physical parameters brought about by 3D culture on MSCs to commit to chondrogenesis is so strong that this can occur even without the addition of biochemical factors including transforming growth factor β (TGFβ), the standard for this protocol.[40]

In contrast to MSC chondrogenesis where 3D multicellular non-adherent culture is a prerequisite, 3D culture to explore other functional capabilities of MSCs was first attempted in the late 2000s. A frequent result after MSC 3D spheroid formation is increased expression of pluripotency factors Oct4, Sox2, and Nanog,[57-59] but the functional role of these findings with regard to MSCs is still of some debate.[60-62] Multilineage differentiation capacity in terms of both adipogenic and osteogenic potential was also found to be increased in numerous studies;[58,59,63,64] but strikingly, none of these reports evaluated chondrogenic potential, perhaps because studies were performed in serum-containing conditions. In contrast, one study found just chondrogenic capacity to be enhanced after 3D spheroid formation.[57] Of the differentiation capacity found to be enhanced after 3D spheroid formation in serum conditions, increased osteogenesis has been most frequently documented in both in vitro and in vivo models,[65-67] with one recent study demonstrating 3D spheroid MSCs could be rapidly induced into osteocytes, an even more mature cell type than osteoblasts.[68] The strong osteogenic propensity of serum-cultured 3D MSCs is such that, in the few 3D MSC studies using non-human cells, similar results were found as well.[69,70] 3D culture profoundly changes cell shape and cytoskeletal dynamics, and these parameters are known to influence MSC lineage commitment especially osteogenesis;[71] in fact, spontaneous osteogenesis can occur when MSCs are cultured in 3D microcarriers as a result of cytoskeletal alterations.[72] Also, similar to the early report on MSC-related osteoprogenitors enhancing osteogenesis after spontaneous spheroid formation,[42] these MSC 3D studies were carried out in serum-containing conditions, implicating the importance of serum on influencing MSC commitment into osteoblasts versus chondrocytes.[73] Increased protein expression of ECM molecules,[69] osteogenic integrins,[67] and cadherins[66] were all found to be involved in the enhanced osteogenic capacity of 3D MSC spheroids. Such results further support that the 3D

spheroid culture can recapitulate aspects of in vivo ECM/niche-cell and cell-cell adhesion and interactions less evident in 2D monolayer culture.[39,41,74,75]

One of the most consistent findings when MSCs are cultured as 3D spheroids appear to be increased cell viability and survival.[76] This was very comprehensively evaluated in a report which not only assessed MSC spheroids but also human ESC spheroids.[77] The enhanced cell survival can be seen across different culture conditions, including in minimal or serum-free conditions[39,58,78] as well as hypoxia.[79,80] Most studies found that MSCs cultured as 3D spheroids remain in a more quiescent, less proliferative state compared to 2D monolayer culture due to metabolic shifts and deregulation of cell cycle genes.[74,77,81] While one report found increased apoptosis with 3D spheroid formation,[82] nearly all other reports showed either maintenance of cell viability or decreased apoptosis in vitro as well as after in vivo transplantation.[58,59,79,80,83] A few studies have delved into the mechanisms involved and identified the upregulation of anti-apoptotic genes such as Bcl-2 and the downregulation of apoptotic genes like Bax in MSC 3D spheroids.[77,84] Collectively, these results implicate the higher relevance of 3D culture to native in vivo states/uncultured cells which by transcriptomic and epigenetic profiling analyses are less proliferative and more quiescent than 2D monolayer-cultured cells.[5]

3D MSC Culture Enhance Immunomodulation, Angiogenesis, and Paracrine Activities

While not an essential criterion, the strong immunomodulatory properties of nearly all sources of human MSCs have become one of the most clinically relevant functions of these stem/progenitor cells, by not only broadening the application of MSCs toward immune and inflammatory diseases but also allowing for immunologically unmatched use of these cells as off-the-shelf products.[85] Interestingly, among the first reports to explore 3D MSC culture and functional outcomes was focused on immunomodulation.[81] More in-depth research by the same group demonstrated specific mechanisms involved in the increased anti-inflammatory and immunomodulatory effects of 3D spheroid MSCs, which included triggering of caspase-dependent interleukin (IL)-1 signaling and the secretion of major immunomodulatory factors including prostaglandin E_2 (PGE_2) and tumor necrosis factor-stimulated gene 6 (TSG6),[86] as well as suppression of inflammatory cytokines such as tumor necrosis factor-α (TNFα).[81] The stronger immunomodulatory function of 3D cultured-MSC spheroids has been quite consistently reported,[58,87,88] and compared to 2D culture, priming MSC spheroids with inflammatory factors such as interferon-γ (IFNγ)[89,90] or IL-1[76,91] also leads to increased immunomodulatory effects in vitro. In vivo, 3D spheroid MSCs led to better resolution of murine peritonitis[81] and colitis.[77] Moreover, 2 recent studies focusing on different types of arthritis have demonstrated better outcome with 3D spheroid MSCs: (1) in a murine rheumatoid arthritis model, just injection of the conditioned medium of 3D MSC spheroids resulted in a better outcomes than the application of the cells themselves whether 2D- or 3D-cultured;[92] and (2) in a non-human primate model of osteoarthritis, injection of either xenogeneic or allogeneic 3D MSC spheroids decreased joint inflammation and improved disease outcome.[93] While functional improvement of MSC immunomodulation was clearly demonstrated in all these studies, mechanistic understanding of how cell dimensionality can alter immune function awaits further elucidation.

Similar to immunomodulation, the angiogenic and wound-healing capacity of MSCs are well reported despite not being one of the Minimal Criteria. Paracrine factors are also largely responsible for many of these therapeutic effects and even in the first reports, increased vascular endothelial growth factor (VEGF) secretion after 3D spheroid culture was consistently seen to enhance these properties;[37,75,79,80,84,94,95] other angiogenic and/or mitogenic factors especially hepatocyte growth factor (HGF) and basic fibroblast growth factor (bFGF) were also frequently reported to be upregulated.[37,74,79,84,94,95] A number of in vivo ischemic disease models demonstrated therapeutic effects of 3D MSC transplantation, including in ischemic limb injury,[79,84] ischemic kidney injury,[75] and ischemic heart disease.[96] There are also many studies showing 3D MSC spheroid application improving wound healing and involving angiogenesis in mouse models.[74,83,95] Mechanistically, this appears to be due to the strong upregulation of hypoxia-related pathways in 3D MSC spheroids as evidenced by transcriptomic profiling,[37] as hypoxia is one of the strongest inducers of angiogenesis.[97] Collectively, these studies and immune-related studies demonstrate that, compared to 2D monolayer culture, 3D spheroid culture further enhances the paracrine functions of MSCs to have strong translational value.

3D MSC Culture Modulates ECM Molecules: Implications for Lineage Commitment and Stemness/Senescence

A number of studies have shown that the ECM can regulate stem cell fate, especially in MSCs.[98] Conversely, MSCs are known to secrete a number of ECM molecules and remodeling enzymes due to the capacity of these stem cells to differentiate into tissues with significant ECM components. While such studies are beginning to be conducted using 3D-cultured MSC spheroids, most reports especially report including in vivo evaluation have largely used standard 2D culture systems; we have therefore summarized 2D-cultured MSC-ECM molecule studies along with 3D spheroid studies to better present the potential functional application of 3D MSC spheroid culture in modulating ECM molecules (Supplementary Table S1; Fig. 1). Upregulation of collagen I, fibronectin 1, and laminin were observed in 3D spheroid compared to 2D monolayer culture, and all 3 molecules are involved in increasing survival, proliferation, paracrine effects as well as stem cell selection/enrichment of MSCs.[75] Collagen V and collagen VI were also highly expressed in MSC spheroids and reported to enhance proliferation.[95] These results indicate that the enhancement of MSC stem cell properties by 3D spheroid culture could contribute to the expression of specific ECM components including collagens I, V, VI, as well as fibronectin and laminin. Some ECM molecules reported to modulate MSC differentiation are also observed in MSC spheroids. Upregulation of collagen V,[99] laminin,[100] and perlecan[101] was seen in MSC spheroids; in 2D studies, these ECM molecules respectively were seen to promote chondrogenesis, neurite outgrowth, and osteogenesis while blocking adipogenesis. Interestingly, collagen IV, which in 2D studies is upregulated during adipogenic induction, is less expressed in 3D MSC spheroid cultures.[102] Since it has been reported that senescent MSCs activate adipogenesis but

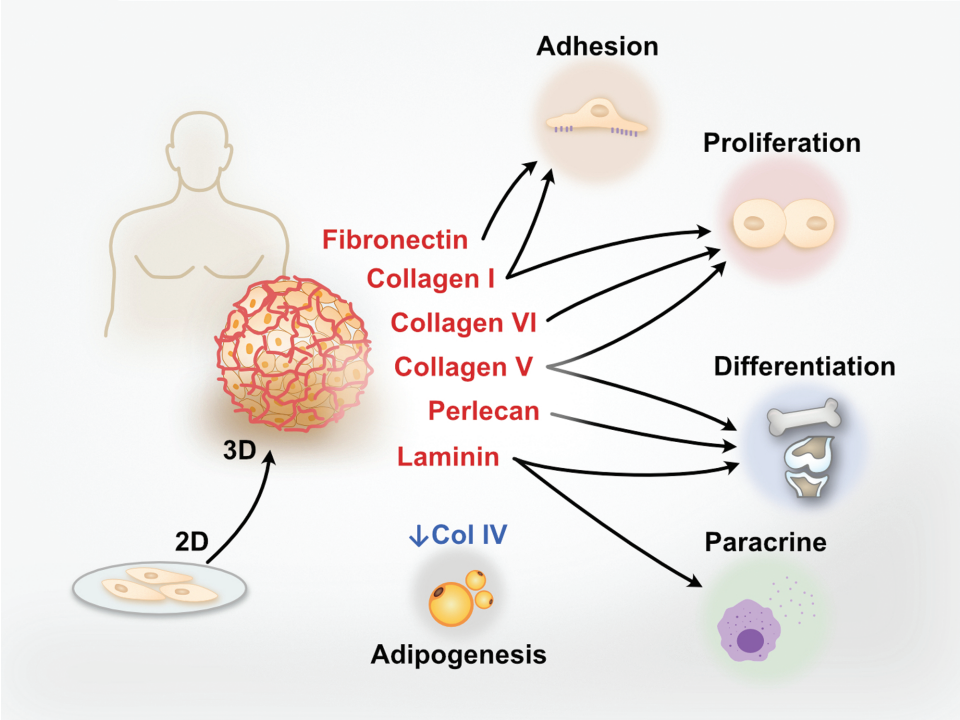

Figure 1. Effects and expression of ECM molecules in 3D-cultured MSC spheroids. MSCs are known to secrete a number of ECM molecules which can regulate stem cell fate. Compared to 2D monolayer system, ECM molecules including collagen I, IV, V VI, as well as fibronectin, laminin, and perlecan have been reported upregulated or highly expressed in human MSC 3D spheroids. The effects of these ECM molecules on MSCs examined through 2D monolayer model can be categorized to enhance cell adhesion, proliferation, differentiation, and paracrine effects according to previous research. In contrast, collagen IV, which upregulated during adipogenic differentiation, was found less expressed in MSC spheroids.

suppress osteogenesis,[103] the lower expression level of collagen IV in 3D MSC spheroids is in line with the ability to maintain stemness and avoiding senescence.

Disease-Specific 3D MSC Spheroid In Vivo Studies: Recent Advances and Implications for Therapeutic Applications

Because culturing MSCs in 3D spheroid conditions is a newer methodology, there are much fewer studies in general compared to studies using standard 2D culture methods (Supplementary Table S2). However, in the relatively smaller pool of in vivo disease model studies using 3D MSC spheroid cultures, a surprisingly large proportion have focused on the differentiation capacity towards osteogenesis in the repair of bony defects using rodent calvarial defect models[65,70,104] and femur fracture model;[88] one recent study specifically evaluated 3D MSC spheroids for use toward calvarial defects in aged mice,[105] which is in line with the overall in vitro finding of increased stemness/decreased senescence of 3D-cultured MSCs. A recent study evaluated the use of matrilin-3-primed MSC spheroids in a rat model of intervertebral disc degeneration.[106] Also recently, secretome from 3D MSC spheroids have also been found to improve a mouse model of rheumatoid arthritis;[92] more significantly and also very recently in a non-human primate model of osteoarthritis, direct intra-articular injection of either human BM- or ESC-MSC spheroids have also demonstrated therapeutic improvement.[93] In these joint-related studies, some of the therapeutic effects could be attributed to immunomodulation since inflammation is a known component of any type of arthritis.

A significant number of in vivo studies have focused on paracrine properties of 3D MSC spheroids, with the earliest study revealing enhanced immunomodulatory properties in a mouse model of peritonitis.[81] Subsequently, others have found similar results in a mouse model of colitis,[77] and more recently in a mouse model of pulmonary inflammation.[107] Another important MSC paracrine function is angiogenesis, and 3D spheroid administration has been evaluated in several rodent limb ischemia models.[79,84,108] Wound-healing is a related angiogenic outcome and has been studied using either healthy[83,95] or diabetic rodent models.[74] Other organ-ischemia models likely benefitting from both enhanced angiogenic and immunomodulatory functions in addition to possible differentiation effects of 3D MSC spheroids include acute kidney injury in rats[75] and a large-animal pig model of chronic myocardial infarction.[96] More recently, 2 studies have focused on the repair of neurological injury using mouse models of neurogenic pain[109] and spinal cord injury.[110] A recent highly translational study using healthy non-human primates sought to understand the distribution and safety profile of intravenous administration of 2 types of MSCs—BM and human ESCs—cultured as 3D spheroids.[35] Collectively, the increasing numbers of in vivo disease model studies and large animals demonstrate the strong interest and potential of 3D MSC spheroids for clinical use in a broad range of disease indications.

Conclusions and Future Considerations

The increasing numbers of accumulated reports strongly support that 3D spheroid culture for MSCs has therapeutically

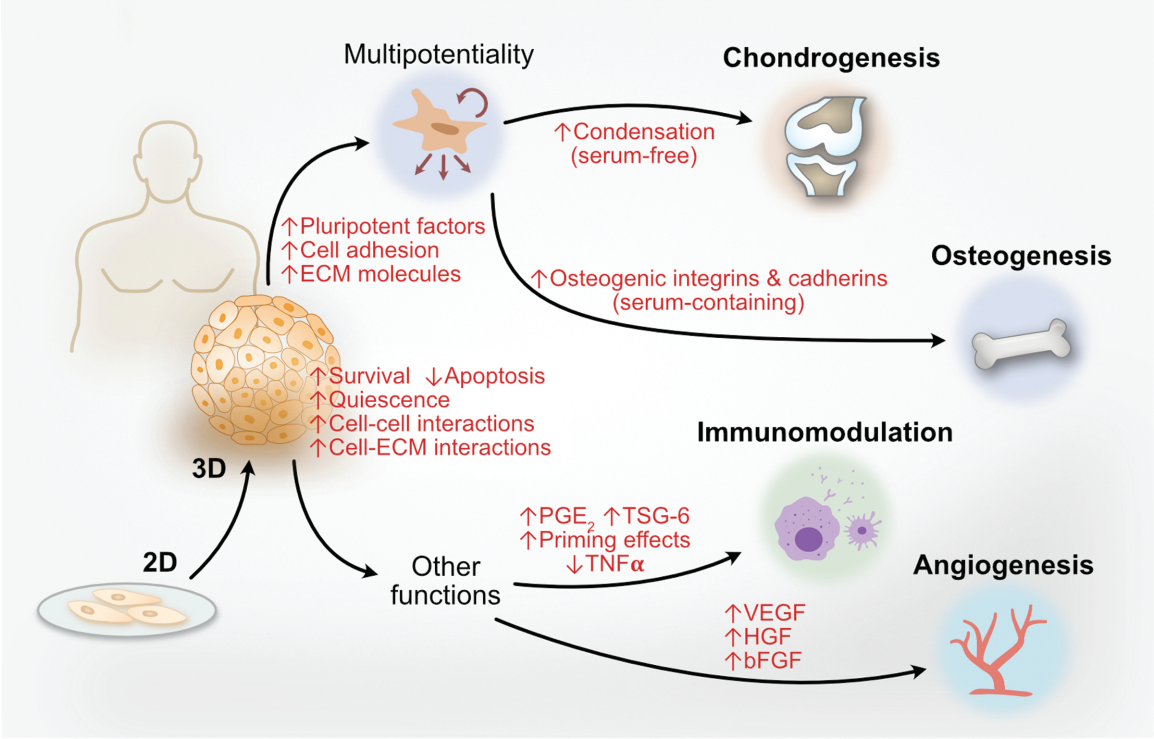

Figure 2. 3-Dimensional (3D) multicellular spheroid culture of human mesenchymal stem cells (MSCs) induce profound biological changes. Compared to 2D monolayer-cultured cells, 3D spheroid MSCs demonstrate improved cell viability/survival, decreased apoptosis, and increased cellular quiescence. Cell-cell and cell-extracellular matrix (ECM) interactions are increased as well. In terms of multilineage/multipotential differentiation capacity, MSC chondrogenesis requires 3D serum-free conditions to increase condensation, whereas 3D MSC spheroid culture under typical serum-containing conditions increase pluripotency factor expression as well cell adhesion and ECM proteins, especially for osteogenesis. Immunomodulation and angiogenic/wound healing functions are improved as well in 3D MSC spheroids, largely through the increased expression of many paracrine factors. PGE_2, prostaglandin E_2; TSG6, tumor necrosis factor-stimulated gene 6; TNFα, tumor necrosis factor-α; VEGF, vascular endothelial growth factor; HGF, hepatocyte growth factor; bFGF, basic fibroblast growth factor.

useful outcomes, and may also recapitulate many aspects of in vivo MSC biology (Fig. 2). As exciting and interesting as the results are from 3D MSC spheroid culture, one of the biggest concerns may be the methodology itself: culture methods in 3D are by nature more complex than 2D monolayer culture, with more room to adjust existing parameters as well as add new ones.[111] Even when the 3D in vitro cell culture methodology is limited to multicellular spheroids, emerging data is showing that there are profound metabolic and proliferative/survival differences between cells within different locations of the spheroid[112,113] (and recently reviewed in[114]); even cell size is altered, which in addition to obviously affecting biophysical parameters, appears to also have a translational impact.[115] In addition, tissue-specific functional propensity of MSCs cultured in standard 2D conditions has emerged after decades of accumulated reports.[26] While the relatively lower numbers of 3D-cultured MSC spheroid reports make it difficult currently to discern tissue-specific differences in functional outcome, this important MSC-specific variability should be evaluated in future 3D studies as clinical efficacy may be implicated. Clearly, rigorous examination and execution of in vitro 3D culture conditions are critical for broad use of such innovative methods. Recent advances in profiling technologies—both at the gene expression as well as protein level—are already providing more precise and granular information into the broad and profound changes brought about by the 3D spheroid culture of MSCs.[116,117] It is anticipated in this rapidly advancing field that such tools and other novel technologies will continue to yield important data revealing how nuanced changes in 3D culture methodology can shape MSC biology for a better understanding of its original in vivo niche, as well as improve therapeutic outcome after ex vivo expansion.

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Conflict of Interest

B.L.Y. and C-C.H. are co-inventors on a MSC chondrogenesis patent currently in application. All of the other authors declared no potential conflicts of interest.

Author Contributions

B.L.Y., M.L.Y.: conception, manuscript writing, final approval, and funding; C.C.H.: data research, organization, and manuscript writing; P.J.H, C.C.C., L.T.W.: data organization.

Data Availability

No new data were generated or analyzed in support of this research.

Supplementary Material

Supplementary material is available at *Stem Cells Translational Medicine* online.

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