applied sciences

MDPI

Review

Cellular Spheroids of Mesenchymal Stem Cells and Their Perspectives in Future Healthcare

Hao-Wei Han [1], Shigetaka Asano [2] and Shan-hui Hsu [1,3,4,*]

[1] Institute of Polymer Science and Engineering, National Taiwan University, Taipei 10617, Taiwan; hwhan@ntu.edu.tw
[2] Consolidated Research Institute for Advanced Science and Medical Care, Waseda University, Tokyo 169-8555, Japan; asgtkmd@waseda.jp
[3] Center of Tissue Engineering and 3D Printing, National Taiwan University, Taipei 10617, Taiwan
[4] Institute of Cellular and System Medicine, National Health Research Institutes, Zhunan 35053, Taiwan
* Correspondence: shhsu@ntu.edu.tw; Tel.: +886-2-3366-5313; Fax: +886-2-3366-5237

Received: 29 November 2018; Accepted: 2 February 2019; Published: 13 February 2019

Abstract: Intrinsic cellular properties of several types of cells are dramatically altered as the culture condition shifts from two-dimensional (2D) to three-dimensional (3D) environment. Currently, several lines of evidence have demonstrated the therapeutic potential of mesenchymal stem cells (MSCs) in regenerative medicine. MSCs not only replenish the lost cells, they also promote the regeneration of impaired tissues by modulating the immune responses. Following the development of 3D cell culture, the enhanced therapeutic efficacy of spheroid-forming MSCs have been identified in several animal disease models by promoting differentiation or trophic factor secretion, as compared to planar-cultured MSCs. Due to the complicated and multifunctional applications in the medical field, MSCs are recently named as medicinal signaling cells. In this review, we summarize the predominant differences of cell–environment interactions for the MSC spheroids formed by chitosan-based substrates and other scaffold-free approaches. Furthermore, several important physical and chemical factors affecting cell behaviors in the cell spheroids are discussed. Currently, the understanding of MSCs spheroid interactions is continuously expanding. Overall, this article aims to review the broad advantages and perspectives of MSC spheroids in regenerative medicine and in future healthcare.

Keywords: mesenchymal stem cells; 3D cell culture; cellular spheroids; chitosan

1. Three-Dimensional (3D) Cell Culture Systems

Cell–cell and cell–environment interactions cooperatively determine the physiological phenomena in vivo. Many cell growth mechanisms and signal transduction pathways occurring in cells have been unveiled by the traditional two-dimensional (2D) culture system. Plastic-based culture materials remain the predominant cell culture platforms to date, yet some concerns have been raised with the accumulating experimental evidence.

A major drawback of cell culture on 2D plastic plates is the planar cell–cell interaction that has the insufficient and inappropriate cell–environment crosstalk. In vivo, cells are grown and stacked within a three-dimensional (3D) space, and extracellular matrix (ECM) secreted by cells constitutes the supporting framework to further organize tissues and organs. Therefore, some important cell–cell and cell–environment interactions may be ignored in a planar culture platform. Primary cells, cancer cells, and stem cells have been cultured in a 3D environment, and many interesting findings have been uncovered. In general, the definition of 3D cell culture systems is that cells are cultured on/in the 3D scaffolds composed by natural or synthetic materials, or they are organized into cellular spheroids. With the continuing development of 3D cell culture, it is now generally agreed that the cell physiology and cell behavior are dramatically distinct for cells cultured within a 2D or 3D environment [1–3].

2. 3D Culture of Cancer Cells or Stem Cells

Cancer cells are the most common cell types that have been investigated by 3D culture systems, and obvious change of gene expression as well as intracellular signaling in these 3D-cultured cancer cells have been shown in the literature, as compared with those cultured on tissue culture polystyrene (TCPS) plates. The overall change of transcriptome has been analyzed by bioinformatic approaches in several types of cancers. For glioblastoma multiforme (GBM) cells, the change of expression levels of ~10 thousand genes has been demonstrated after culture in 3D poly (lactic acid) (PLA) scaffolds [4]. The whole gene expression profiles of lung carcinoma and squamous cell carcinoma cultured in Matrigel were also analyzed. The expression levels of genes related to cell adhesion and immune response were changed when the culture environment of cancer cells was changed from 2D to 3D, suggesting the alternation of tumorigenicity of these 3D-cultured cancer cells [5]. Regarding the cell growth, the proliferation rate of ALDH+ (aldehyde dehydrogenase, a classical marker for cancer stem cells) breast cancer stem cells was amplified (~2-fold after 3 days of culture) by PLA porous scaffolds vs. those cultured on 2D plastic plates [6]. Spheroid-forming lung and liver cancer cells generated on biomaterial-based flat substrates or cellulosic scaffolds have increased cancer stemness, cell mobility, and chemoresistance [7,8].

Stem cell transplantation is the most promising strategy in regenerative medicine to achieve the functional recovery of impaired tissues or organs. With regard to stem cell culture, the stemness maintenance and differentiation capacities are of critical importance. Several positive effects have been demonstrated for 3D-cultured stem cells. For example, human embryonic stem cells (hESCs) have been successfully amplified in the form of suspended 3D spheroids, and these massively produced hESCs maintain their pluripotent properties to differentiate into the cells belonging to all three germ layers [9]. With the appropriate induction, floating-cultured ESCs self-organize into several different 3D organoids such as neural retina, liver buds, and inner ear [10], whereas it is inapproachable for the traditional 2D culture system.

Mesenchymal stem cells (MSCs) cultured in 3D space are intensively examined as well, though MSCs are conventionally defined as plastics-adherent. As compared to 2D culture, several benefits to MSCs have been identified when they are grown in a 3D environment. The benefits include the promotion of self-renewal and differentiation activities as well as up-regulation of paracrine secretion [11]. In addition to these positive effects, the enhancement of therapeutic potential of 3D-cultured MSCs was proven in cardiac and neural disorders [12]. Neural stem cells (NSCs) have been proposed to treat the neurodegenerative diseases, and the clinical trials have already started [13]. The proliferation and differentiation of NSCs were both observed in the scaffold-based or scaffold-free 3D culture system [14,15]. Furthermore, the differentiation potential of adherent and spheroid-forming (by non-adherent plates) neural progenitors was compared in a recent study. Researchers found that the number of neural progenitors with higher differentiation potential was greater in the spheroid-forming group, as compared to the 2D-cultured neural progenitors. Meanwhile, the differentiated neurons derived from 3D neural spheroids also displayed the longer neurites (~1.5-fold increase) after induction [16]. These evidences indicate the advantages of culturing neural progenitors in a 3D spheroid.

The accumulated evidence indicates that the properties of cancer cells and stem cells are significantly distinct after culturing in 2D vs. 3D spaces. Due to the biomimetic features, the 3D-cultured cells have been tested in tissue regeneration, drug screening, and other medical applications [17,18]. Furthermore, 3D culture platforms are convenient tools for clarifying the interactions of cell–cell and cell–environment in normal and cancer tissues in vivo [19,20]. Current 3D culture systems can be divided into the scaffold-free and scaffold-based platforms. The differences in cell–substrate interaction between these two types of approaches may result in the specific effects on the alteration of cellular physiology. For the scaffold-based platforms, cells are attached and grown in or on the porous scaffolds. On the contrary, for the scaffold-free platforms, cells are organized into the cell spheroids and floated in the culture medium due to the lack of tangible supporting materials.

Cells cultured on the chitosan (CS)-based substrates are unique because they are transiently attached on the surface but only loosely attached (floated) after forming the spheroids [21]. It is valuable to explore the relationship between the environmental factors and behavior changes for the cells grown in the common 3D culture systems.

3. Cell–Material Interface and Interaction in Scaffold-Free Stem Cell 3D Culture Systems

Scaffold-free culture platforms are simple, rapid, and low-cost methods to culture stem cells in a 3D environment, and thus are suitable for biomedical applications requiring high throughput property. In these systems, stem cells are cultured in a non- or low-adherent environment, and then are compelled to organize into the suspended cell spheroids. Hanging drop is the easiest approach to generate cell spheroids and does not require any additional or specific culture materials. Spheroids formed by hanging drop are aggregated by gravitational force and maintained in a liquid environment [22]. Due to the lack of direct contact with solid supporting interfaces, the cell–environment interaction only exists within the interior of cell spheroids. A few other similar approaches, such as pellet culture and low-attachment culture also demonstrate the spontaneous secretion of ECM proteins from the spheroid-forming cells [23–25], indicating that the composition of ECM proteins is the crucial environmental factor for the regulation of cell properties in these types of 3D culture systems.

Biomaterial-based planar substrates are major systems to generate cellular spheroids as well, such as agarose and CS-based substrates [26,27]. Agar/agarose system belongs to the non-adherent spheroid-forming approach. Therefore, the cell–substrate interaction is also limited in the agar/agarose culture system. In contrast, the carbohydrate structure of chitosan is similar to the glycosaminoglycans (GAGs) in the ECM [27]. Meanwhile, the cell–chitosan contact has been demonstrated by experiments [21]. Therefore, the cell–environment interactions in the CS-coated system are more complicated than those of hanging drop and low-attachment culture approaches.

Several kinds of stem cells are assembled into cellular spheroids or co-spheroids and then used to demonstrate various medical applications. The physiological features of cell spheroids may be different due to the approaches of spheroid formation. Some distinct effects are found in MSC spheroids formed by hanging drop, low-adherent culture, or CS-based substrates, as compared to the planar-cultured MSCs. Hanging-drop-derived MSC spheroids displayed over 100-fold increase in the expression of anti-inflammatory [TNFα stimulated gene/protein 6 (TSG-6)] and anti-cancer [IL-24 and TNFα-related apoptosis inducing ligand (TRAIL)] genes, compared with the adherent MSCs on TCPS [28]. The stemness of MSCs was well maintained in the spheroids generated by the hanging drop approach, and the expression levels of stemness markers including Oct4, Sox2, and Nanog were higher than those of 2D-cultured MSCs [29]. On the other hand, MSC spheroids formed by low-adherent plates secreted more angiogenic factors, such as vascular endothelial growth factor (VEGF) as well as stromal cell derived factor (SDF), and the cell proliferation of MSC spheroids displayed ~2-fold increase as compared to 2D-cultured MSCs in vivo after transplantation to the mice ischemic hindlimb [30]. Moreover, MSCs derived from human adipose and placenta tissues displayed increased stemness and chondrogenic differentiation potential after culture on CS and CS-based substratum [21]. Meanwhile, by the microarray technique, the changes of expression levels of ~300 genes were discovered for the MSC spheroids on CS substrates compared with the MSC grown on TCPS, indicating the substantial alternation for the MSC behavior after the spheroid formation [31].

Based on the findings mentioned above, the cell behavior of MSCs is dramatically changed as the culture environment shifts from 2D to 3D. Furthermore, the cell properties of MSCs may be different in the spheroids generated by the distinct forming approaches. Further direct evidence is that the human MSCs expressed more focal adhesion proteins and displayed stronger intercellular crosstalk when they were cultured on graphene-incorporated CS membrane rather than pristine CS substrates [32]. Therefore, the accumulating evidences provide important information for selecting the appropriate culture materials to induce the specific differentiation of MSCs or to trigger the unique regulation of MSC behavior, depending on the experimental requirement.

4. Spheroid Culture of MSCs

MSCs have been considered as the multifunctional agents for treating several types of degenerative diseases and immunological disorders [33]. Recently, the therapeutic efficacy of MSC spheroids has been tested in several animal disease models including wound healing as well as bone, cardiovascular, kidney, and liver disorders. Spheroid-forming MSCs secrete more bioactive factors such as fibroblast growth factor 1 (FGF1), vascular endothelial growth factor (VEGF), and prostaglandin E2 (PGE_2) to promote angiogenesis and reduce immune response, leading to the enhancement of wound regeneration [34,35]. In a murine skin wound model, the injured site treated with MSC spheroids displayed higher regenerative and angiogenic rates [34]. In addition, the therapeutic efficiency of MSC spheroids was better than dispersed cells for the 5-fluorouracil-induced oral mucositis as well as weight loss [36], indicating the potential of MSC spheroids in alleviating the side effects for cancer patients after chemotherapy.

MSC spheroids have been employed to in vivo cartilage and bone regeneration. Transplantation of MSC aggregates generated by hanging drop induced cartilage regeneration in the rabbit femur osteochondral defect model through the secretion of cartilage matrix [37]. On the other hand, MSC spheroids also promoted the osteogenesis and bone regeneration in the rat calvarial and femur defect models [38,39]. Regarding the cardiovascular diseases, the therapeutic functions of MSC spheroids may be divided into cardiomyocyte regeneration and neovascularization. Adipose-derived MSC spheroids displayed higher cardiomyogenic potential as compared to 2D-cultured MSCs, and the functional recovery of impaired heart was demonstrated after injection of MSC spheroids in a chronic myocardial infarction rat model [40]. Moreover, the myocardial infarction of porcine can be obviously repaired by implantation of adipose-derived MSC spheroids [41]. Regarding the liver regeneration, 90% hepatectomized rats transplanted with the alginate hydrogel-encapsulated bone marrow cells displayed ~5-fold survival rate and ~1.3-fold liver weight, as compared to those transplanted with free bone marrow cells [42]. Another similar strategy to improve the survival rate of 90% hepatectomized rats was transplantation of hydrogel-embedded bone marrow MSCs into the spleen. In the latter case, the encapsulated MSCs rather than free MSCs continuously provided hepatotrophic factors with stable concentration, and resulted in ~70% increase to the free MSC group for the survival rate of hepatectomized rats [43]. A more recent report demonstrated that the alginate-embedded MSCs differentiated into the liver cells and secreted IL-6 and other growth factors after transplantation into the spleen. Meanwhile, the secretion of the cytokines and growth factors for the encapsulated MSCs was ~2-fold higher than free MSCs two days after surgery [44]. The above findings indicate that the therapeutic efficacy of MSCs can be obviously promoted when the MSCs are grown in an appropriate 3D environment. Furthermore, by the promotion of angiogenesis, paracrine secretion, cell differentiation, anti-inflammation, anti-apoptosis, or anti-oxidation, the greater therapeutic potential of MSC spheroids has also been demonstrated in kidney [23] and liver [45,46] injuries, as compared with monolayer MSCs. These groups of evidence reveal the promising roles of spheroid-forming MSCs in future healthcare, especially in the functional recovery of damaged organs.

Currently, the novel and advanced strategies used to generate MSC spheroids and to induce their differentiation toward specific cell types are being continuously developed, such as magnetic levitation and micro-fluidic system [47,48]. Meanwhile, several novel events have been discovered in the MSC spheroids and co-spheroids. Rat adipose-derived MSCs were cultured on agarose plate and self-assembled into microspheres. After induction, the expression of endothelial and osteoblast markers were both detected in the MSC spheroids, suggesting that it is a potential and simple method to fabricate a vascularized bone tissue [49]. Activity of angiogenic promotion for MSCs was proven in another MSC and endothelial cell (EC) co-culture experiment. ECs organized into a network structure in the MSC/EC heterotypic co-spheroids formed by the non-adherent U-bottomed plates. Replacing the MSCs by fibroblasts decreased the length and width of EC networks as well as branch formation. By the use of different inhibitors, platelet-derived growth factor (PDGF)-involved signaling pathway has been found as the predominant regulator for the EC alignment in this model [50].

Positive effects for the cells co-cultured with MSCs in 3D co-spheroids have been mentioned in other literature. In the conventional culture, the islet cells are difficult to maintain. Although insulin secretion was not stimulated for islet cells, a current finding has demonstrated that the survival rates of islet cells were largely enhanced by forming the co-spheroids with MSCs on a special glass micromold [51]. Moreover, the proliferation of hematopoietic stem/progenitor cell (HSPC) was promoted by MSCs in a monolayer co-culture condition. However, this support is still too weak to produce sufficient HSPCs for clinical usage. Currently, co-culture of HSPCs and MSCs has been evaluated in a 3D spheroid by using non-adherent Pluronic-coated polydimethylsiloxane (PDMS) microwell platforms. The results demonstrated that the number of CD34-positive cells was increased on PDMS microwells; nevertheless, the co-culture strategy and system still needed further improvement before being subjected to clinical applications [52].

For cell spheroid preparation, the major advantage of scaffold-free systems is the convenient collection of the formed spheroids. However, the drawbacks of these systems mentioned above, such as hanging-drop, low-adherent plate, micro-fluidic system, and PDMS microwells, are deficient in environmental interaction during and after the spheroid formation. CS-based substrates also belong to the scaffold-free platforms; however, the transient interaction of MSCs and chitosan surface has been observed. An illustration demonstrating the processes of MSC spheroid/co-spheroid formation on CS substrates and other common scaffold-free methods is shown in Figure 1. In a previous study, human adipose-derived MSCs self-organized into cell spheroids on the CS-coated surfaces. MSCs displayed better chondrogenic differentiation when cultured on CS substrates as compared to those on TCPS. During the spheroid formation, MSCs adhered on the CS substrates at initial culture period, and then gradually merged and self-assembled into the cell spheroids, revealing the existence of crosstalk between the cells and CS substrates [21]. Rho/ROCK signaling pathway regulates the cytoskeleton rearrangement and cell adhesion/migration [53]. After the treatment of a ROCK inhibitor, Y-27632, MSCs still adhered and migrated on the CS substrates; however, MSC spheroids were not further organized. Meanwhile, CD44, a stemness marker of MSCs, was required for the movement and spheroid formation of MSCs on CS substrates. As blocking the formation of MSC spheroids by ROCK inhibitor or CD44 antibody, the overall stemness of MSCs was reduced [21], suggesting the positive correlation between the spheroid formation and stemness maintenance for MSCs cultured on CS-based substrates. Furthermore, based on the observation on the relationship between MSC spheroid formation and their stemness, a further study has demonstrated that CS substrates can be employed as a rapid platform for selecting MSCs from adipose tissues. Adipose-derived MSCs isolated by CS substrates displayed higher proliferation rates and stemness, as compared to those collected by conventional plastic adherence approach. The expression of several stemness markers, such as Oct4, Sox2, and Nanog, as well as MSC marker, CD271 [54], were well maintained in the MSC spheroids generated by CS substrates [55].

After chemical grafting with other biomaterial compounds such as hyaluronan on CS membrane, several positive effects exerted on MSCs were further amplified, as compared to original CS substrates [21]. Furthermore, the composite CS materials, for example, CS-gelatin films and CS-collagen microbeads have been also tested as the culture materials and cell carriers for MSCs [56,57]. Due to the ECM-mimicking and high biocompatibility, these biomaterial-based systems are promising approaches to maintain, expand, and even differentiate MSCs into the desired cell types.

Similar to the effects occurred in other scaffold-free co-culture systems, MSCs also played a supporting role in the heterotypic co-spheroids formed by CS substrates. MSCs isolated from rat adipose tissues improved the survival rates and promoted the differentiation of co-cultured NSCs in the co-spheroids on CS substrates. Meanwhile, the expression of neural-related markers was up-regulated in MSCs, indicating the increasing neural differentiation tendency for MSCs [15]. These evidences further strengthen the assistant role of MSC spheroids or co-spheroids in regenerative medicine.

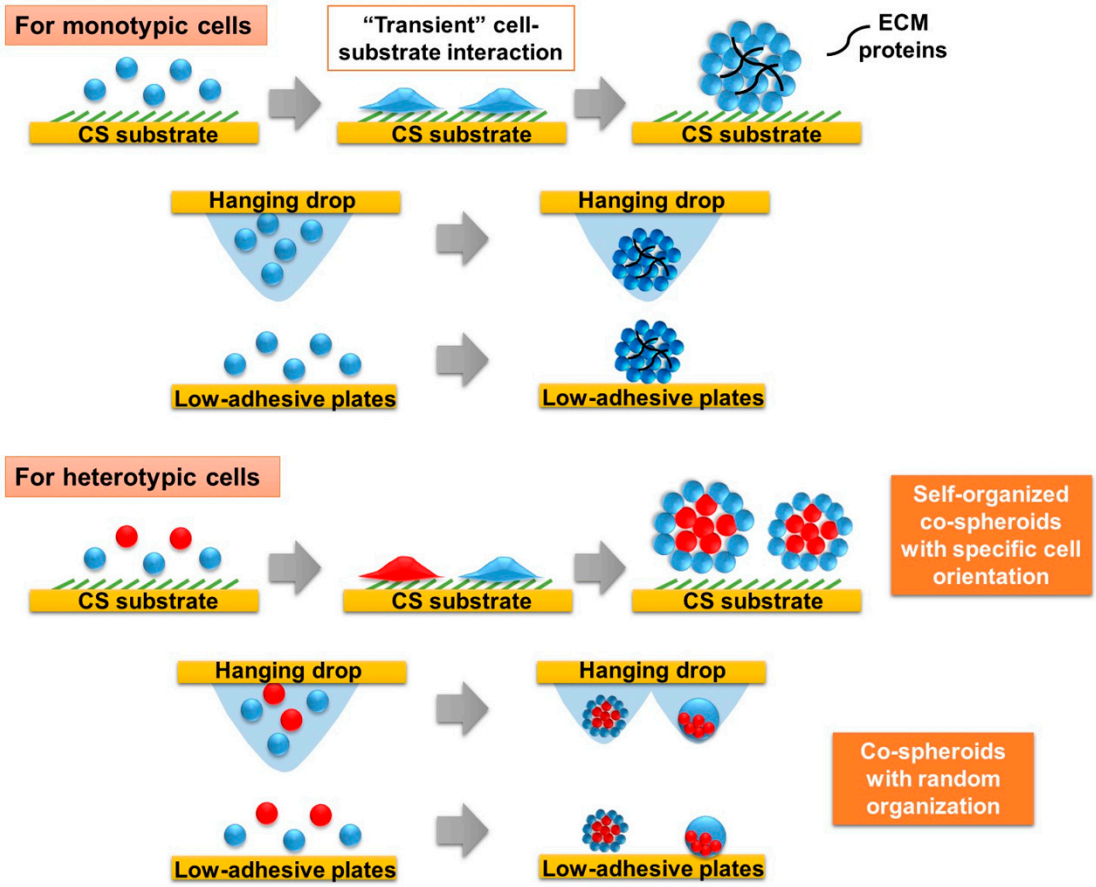

Figure 1. Formation of mesenchymal stem cells (MSC) spheroids/co-spheroids by chitosan (CS) substrates and other common scaffold-free methods. During the monotypic or heterotypic spheroid formation on CS substrates, cells transiently adhere and interact with CS substrates, and then merge and further organize into cell spheroids. On the contrary, cells directly assemble into cell spheroids without cell–substrate interaction in other scaffold-free methods (hanging drop and low-adhesive methods). Furthermore, for the heterotypic spheroid formation, cell–cell and cell–substrate interactions result in the self-assembly of heterotypic cells and specific cell arrangement in the co-spheroids on CS substrates. However, cells are forced to form co-spheroids with random organization by other scaffold-free methods. ECM: extracellular matrix.

5. Physical and Chemical Factors Affecting Cell Behaviors in Scaffold-Free 3D Culture Systems

In 2D culture systems, the predominant physical factors affecting cell behaviors are the stiffness and topology of culture materials [58]. For scaffold-based 3D culture platform, such as hydrogel-encapsulated system, the differentiation potential of encapsulated MSCs is also directly affected by the stiffness of hydrogel. The hydrogels with higher and lower stiffness induced the differentiation of embedded MSCs toward myocytes/osteocytes and adipocytes/chondrocytes, respectively. Meanwhile, the differentiation tendencies of these MSCs were regulated by transforming growth factor β (TGF-β)- and integrin-involved signaling, revealing the relationship between the cell–environment interaction and cell fate determination [59,60].

For common scaffold-free 3D culture systems, the direct contact of cells and culture materials is very limited. In the hanging drop, cells are maintained and suspended in the culture medium, and no cell–substrate interaction exists. For the non- or low-adherent spheroid-forming system, cells "touch" rather than "interact" to the surfaces of culture materials. In a CS-based system, the cell adhesion is observed transiently. Therefore, the overall influences of surface stiffness and topology of culture materials in scaffold-free 3D culture systems are much slighter than those in 2D and scaffold-based 3D culture platforms. Based on the different approaches for spheroid formation, other extrinsic physical

stimulation also affects the MSC behaviors. For instance, several evidences have demonstrated the functions of fluid flow in MSC differentiation. It has been reported that the osteodifferentiation of dexamethasone-treated MSCs was enhanced by the shear stress produced by a flow perfusion system during the period of culture [61]. A recent report also demonstrated that several indicators of osteodifferentiation of MSCs, including alkaline phosphatase (ALP) activity, type I collagen expression, and intracellular calcium ion concentration, were up-regulated in the MSCs after the stimulation of mechanical stress (4000 microstrain). Meanwhile, the mechanical stress-induced osteodifferentiation of MSCs was related to the activation of p38 mitogen-activated protein kinase (MAP kinase) [62]. Furthermore, the static or dynamic perfusion fluid also regulates the transportation of nutrients, waste, and gas in the culture systems. Thus, these factors have to be carefully concerned, especially in a micro-fluidic 3D system. Moreover, the hydrostatic pressure is also mentioned in the regulation of the cytoskeletal composition and MSC differentiation [63]. In scaffold-free 3D culture platforms, besides the gravity, the water pressure is the only resource of external pressure for the cultured cells. Therefore, it may be another choice to control the differentiation of MSCs by adjusting the hydrostatic pressure given to the spheroid-forming cells.

Physical factors affecting the MSC behaviors within the spheroids are generally related to the diameter and cell density of spheroids. With the growth or aggregation of cells, the diameter or cell density of spheroids is gradually increased and internal acidity may be changed due to the metabolic status. The internal material transfer may be blocked or decelerated by the size expansion of spheroids. However, it has been demonstrated that the cell viability of MSC spheroids or co-spheroids was over 80% when the sizes of these spheroids were larger than 300 μm [64,65], suggesting that the tight interaction between the cells would establish a strong communication network to support the cell vitality in the spheroids. Oxygen gradient is also a common issue in the study of cell spheroids. It is generally considered that the cells within the core of spheroids have to face the hypoxic state [66]. Recently, an approach to estimate the oxygen tension in the MSC spheroids has been developed. By a simulation approach, the researchers concluded that the change of oxygen concentration from the center to the surface was lower than 10% when the spheroid diameter was ~700 μm. Meanwhile, the cell density and metabolism were both decreased by the increment of spheroid sizes, indicating that the cells would self-adjust the physiological status to avoid cell death through promoting mass transfer and reducing energy consumption [67]. It may be a mechanism for maintaining the cells with high viability in the spheroids.

The change of signal transduction is the major responses to the environmental chemical factors. Certainly, a part of these chemical effects was also derived from the mechanical stimulation. Ion strength may be one of chemical factor regulating MSC behaviors. To date, ion concentration in the spheroids has not been explored. It has been reported that the mechanical stress affected the activation of calcium ion channels on the cell membrane of MSCs, and the calcium ion-involved signaling played important roles in the differentiation of MSCs [68,69]. On the other hand, the cytoskeleton would be dramatically rearranged during the spheroid formation on CS substrates. Blocking the cytoskeleton rearrangement by the treatment of a Rho-associated coiled-coil containing protein kinase (ROCK) inhibitor interrupted the self-assembly of MSC spheroids [21]. Meanwhile, ROCK signaling was also found to regulate the calcium ion-involved actin remodeling [70]. Therefore, the activation of calcium channels and the change of calcium influx may be the key regulators for the spheroid formation and the subsequent MSC differentiation on CS substrates.

Integrin-involved signaling may be a crucial factor for regulating the physiology of cell spheroids. Integrins are the important anchor proteins for cells bound to ECM. We have previously mentioned that ECM proteins are secreted by spheroid-forming cells. Thus, the combination of alpha and beta units of integrin on the membrane of MSCs as well as which ECM proteins they interact with may determine the stemness or differentiation potential of MSCs in the spheroids. For example, human placenta MSC spheroids displayed lower expression of integrin β1 compared to normal 2D culture, leading to a decrease of cell–ECM interaction and enhancement of MSC stemness. By other

approaches to release cytoskeleton tension, the stemness of MSCs was promoted as well, revealing the negative relationship between stemness and cytoskeleton tension in MSCs [71]. In addition to cell–matrix interaction, the cadherin-mediated cell–cell interaction cannot be ignored. Cadherin is a kind of calcium-dependent adhesive molecule between neighboring cells. When calcium ions bind on the extracellular region of cadherin, the cadherin forms a homodimer and then binds to another cadherin homodimer on the neighboring cells. Intercellular cadherin interaction regulates the cell–cell adhesion, cell migration, and multiple types of cell signaling by association with the actin filaments [72]. The up-regulated expression levels of N-cadherin and/or E-cadherin were observed in MSC spheroids formed by different scaffold-free 3D culture systems including hanging drop method and CS substrates, comparing to those cultured on TCPS [71,73], indicating the involvement of cadherin-mediated signaling in the formation of MSC spheroids. Meanwhile, the calcium ions were chelated by the CS substrates [73]. Combined with several findings mentioned above, we propose a mechanism of MSC spheroid formation on CS substrates. At initial seeding, MSCs adhere on the surface of CS substrates because of the activation of cadherin proteins by calcium ions chelated on the CS substrates. Cell tension generated by the interaction between MSCs and CS membranes leads to the activation of calcium channels on the membrane of MSCs, resulting in the change of calcium concentration within MSCs. The intracellular calcium-mediated signaling, especially in the regulation of cytoskeleton organization, was affected. Finally, MSCs detached from CS substrates and rapidly migrated as well as self-assembled into spheroids.

Several behavior changes of MSCs are observed when they are organized into the spheroids, such as enhancement of cell proliferation and differentiation. However, the underlying molecular signaling is still rarely investigated in the cells. With the accumulating evidence, we will determine what happens in the cells during the spheroid formation, and this information will be useful for generating the desired or customized MSC spheroids in the future.

6. Perspectives of MSCs Spheroids in Healthcare

Because of their unique functions and applications, MSCs have been recently named by Professor Arnold Caplan as medicinal signaling cells [74]. MSCs are not only capable of differentiating into osteocytes, chondrocytes, as well as adipocytes, they secrete several trophic proteins to modulate immune response and to promote tissue regeneration, indicating the versatile roles of MSCs in regenerative medicine [75]. Based on the accumulating experimental evidence, the approaches to induce the specific differentiation of MSCs might be gradually established in the near future. For clinical applications, the origins and amplification protocols have to be concerned in MSC therapies. The heterogeneity of MSCs from different individuals and tissues has been demonstrated [76]. Therefore, for treating the specific disorders, the most suitable origin of MSCs has to be determined. Continuous improvement of biotechnology in the study of secretome, epigenetics, genomics, and proteomics is helpful for clarifying the differentiation and therapeutic capacities for MSCs derived from different origins [77]. To obtain a sufficient number of MSCs for medical applications, an appropriate expansion procedure is required. During the MSC amplification, the maintenance of stemness and differentiation capacities is a critical issue. As mentioned in the previous sections, the differentiation capacities of spheroid-forming MSCs are enhanced, and their differentiation tendencies are further controllable by the planar culture substratum combined with specific induction. Furthermore, MSC spheroids generated by scaffold-free culture platforms are more convenient than those grown within the 3D scaffolds for actual applications, such as intravenous injection. Therefore, the 3D MSC spheroids assembled on a planar scaffold-free culture substrate are promising therapeutic tools or agents to restore the functions of damaged organs in the era of regenerative medicine.

Recently, the relatively common and reliable origins of MSCs are bone marrow and adipose tissues. However, surgical requirement remains for isolating MSCs from these tissues. Circulating MSCs may be a great another resource. The similar properties of circulating MSCs have been identified as compared to the bone marrow-derived MSCs. The expression levels of surface markers such as

CD44, CD90, and CD73 were almost identical in the circulating MSCs and bone marrow-derived MSCs [78]. The critical drawback of circulating MSCs is low quantity. Therefore, a reliable protocol or strategy used for isolating MSCs from blood and further amplifying in vitro is essential for in vivo applications of circulating MSCs. According to the current evidence, CS-based substrates may be a potential platform to isolate and expand the MSCs from blood circulation.

In addition to regenerative medicine, MSCs also display potential in acting as diagnostic tools. For example, the patients with hypertrophic cardiomyopathy had increased circulating MSCs in the blood stream, comparing to the normal individuals [79]. As mentioned above, the cellular properties of MSCs derived from the respective individuals are different. After the development of a reliable protocol used for MSC isolation and amplification, circulating MSCs and other MSCs would be characterized from the individuals with different healthy status, age, nationality, or lifestyle, and this database of MSCs may be further organized into a smart healthcare platform in the future (Figure 2).

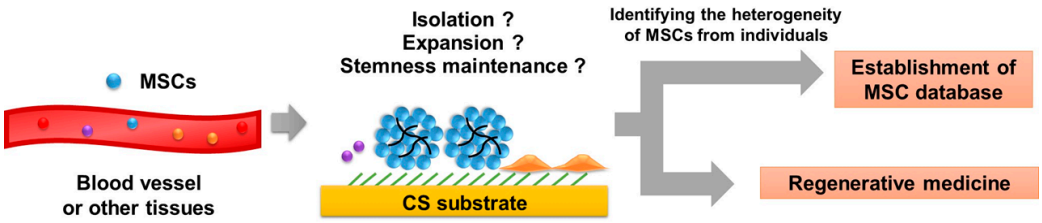

Figure 2. Perspectives of MSCs in medical application. Due to the specific interaction of MSCs and CS substrates, MSCs may be rapidly isolated and expanded by CS substrates and maintain their stemness. After amplification and characterization, sufficient MSCs can be applied to regenerative medicine or used as a diagnostic indicator for future healthcare.

Author Contributions: H.-W.H., S.A., and S.-h.H. wrote the paper.

Acknowledgments: This work was supported by the Program for Regenerative Medicine (MOST 107-2321-B-002-040), funding of National Taiwan University for the postdoctoral fellowship (NTU-107L4000), and funding of National Health Research Institutes (Central Government S & T Grant 107-0324-01-19-03).

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Ravi, M.; Paramesh, V.; Kaviya, S.R.; Anuradha, E.; Solomon, F.D. 3D cell culture systems: Advantages and applications. *J. Cell. Physiol.* **2015**, *230*, 16–26. [CrossRef] [PubMed]
2. Fang, Y.; Eglen, R.M. Three-dimensional cell cultures in drug discovery and development. *SLAS Discov.* **2017**, *22*, 456–472. [PubMed]
3. McKee, C.; Chaudhry, G.R. Advances and challenges in stem cell culture. *Colloids Surf. B Biointerfaces* **2017**, *159*, 62–77. [CrossRef] [PubMed]
4. Ma, L.; Zhang, B.; Zhou, C.; Li, Y.; Li, B.; Yu, M.; Luo, Y.; Gao, L.; Zhang, D.; Xue, Q.; et al. The comparison genomics analysis with glioblastoma multiforme (GBM) cells under 3D and 2D cell culture conditions. *Colloids Surf. B Biointerfaces* **2018**, *172*, 665–673. [CrossRef] [PubMed]
5. Zschenker, O.; Streichert, T.; Hehlgans, S.; Cordes, N. Genome-wide gene expression analysis in cancer cells reveals 3D growth to affect ECM and processes associated with cell adhesion but not DNA repair. *PLoS ONE* **2012**, *7*, e34279. [CrossRef] [PubMed]
6. Polonio-Alcala, E.; Rabionet, M.; Guerra, A.J.; Yeste, M.; Ciurana, J.; Puig, T. Screening of additive manufactured scaffolds designs for triple negative breast cancer 3D cell culture and stem-like expansion. *Int. J. Mol. Sci.* **2018**, *19*, 3148. [CrossRef]
7. Huang, Y.J.; Hsu, S.H. Acquisition of epithelial-mesenchymal transition and cancer stem-like phenotypes within chitosan-hyaluronan membrane-derived 3D tumor spheroids. *Biomaterials* **2014**, *35*, 10070–10079. [CrossRef] [PubMed]

8. Wu, G.; Zhan, S.; Rui, C.; Sho, E.; Shi, X.; Ding, Y. Microporous cellulosic scaffold as a spheroid culture system modulates chemotherapeutic responses and stemness in hepatocellular carcinoma. *J. Cell. Biochem.* **2018**. [CrossRef] [PubMed]
9. Li, X.; Ma, R.; Gu, Q.; Liang, L.; Wang, L.; Zhang, Y.; Wang, X.; Liu, X.; Li, Z.; Fang, J.; et al. A fully defined static suspension culture system for large-scale human embryonic stem cell production. *Cell Death Dis.* **2018**, *9*, 892. [CrossRef] [PubMed]
10. Koehler, K.R.; Hashino, E. 3D mouse embryonic stem cell culture for generating inner ear organoids. *Nat. Protoc.* **2014**, *9*, 1229–1244. [CrossRef] [PubMed]
11. Cesarz, Z.; Tamama, K. Spheroid culture of mesenchymal stem cells. *Stem Cells Int* **2016**, *2016*, 9176357. [CrossRef] [PubMed]
12. Petrenko, Y.; Sykova, E.; Kubinova, S. The therapeutic potential of three-dimensional multipotent mesenchymal stromal cell spheroids. *Stem Cell Res. Ther.* **2017**, *8*, 94. [CrossRef] [PubMed]
13. Wang, Z.; Luo, Y.; Chen, L.; Liang, W. Safety of neural stem cell transplantation in patients with severe traumatic brain injury. *Exp. Ther. Med.* **2017**, *13*, 3613–3618. [CrossRef] [PubMed]
14. Cunha, C.; Panseri, S.; Villa, O.; Silva, D.; Gelain, F. 3D culture of adult mouse neural stem cells within functionalized self-assembling peptide scaffolds. *Int. J. Nanomed.* **2011**, *6*, 943–955. [CrossRef] [PubMed]
15. Han, H.W.; Hsu, S.H. Chitosan derived co-spheroids of neural stem cells and mesenchymal stem cells for neural regeneration. *Colloids Surf. B Biointerfaces* **2017**, *158*, 527–538. [CrossRef] [PubMed]
16. Chandrasekaran, A.; Avci, H.X.; Ochalek, A.; Rosingh, L.N.; Molnar, K.; Laszlo, L.; Bellak, T.; Teglasi, A.; Pesti, K.; Mike, A.; et al. Comparison of 2D and 3D neural induction methods for the generation of neural progenitor cells from human induced pluripotent stem cells. *Stem Cell Res.* **2017**, *25*, 139–151. [CrossRef] [PubMed]
17. Langhans, S.A. Three-dimensional in vitro cell culture models in drug discovery and drug repositioning. *Front. Pharmacol.* **2018**, *9*, 6. [CrossRef]
18. Antoni, D.; Burckel, H.; Josset, E.; Noel, G. Three-dimensional cell culture: A breakthrough in vivo. *Int. J. Mol. Sci.* **2015**, *16*, 5517–5527. [CrossRef]
19. Shoval, H.; Karsch-Bluman, A.; Brill-Karniely, Y.; Stern, T.; Zamir, G.; Hubert, A.; Benny, O. Tumor cells and their crosstalk with endothelial cells in 3D spheroids. *Sci. Rep.* **2017**, *7*, 10428. [CrossRef]
20. Han, H.W.; Hsu, S.H. Chitosan-hyaluronan based 3D co-culture platform for studying the crosstalk of lung cancer cells and mesenchymal stem cells. *Acta Biomater.* **2016**, *42*, 157–167. [CrossRef]
21. Huang, G.S.; Dai, L.G.; Yen, B.L.; Hsu, S.H. Spheroid formation of mesenchymal stem cells on chitosan and chitosan-hyaluronan membranes. *Biomaterials* **2011**, *32*, 6929–6945. [CrossRef] [PubMed]
22. Foty, R. A simple hanging drop cell culture protocol for generation of 3D spheroids. *J. Vis. Exp.* **2011**. [CrossRef] [PubMed]
23. Xu, Y.; Shi, T.; Xu, A.; Zhang, L. 3D spheroid culture enhances survival and therapeutic capacities of MSCs injected into ischemic kidney. *J. Cell. Mol. Med.* **2016**, *20*, 1203–1213. [CrossRef] [PubMed]
24. Santos, J.M.; Camoes, S.P.; Filipe, E.; Cipriano, M.; Barcia, R.N.; Filipe, M.; Teixeira, M.; Simoes, S.; Gaspar, M.; Mosqueira, D.; et al. Three-dimensional spheroid cell culture of umbilical cord tissue-derived mesenchymal stromal cells leads to enhanced paracrine induction of wound healing. *Stem Cell Res. Ther.* **2015**, *6*, 90. [CrossRef] [PubMed]
25. Redondo-Castro, E.; Cunningham, C.J.; Miller, J.; Cain, S.A.; Allan, S.M.; Pinteaux, E. Generation of Human Mesenchymal Stem Cell 3D Spheroids Using Low-binding Plates. *Bio Protoc.* **2018**, *8*, e2968. [CrossRef] [PubMed]
26. Costa, E.C.; de Melo-Diogo, D.; Moreira, A.F.; Carvalho, M.P.; Correia, I.J. Spheroids formation on non-adhesive surfaces by liquid overlay technique: Considerations and practical approaches. *Biotechnol. J.* **2018**, *13*, 1700417. [CrossRef] [PubMed]
27. Cui, X.; Hartanto, Y.; Zhang, H. Advances in multicellular spheroids formation. *J. R. Soc. Interface* **2017**, *14*, 20160877. [CrossRef]
28. Bartosh, T.J.; Ylostalo, J.H.; Mohammadipoor, A.; Bazhanov, N.; Coble, K.; Claypool, K.; Lee, R.H.; Choi, H.; Prockop, D.J. Aggregation of human mesenchymal stromal cells (MSCs) into 3D spheroids enhances their antiinflammatory properties. *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 13724–13729. [CrossRef]

29. Lou, Y.L.; Guo, D.W.; Zhang, H.; Song, L.J. Effectiveness of mesenchymal stems cells cultured by hanging drop vs. conventional culturing on the repair of hypoxic-ischemic-damaged mouse brains, measured by stemness gene expression. *Open Life Sci.* **2016**, *11*, 519–523. [CrossRef]
30. Lee, J.H.; Han, Y.S.; Lee, S.H. Long-duration three-dimensional spheroid culture promotes angiogenic activities of adipose-derived mesenchymal stem cells. *Biomol. Ther.* **2016**, *24*, 260–267. [CrossRef]
31. Yeh, H.Y.; Liu, B.H.; Sieber, M.; Hsu, S.H. Substrate-dependent gene regulation of self-assembled human MSC spheroids on chitosan membranes. *BMC Genom.* **2014**, *15*, 10. [CrossRef] [PubMed]
32. Kim, J.; Kim, Y.-R.; Kim, Y.; Lim, K.T.; Seonwoo, H.; Park, S.; Cho, S.-P.; Hong, B.H.; Choung, P.-H.; Chung, T.D.; et al. Graphene-incorporated chitosan substrata for adhesion and differentiation of human mesenchymal stem cells. *J. Mater. Chem. B* **2013**, *1*, 933–938. [CrossRef]
33. Law, S.; Chaudhuri, S. Mesenchymal stem cell and regenerative medicine: Regeneration versus immunomodulatory challenges. *Am. J. Stem Cells* **2013**, *2*, 22–38. [PubMed]
34. Hsu, S.H.; Hsieh, P.S. Self-assembled adult adipose-derived stem cell spheroids combined with biomaterials promote wound healing in a rat skin repair model. *Wound Repair Regen.* **2015**, *23*, 57–64. [CrossRef] [PubMed]
35. Murphy, K.C.; Whitehead, J.; Zhou, D.; Ho, S.S.; Leach, J.K. Engineering fibrin hydrogels to promote the wound healing potential of mesenchymal stem cell spheroids. *Acta Biomater.* **2017**, *64*, 176–186. [CrossRef] [PubMed]
36. Zhang, Q.; Nguyen, A.L.; Shi, S.; Hill, C.; Wilder-Smith, P.; Krasieva, T.B.; Le, A.D. Three-dimensional spheroid culture of human gingiva-derived mesenchymal stem cells enhances mitigation of chemotherapy-induced oral mucositis. *Stem Cells Dev.* **2012**, *21*, 937–947. [CrossRef] [PubMed]
37. Suzuki, S.; Muneta, T.; Tsuji, K.; Ichinose, S.; Makino, H.; Umezawa, A.; Sekiya, I. Properties and usefulness of aggregates of synovial mesenchymal stem cells as a source for cartilage regeneration. *Arthritis Res. Ther.* **2012**, *14*, R136. [CrossRef]
38. Suenaga, H.; Furukawa, K.S.; Suzuki, Y.; Takato, T.; Ushida, T. Bone regeneration in calvarial defects in a rat model by implantation of human bone marrow-derived mesenchymal stromal cell spheroids. *J. Mater. Sci. Mater. Med.* **2015**, *26*, 254. [CrossRef]
39. Yanagihara, K.; Uchida, S.; Ohba, S.; Kataoka, K.; Itaka, K. Treatment of Bone Defects by Transplantation of Genetically Modified Mesenchymal Stem Cell Spheroids. *Mol. Ther. Methods Clin. Dev.* **2018**, *9*, 358–366. [CrossRef]
40. Liu, B.H.; Yeh, H.Y.; Lin, Y.C.; Wang, M.H.; Chen, D.C.; Lee, B.H.; Hsu, S.H. Spheroid formation and enhanced cardiomyogenic potential of adipose-derived stem cells grown on chitosan. *BioRes. Open Access* **2013**, *2*, 28–39. [CrossRef]
41. Emmert, M.Y.; Wolint, P.; Wickboldt, N.; Gemayel, G.; Weber, B.; Brokopp, C.E.; Boni, A.; Falk, V.; Bosman, A.; Jaconi, M.E.; et al. Human stem cell-based three-dimensional microtissues for advanced cardiac cell therapies. *Biomaterials* **2013**, *34*, 6339–6354. [CrossRef] [PubMed]
42. Liu, Z.C.; Chang, T.M.S. Transdifferentiation of bioencapsulated bone marrow cells into hepatocyte-like cells in the 90% hepatectomized rat model. *Liver Transplant.* **2006**, *12*, 566–572. [CrossRef] [PubMed]
43. Liu, Z.C.; Chang, T.M.S. Preliminary study on intrasplenic implantation of artificial cell bioencapsulated stem cells to increase the survival of 90% hepatectomized rats. *Artif. Cell Blood Substit. Biotechnol.* **2009**, *37*, 53–55. [CrossRef] [PubMed]
44. Liu, Z.C.; Chang, T.M.S. Intrasplenic transplantation of bioencapsulated mesenchymal stem cells improves the recovery rates of 90% partial hepatectomized rats. *Stem Cells Int.* **2012**, *2012*, 697094. [CrossRef] [PubMed]
45. Talaei-Khozani, T.; Borhani-Haghighi, M.; Ayatollahi, M.; Vojdani, Z. An in vitro model for hepatocyte-like cell differentiation from Wharton's jelly derived-mesenchymal stem cells by cell-base aggregates. *Gastroenterol. Hepatol. Bed Bench* **2015**, *8*, 188–199. [PubMed]
46. Sun, Y.; Wang, Y.; Zhou, L.; Zou, Y.; Huang, G.; Gao, G.; Ting, S.; Lei, X.; Ding, X. Spheroid-cultured human umbilical cord-derived mesenchymal stem cells attenuate hepatic ischemia-reperfusion injury in rats. *Sci. Rep.* **2018**, *8*, 2518. [CrossRef]
47. Turker, E.; Arslan-Yildiz, A. Recent advances in magnetic levitation: A biological approach from diagnostics to tissue engineering. *ACS Biomater. Sci. Eng.* **2018**, *4*, 787–799. [CrossRef]
48. Lv, D.; Hu, Z.; Lu, L.; Lu, H.; Xu, X. Three-dimensional cell culture: A powerful tool in tumor research and drug discovery. *Oncol. Lett.* **2017**, *14*, 6999–7010. [CrossRef] [PubMed]

49. Saburina, I.N.; Gorkun, A.A.; Fidarov, A.F.; Kolokol'tsova, T.D.; Zurina, I.M.; Kosheleva, N.V.; Ustinova, E.E.; Repin, V.S. Induction of Vasculo- and Osteogenesis in Spheroids Formed by Adipose-Derived Stromal Cells. *Bull. Exp. Biol. Med.* **2018**, *166*, 163–169. [CrossRef] [PubMed]
50. Marshall, J.; Barnes, A.; Genever, P. Analysis of the intrinsic self-organising properties of mesenchymal stromal cells in three-dimensional co-culture models with endothelial cells. *Bioengineering* **2018**, *5*, 92. [CrossRef] [PubMed]
51. Rawal, S.; Williams, S.J.; Ramachandran, K.; Stehno-Bittel, L. Integration of mesenchymal stem cells into islet cell spheroids improves long-term viability, but not islet function. *Islets* **2017**, *9*, 87–98. [CrossRef] [PubMed]
52. Futrega, K.; Atkinson, K.; Lott, W.B.; Doran, M.R. Spheroid coculture of hematopoietic stem/progenitor cells and monolayer expanded mesenchymal stem/stromal cells in polydimethylsiloxane microwells modestly improves in vitro hematopoietic stem/progenitor cell expansion. *Tissue Eng. Part C Methods* **2017**, *23*, 200–218. [CrossRef] [PubMed]
53. Amano, M.; Nakayama, M.; Kaibuchi, K. Rho-kinase/ROCK: A key regulator of the cytoskeleton and cell polarity. *Cytoskeleton* **2010**, *67*, 545–554. [CrossRef] [PubMed]
54. Alvarez-Viejo, M.; Menendez-Menendez, Y.; Otero-Hernandez, J. CD271 as a marker to identify mesenchymal stem cells from diverse sources before culture. *World J. Stem Cells* **2015**, *7*, 470–476. [CrossRef] [PubMed]
55. Huang, G.S.; Tseng, T.C.; Dai, N.T.; Fu, K.Y.; Dai, L.G.; Hsu, S.H. Fast isolation and expansion of multipotent cells from adipose tissue based on chitosan-selected primary culture. *Biomaterials* **2015**, *65*, 154–162. [CrossRef] [PubMed]
56. Cheng, N.C.; Chang, H.H.; Tu, Y.K.; Young, T.H. Efficient transfer of human adipose-derived stem cells by chitosan/gelatin blend films. *J. Biomed. Mater. Res. B Appl. Biomater.* **2012**, *100*, 1369–1377. [CrossRef]
57. Wang, L.; Rao, R.R.; Stegemann, J.P. Delivery of mesenchymal stem cells in chitosan/collagen microbeads for orthopedic tissue repair. *Cells Tissues Organs* **2013**, *197*, 333–343. [CrossRef]
58. Wu, Y.; Yang, Z.; Law, J.B.; He, A.Y.; Abbas, A.A.; Denslin, V.; Kamarul, T.; Hui, J.H.; Lee, E.H. The Combined effect of substrate stiffness and surface topography on chondrogenic differentiation of mesenchymal stem cells. *Tissue Eng. Part A* **2017**, *23*, 43–54. [CrossRef]
59. Huebsch, N.; Arany, P.R.; Mao, A.S.; Shvartsman, D.; Ali, O.A.; Bencherif, S.A.; Rivera-Feliciano, J.; Mooney, D.J. Harnessing traction-mediated manipulation of the cell/matrix interface to control stem-cell fate. *Nat. Mater.* **2010**, *9*, 518–526. [CrossRef]
60. Park, J.S.; Chu, J.S.; Tsou, A.D.; Diop, R.; Tang, Z.; Wang, A.; Li, S. The effect of matrix stiffness on the differentiation of mesenchymal stem cells in response to TGF-beta. *Biomaterials* **2011**, *32*, 3921–3930. [CrossRef]
61. Datta, N.; Pham, Q.P.; Sharma, U.; Sikavitsas, V.I.; Jansen, J.A.; Mikos, A.G. In vitro generated extracellular matrix and fluid shear stress synergistically enhance 3D osteoblastic differentiation. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 2488–2493. [CrossRef] [PubMed]
62. Li, S.; Wang, J.; Han, Y.; Li, X.; Liu, C.; Lv, Z.; Wang, X.; Tang, X.; Wang, Z. Carbenoxolone inhibits mechanical stress-induced osteogenic differentiation of mesenchymal stem cells by regulating p38 MAPK phosphorylation. *Exp. Ther. Med.* **2018**, *15*, 2798–2803. [CrossRef] [PubMed]
63. Steward, A.J.; Kelly, D.J. Mechanical regulation of mesenchymal stem cell differentiation. *J. Anat.* **2015**, *227*, 717–731. [CrossRef] [PubMed]
64. Saleh, F.A.; Whyte, M.; Genever, P.G. Effects of endothelial cells on human mesenchymal stem cell activity in a three-dimensional in vitro model. *Eur. Cell Mater.* **2011**, *22*, 242–257. [CrossRef] [PubMed]
65. Zhang, S.; Liu, P.; Chen, L.; Wang, Y.; Wang, Z.; Zhang, B. The effects of spheroid formation of adipose-derived stem cells in a microgravity bioreactor on stemness properties and therapeutic potential. *Biomaterials* **2015**, *41*, 15–25. [CrossRef] [PubMed]
66. Edmondson, R.; Broglie, J.J.; Adcock, A.F.; Yang, L. Three-dimensional cell culture systems and their applications in drug discovery and cell-based biosensors. *Assay Drug Dev. Technol.* **2014**, *12*, 207–218. [CrossRef]
67. Murphy, K.C.; Hung, B.P.; Browne-Bourne, S.; Zhou, D.; Yeung, J.; Genetos, D.C.; Leach, J.K. Measurement of oxygen tension within mesenchymal stem cell spheroids. *J. R. Soc. Interface* **2017**, *14*, 20160851. [CrossRef]
68. Campbell, J.J.; Bader, D.L.; Lee, D.A. Mechanical loading modulates intracellular calcium signaling in human mesenchymal stem cells. *J. Appl. Biomater. Biomech.* **2008**, *6*, 9–15.

69. Uzieliene, I.; Bernotas, P.; Mobasheri, A.; Bernotiene, E. The role of physical stimuli on calcium channels in chondrogenic differentiation of mesenchymal stem cells. *Int. J. Mol. Sci.* **2018**, *19*, 2998. [CrossRef]
70. Formigli, L.; Meacci, E.; Sassoli, C.; Squecco, R.; Nosi, D.; Chellini, F.; Naro, F.; Francini, F.; Zecchi-Orlandini, S. Cytoskeleton/stretch-activated ion channel interaction regulates myogenic differentiation of skeletal myoblasts. *J. Cell. Physiol.* **2007**, *211*, 296–306. [CrossRef]
71. Zhou, Y.; Chen, H.; Li, H.; Wu, Y. 3D culture increases pluripotent gene expression in mesenchymal stem cells through relaxation of cytoskeleton tension. *J. Cell. Mol. Med.* **2017**, *21*, 1073–1084. [CrossRef] [PubMed]
72. Mege, R.M.; Ishiyama, N. Integration of cadherin adhesion and cytoskeleton at adherens junctions. *Cold Spring Harb. Perspect. Biol.* **2017**, *9*, a028738. [CrossRef] [PubMed]
73. Huang, G.S.; Hsieh, P.S.; Tseng, C.S.; Hsu, S.H. The substrate-dependent regeneration capacity of mesenchymal stem cell spheroids derived on various biomaterial surfaces. *Biomater. Sci.* **2014**, *2*, 1652–1660. [CrossRef]
74. Caplan, A.I. Mesenchymal stem cells: Time to change the name! *Stem Cells Transl. Med.* **2017**, *6*, 1445–1451. [CrossRef] [PubMed]
75. Fitzsimmons, R.E.B.; Mazurek, M.S.; Soos, A.; Simmons, C.A. Mesenchymal stromal/stem Cells in regenerative medicine and tissue engineering. *Stem Cells Int.* **2018**, *2018*, 8031718. [CrossRef] [PubMed]
76. Phinney, D.G. Functional heterogeneity of mesenchymal stem cells: Implications for cell therapy. *J. Cell Biochem.* **2012**, *113*, 2806–2812. [CrossRef] [PubMed]
77. McLeod, C.M.; Mauck, R.L. On the origin and impact of mesenchymal stem cell heterogeneity: New insights and emerging tools for single cell analysis. *Eur. Cell Mater.* **2017**, *34*, 217–231. [CrossRef] [PubMed]
78. Xu, L.; Li, G. Circulating mesenchymal stem cells and their clinical implications. *J. Orthop. Transl.* **2014**, *2*, 1–7. [CrossRef]
79. Marketou, M.E.; Parthenakis, F.I.; Kalyva, A.; Pontikoglou, C.; Maragkoudakis, S.; Kontaraki, J.E.; Zacharis, E.A.; Patrianakos, A.; Chlouverakis, G.; Papadaki, H.A.; et al. Circulating mesenchymal stem cells in patients with hypertrophic cardiomyopathy. *Cardiovasc. Pathol.* **2015**, *24*, 149–153. [CrossRef]