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Spheroids of stem cells as endochondral templates for improved bone engineering

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1. ABSTRACT

Osteodegenerative disease and bone fractures lead to bone damage or loss, requiring new bone formation to replace the damaged tissues. Classical 'top-down' tissue engineering relies on seeding cell suspensions into biomaterial scaffolds, and then guiding cell fate by growth factors. However, complex tissue fabrication using this approach has important limitations. 'Bottom-up' tissue engineering has the potential to overcome the drawbacks of the top-down approach, by using 'building blocks' of cell spheroids for tissue biofabrication without a scaffold. Spheroids are 3D structures that resemble the physiological tissue microenvironment and can be produced in vitro by different methods. Spheroids of mesenchymal stem cells (MSC) and adipose stem cells (ASC) have regenerative properties. Here we review, the use of spheroids as 'building blocks' in the 3D bioprinting of large-scale bone tissue and as a promising alternative for the treatment of osteodegenerative diseases and in bone engineering, including endochondral ossification (or developmental engineering).

2. INTRODUCTION

Bone is a metabolically active tissue that can adapt to the loading conditions imposed by the skeletal system. This adaptability allows the skeleton to effectively protect and support the body organs during embryonic development (1). Bone is also responsible for hematopoiesis and mineral homeostasis, among other functions (2).

In the United States there are six million patients with bone lesions every year and approximately 10% of these fractures do not reach full regeneration due to tissue loss, failed fixation, infection or inadequate vascularization (3). Currently, the most common treatments for fractures are based on the use bone autografts; however, this approach has serious limitations such as scarce tissue supply, donor site morbidity, infections and loss of cell integration (4). Allograft transplants have also been used as an alternative therapy for bone lesions, but have recently been associated with disease transmission and host immune rejection (4).

Table 1. Studies on osteogenic induction in spheroids of adipose stem cells (ASC) and mesenchymal stem cells (MSC)

Cell source	Spheroid production method	Factors used for differentiation induction	Main outcomes	Reference
MSC	Pellet culture	Chondrogenic medium: 10 ng ml^{-1} rhTGF-β1, 10^{-7} M dexamethasone and 2.5 x 10^{-4} M ascorbic acid. Osteogenic medium: 7.0 x10^{-3} M β-glycerophosphate, 10^{-8} M dexamethasone and 2.5 x 10^{-4} M ascorbic acid	Production of a chondro-osseous organoid reminiscent of the pre-invasion endochondral ossification pattern, where a bony collar is found around cartilage	76
MSC	Suspension culture in 96- well plates	50 mg/mL L-ascorbic acid, 10 nM β-glycerolphosphate, 10 nM dexamethasone	Recapitulated *in vivo* bone formation, while providing a reproducible and versatile *in vitro* model of osteogenesis	84
MSC	Spinner culture, hanging drop, 96-well non-adhesive culture plates and polypropylene tubes	3 × 10^{-4} M l-ascorbic acid phosphate, 1 × 10^{-7} M dexamethasone and 5 × 10^{-3} β-glycerophosphate	Efficient osteogenesis induction in spheroids that are homogenous in size and shape	66
ASC	Hanging drop	Dexamethasone, ascorbic acid 2-phosphate, ITS + 3, fatty acid supplement, NEAA, estradiol, progesterone, hydrocortisone, EGF, PDGF, SCGF-β, TNF-alpha, IL-1β	Revealed the maintenance of developmental plasticity and self-renewal capacity in osteogenic induced ASC spheroids.	85
ASC	Suspension culture in 96-well plates	0.01 mM 1,25-dihydroxyvitamin D3, 50 mM L-ascorbate-2-phosphate, 50 mM dexamethasone, and 10 mM β-glycerophosphate	Spheroids of homogeneous size had high levels of osteogenic differentiation, and increased matrix mineralization both *in vitro* and *in vivo*	11
MSC	Low-binding plates	Ascorbic acid, hydrocortisone, and β -glycerophosphate	Spheroids had increased osteoregenerative properties compared with monolayer cultures	9
MSC	Hanging drop	10 mM β-glycerophosphate, 50 µg/ mL ascorbate-2-phosphate and 100 nM dexamethasone	Spheroids were resistant to apoptosis and had high proangiogenic potential	72
MSC	Liquid overlay	10mM β-glycerophosphate, 50µg/mL ascorbic acid, 10nM dexamethasone, 10nM vitamin K3, 10nM vitamin D3, 1ng/mL TGF-β, 25ng/mL VEGF, 25ng/mL FGF-β	The osteogenic differentiation of spheroids impairs their vascularization capacity *in vivo*	86
MSC	Rotation culture	10 % FBS, 100 nM dexamethasone, 0.0.5 mM l-ascorbic acid-2-phosphate, and 10 mM sodium glycerophosphate	Significant regeneration of bone defects *in vivo* using spheroid implants	70
ASC	Suspension culture in 96-well plates	100 µM ascorbic acid, 10 mM β-glycerophosphate and 1 µM dexamethasone	Regeneration of cartilage and subchondral bone (by 12 months) using spheroids implanted into an osteochondral defect site	74
MSC	Hanging drop	50 mg/ml ascorbate-2-phosphate, 10 mM β-lycerophosphate, and 100 nM dexamethasone	Osteogenic induced spheroids remained capable of mineral production and had increased bone formation and regeneration properties	87
ASC	Atop recombinant elastin-like polypeptide (ELP) conjugated to a charged polyelectrolyte polyethyleneimine (PEI)	50 µM L-ascorbic acid, 10 mM β-glycerophosphate, 0.05 nM dexamethasone and 10% FBS	Superior osteogenic differentiation of ASC cultured as spheroids	36

Bone engineering is a promising strategy for treating bone diseases such as osteoarthritis and osteoporosis, and bone lesions caused by traumas, as well as to reconstruct bone defects from embryogenesis. Traditional approaches to bone engineering rely on a 'top-down' methodology based on the use of scaffolds. MSC are usually seeded on the surface of scaffolds in the presence of osteogenic inductive medium, and are expected to proliferate and differentiate into 3D bone tissue (5). Although the top-down approach has clear potential and allowed considerable progress to be made in bone engineering, recent discoveries about the importance of cell-to-cell and cell-to-extracellular matrix contacts in 3D scaffold-free cultures suggest that tissue engineering without a scaffold – known as the 'bottom-up' approach - can bring further improvement to endochondral tissue engineering (6).

To date, few models of endochondral ossification have been developed, and most studies, both *in vitro* and *in vivo*, involve the use of collagen-based scaffolds to promote endochondral bone formation (7). The use of spheroids formed with human stem cells as a template to recapitulate endochondral ossification has the potential to address some of the challenges of classical bone engineering approaches (Table 1). Spheroids can be formed using different 3D cell culture techniques, such as the 'liquid overlay', or

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the 'hanging drop' methods, or via the use of micro-molded and non-adhesive hydrogels (8). Although spheroids were used initially as tumor models, both MSC and ASC spheroids are currently used for *in vivo* tissue regeneration (9,10,11).

This work reviews the importance of using spheroids for bone regeneration, addressing new perspectives in the field, such as the use of spheroids as a template for endochondral ossification and the importance of 3D bioprinting for large-scale bone tissue engineering.

3. THE BIOLOGY OF ENDOCHONDRAL OSSIFICATION

Long and axial bones of the mammalian skeleton originate from cartilage templates via a complex process called endochondral ossification. During this process, condensed mesenchymal tissue undergoes chondrogenesis directed by the Sox9 protein (12). Initially, chondrocytes produce cartilage extracellular matrix molecules, such as the collagen type II alpha-1 chain (Col2a1) and aggrecan, and proliferate, creating the chondrocyte layer found in the growth plate. This structure defines the shape of skeletal tissues by determining the direction of tissue elongation. Subsequently, chondrocytes stop proliferating, become hypertrophic, mineralize the surrounding matrix and, ultimately, undergo apoptosis. Concomitantly, perichondral cells close to the mineralized hypertrophic chondrocytes turn into osteoblasts, forming the bone collar. In addition, the mineralized cartilaginous matrix is invaded by blood vessels carrying osteoblast precursors, which establish ossification centers (13). MSC, endothelial stem cells and chondrocytes are located in close proximity to each other within the cartilage template before invasion by osteoblasts and bone formation. The proximity between MSC (perivascular cells) and vascular endothelial cells is probably due to direct cell-to-cell contacts, and these cell types share the same paracrine signaling within the blood vessel niche (14).

Several cytokines and growth factors are involved in bone formation, including fibroblast growth factor (FGF), Wnt, transforming growth factor β (TGF-β) and bone morphogenetic protein (BMP) (15-16). Among these, TGF-β and BMP families have important functions in different aspects of skeletogenesis, playing roles in mesenchyme condensation, skeletal morphogenesis, growth plate development and osteoblast differentiation. Furthermore, TGF-β and BMP regulate the homeostasis of postnatal joint cartilage (15-16). BMP and TGF-β interact in various cell signaling pathways - such as Wnt, Hedgehog, Notch and FGF - and mutations in TGF-β and BMP signaling can cause a large range of skeletal disorders in humans. An example of these disorders is Myhre syndrome, where a mutation in the Smad4 protein results in short stature, short hands and feet, facial dysmorphism, muscular hypertrophy, deafness and cognitive delay (15).

To improve the treatment of defects in bone development, there has been increasing interest in cartilage-mediated bone regeneration (endochondral ossification) in the field of tissue engineering, mainly due to the inherent ability of cartilage to form vascularized bone, improving graft integration *in vivo* compared with current bone engineering via intramembranous ossification (17).

4. TISSUE ENGINEERING

4.1. 'Top-down' tissue engineering

Tissue engineering aims to provide alternative approaches for tissue regeneration. Currently, 'gold standard' tissue engineering relies on three principles: (1) the use of scaffolds in the form of a constructed biomaterial designed to mimic the physical properties of the tissue extracellular matrix, while providing functional support to initiate tissue formation; (2) cell seeding on the surface of scaffolds, to adhere and colonize the area to be regenerated *in vivo*; and (3) the provision of soluble growth factors such as vascular endothelial growth factor (VEGF) and FGF, to direct cell fate through proliferation and differentiation (18). The biomaterials and growth factors are expected to interact with the cells to create, *in vivo*, a dynamic environment that enables regeneration (19-20). This approach is part of the classical or 'top-down' tissue engineering and has been useful to build tissues for clinical applications (21).

Top-down tissue engineering can be performed using different cell types. MSC are considered powerful tools for therapeutic strategies in regenerative medicine because they can be isolated from several tissues, are amenable to expansion *in vitro* and can be induced to differentiate into multiple lineages - including cartilage and bone - by BMP and TGF-β soluble factors (22). MSC isolated from bone marrow are commonly used in bone tissue engineering; however, cells originating from other sources, such as subcutaneous adipose tissue (adipose stem cells, or ASC) can also be used for osteogenic differentiation (23).

ASC are considered an attractive cell type for tissue engineering because of their abundance and ease of harvesting, with minimal morbidity of the adipose tissue donor. ASC can be maintained for long periods in culture and have higher proliferation capacity than bone marrow-derived MSC (23). Recent studies showed that human ASC differentiate towards an osteogenic phenotype when cultured in a bioactive

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glass scaffold, as indicated by the expression of bone extracellular matrix markers (osteocalcin and osteopontin), as well as an increase in cell proliferation, viability and alkaline phosphatase (ALP) activity (20-23). However, angiogenesis, which is essential for bone tissue formation, was absent in this model (23).

Different types of biomaterials have been used for bone engineering, including metals, ceramics (i.e., calcium phosphates), organic-inorganic composites and natural polymers, each one showing advantages and disadvantages (24). Metals such as titanium are biocompatible, strong and economic, but are not biodegradable and can induce stress in tissues. On the other hand, bioceramics such as hydroxyapatite and beta-tricalcium phosphate (β-TCP) have been widely used for bone repair due to their bioactivity, which is attributed to their structural and compositional similarity with the mineral phase of bone tissue (24). Also, the bioactivity of ceramics facilitates cell attachment and bone extracellular matrix synthesis (24). However, the use of ceramic implants remains limited because of their poor mechanical properties such as low torsion, bending and shearing resistance.

In addition to metals and ceramics, several studies reported the use of polymeric materials for the generation of bone engineering scaffolds, particularly polycaprolactone (PCL), polylactic acid (PLA) and polyglycolic acid (PGA), which have excellent biocompatibility and biodegradability (4,25). The strategy of combining ceramic materials with polymers, generating composites, aims to improve the bioactivity of scaffolds. The production of biphasic (i.e., organic-inorganic) composites by mixing polymers and ceramic materials combines the high mechanical performance of polymers with the increased compression resistance of ceramics, mimicking the biomechanical properties of bone (25).

While top-down tissue engineering using different scaffold materials can promote the formation of a satisfactory biomechanical microenvironment for tissue regeneration *in vivo*, these strategies often result in non-homogeneous cell seeding, with cell escape and poor cell viability, particularly in the center of the scaffold (26). In addition, the long-term control of the mechanical and physical properties of the graft can be problematic using classic tissue engineering. The biofabrication of complex and larger functional tissues with high cell densities and diverse metabolic requirements is still considered a challenge in the top-down approach, mainly due to the limited diffusion properties of biomimetic scaffolds. The 'bottom-up' approach described below has the potential to address this challenge, because it focuses on the biofabrication of microscale tissue building blocks, followed by the assembly of these blocks into complex engineered tissue constructs (27).

4.2. 'Bottom-up' tissue engineering

Tissue hierarchies are formed from building blocks that enable and regulate system function. Bottom-up tissuMEe engineering aims to mimic this hierarchy, by creating tissues from functional 'building blocks' - represented by cell-encapsulating microscale hydrogels, cell sheets and 3D spheroids - with a defined 3D architecture (27-28). Bottom-up approaches focus on assembling these building blocks to fabricate – or 'biofabricate' - larger constructs that could be used to restore injured tissues (29). In this context, 'biofabrication' is defined as "the automated generation of biologically functional products with structural organization from living cells, bioactive molecules, biomaterials, cell aggregates such as micro-tissues, or hybrid cell-material constructs, through Bioprinting or Bioassembly and subsequent tissue maturation processes" (30).

The main advantages of the bottom-up approach to tissue engineering are: (1) the possibility of scaling by biofabrication methods; (2) the ability to create tissues with much higher cellular densities; and (3) the potential to incorporate a wide range of cell types in the construct (31). An efficient biofabrication process is fundamental for building large cell modules into 3D macroscopic tissues, while maintaining tissue geometries and also the initial conditions of cells (32).

Different types of cell culture can be used as building blocks for bottom-up strategies. In one of these strategies, known as 'cell sheet engineering', 3D tissues are built layer-by-layer from monolayers of cells (33). Cell sheet engineering allows the reconstruction of different types of tissues and organs, including skin, cardiac muscle and liver lobules (34). To allow cell sheet engineering, different methodologies have been applied to promote the nondestructive detachment of cell sheets from culture surfaces, such as the use of thermoresponsive surfaces, hydrogels, and enzymatically degradable substrates (33).

A promising type of building block used in bottom-up tissue engineering is the 'spheroid', a 3D cell structure produced by self-assembly. Spheroids are living materials with controllable composition and biological characteristics (35). One interesting property of spheroids is their ability to undergo fusion when placed together, making it possible to engineer tissues with a predefined structure, such as an intra-organ branched vascular tree (35). Therefore, 3D spheroids represent attractive building blocks for the production of larger tissue constructs.

5. SPHEROIDS

Spheroids are formed when cells do not have a substrate to adhere to and attach to each other

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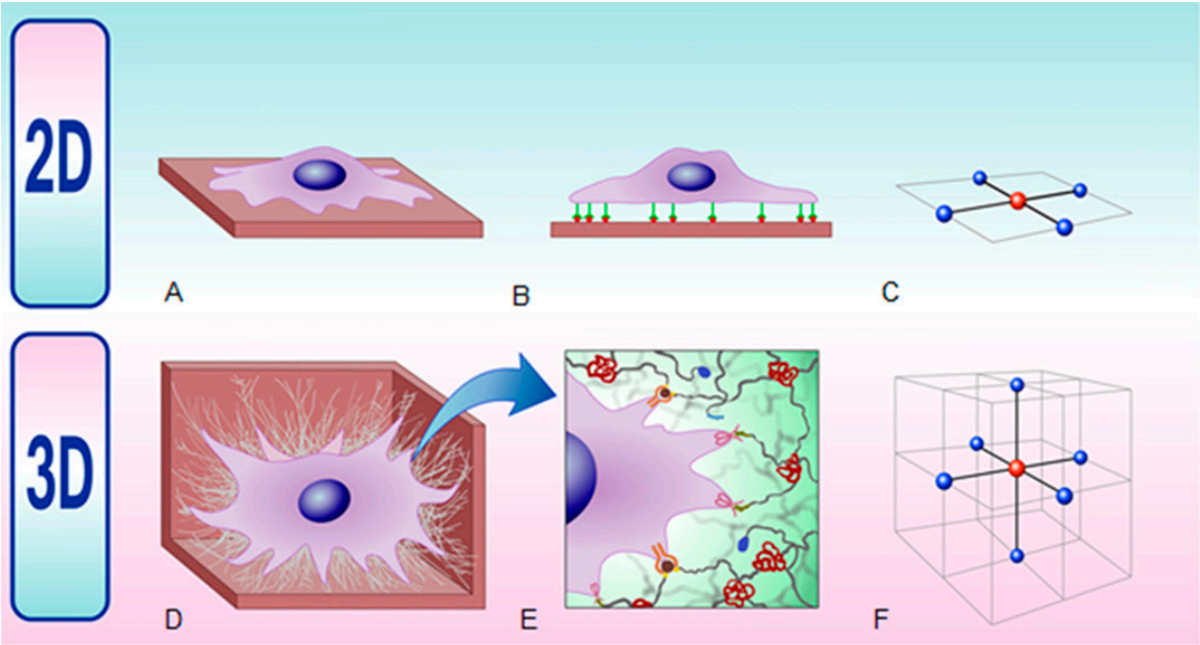

Figure 1. Spheroids show improved cell-cell and cell-extracellular matrix interactions compared with 2D cell culture. Cells cultured in monolayers are forced to adhere to a flat substrate and assume an apical-basal polarity. The expression of adherent molecules, such as N-cadherin, is necessarily polarized, and the secretion of extracellular matrix components is low (A-C). In the spheroid architecture, cell-cell and cell-extracellular matrix interactions occur throughout the cell surface, which improves cytoskeleton dynamics and avoids artificial cell polaritzation. Furthermore, there is improved distribution of adhesion molecules in the plasmatic membrane surface and differences in molecule biosynthesis due to interactions between cells and the extracellular matrix (D-F). (C) and (F) represent the dimensions of cell interactions.

instead, through junctional complexes and adhesion molecules, such as N-cadherin (36), in a process of self-assembly. Spheroid self-assembly mimics natural processes that occur during embryogenesis, morphogenesis and organogenesis. Therefore, spheroids can mimic the architectural and functional characteristics of native tissues; for example, cardiomyocyte spheroids beat with a heart-like rhythm (8). Spheroid-based tissue engineering represents a 'scaffold-free' or 'bottom-up' strategy.

Compared with 2D cell culture, spheroid 3D cultures more closely resemble physiological tissue microenvironments in numerous aspects. Secreted molecules are present at higher concentrations in 3D cultures such as spheroids, which ensures effective communication and facilitates intercellular signaling. Also, cells in 3D cultures are connected with extracellular matrix proteins and with other cells in all dimensions (Figure 1), unlike cells in 2D cultures. Thus, each cell within a spheroid is in contact with a larger number of neighboring cells. The extracellular matrix from spheroids acts as a scaffold and modulator of cell growth, proliferation, regeneration and differentiation (6). Therefore, the formation and morphogenesis of 3D spheroids are dynamic processes regulated by differential cell adhesion, extracellular matrix synthesis and constant remodeling, resembling embryogenesis *in vivo (*37).

Stem cell spheroids represent a new template to study the expansion and differentiation of stem cells, and are currently used in tissue engineering approaches, due to their remarkable regenerative properties. Spheroids of MSC have higher paracrine immunomodulatory capacity (38) and increased secretion of VEGF, basic fibroblast growth factor (bFGF) and angiogenin than 2D cultures (39-40). *In vivo*, spheroids of MSC remain viable in injured tissues, and provide increased secretion of anti-inflammatory and proangiogenic factors to improve repair (41-42). Besides, the differentiation of multipotent MSC into chondrogenic, osteogenic and adipogenic lineages is improved in spheroid cultures when compared with 2D cell monolayers (43,44,45).

In conclusion, spheroids are attractive building blocks for bottom-up tissue engineering because they provide a 3D microenvironment with intensive and direct cell-to-cell contacts, mimicking the biological features of a tissue. Also, spheroids have high regenerative potential when made from adult stem cells and can undergo fusion by biofabrication methods, forming larger constructs. For bottom-up tissue engineering it is mandatory to form spheroids with high cell viability, and homogeneous size and shape. This is particularly important for advanced bottom-up approaches such as bio-assembly and bioprinting, which use spheroid building blocks to

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develop symmetric and functional engineered tissues, such as vascular tubes or a 3D liver (46-47).

5.1. Techniques for spheroid formation

Different techniques can be used to form spheroids. The 'pellet culture' approach uses centrifugal force to concentrate cells in the bottom of a tube, maximizing the opportunity for cell adhesion. This method is often used for bone tissue formation, as it produces aggregates easily; however, it has important disadvantages. In its current format, the method cannot be scaled up. Also, the cells cannot be visualized while they aggregate and there is no N-cadherin upregulation during the assembly process, since cell-cell interactions are forced by centrifugation (8).

Spheroids can also be formed using the 'spinner culture' technique, where cells are transferred to spinner flasks and then cultured on a stirring platform, with stirring being maintained and controlled by an internal magnetic arm. This 3D culture system is similar to the rotating wall vessel, a cylindrical cell culture chamber covered internally with a membrane that allows oxygen to be drawn into the vessel as rotation starts. Both methodologies are dynamic and the rotation of the culture medium improves nutrient diffusion into spheroids. However, it is not possible to visualize spheroid formation using these culture systems, and the spheroids formed are not homogeneous in size (48).

The 'hanging drop' spheroid formation technique is a simple method where cells are allowed to assemble into spheroids at the apex of a droplet of medium. While this method provides control of cell number and spheroid size, the establishment of long-term 3D cultures required for morphogenesis/differentiation assays is problematic using this technique (49-50). The 'liquid overlay' method consists of forming spheroids in culture systems with no-adherent surfaces (such as agarose-coated multi-well plates). However, this technique does not allow for the spheroid shape to be controlled, although spheroid size can be modulated by the number of cells plated in each well (51).

External forces such as electric or magnetic fields can also be used to guide cells to aggregate into spheroids (52-53), although it is difficult to immobilize the aggregates without compromising their integrity, after the field is switched off. The advantages of this method include the possibility to follow cells while they aggregate and the high cell viability when aggregates are successfully imobilized; however, spheroids are not homogeneous in size (53). Recently, Tseng *et al* (54) developed an assay where a suspension of cells is "magnetized" with a mix of biocompatible magnetic nanoparticles. Cells are attracted to form a spheroid at the bottom of a multi-well plate using a cylindrical magnet. The resulting spheroids are homogeneous in size; however, nanoparticles remain inside spheroids.

A different method of spheroid formation involves cultivating cells on a surface made of a thermo-responsive polymer, such as poly (N-isopropylacrylamide) (PIPAAm), which allows the release of cell sheets when the culture temperature is changed to 20ºC for 1 hour, and the polymer changes from hydrophobic to hydrophilic. The small cell sheets released can then be incubated in a non-adhesive surface, where they become compacted into spheroids (55, 56, 8). Thermo-responsive surfaces can be used to produce cultures that combine different cell types, such as hepatocytes and endothelial cells (55).

Microfluidics techonology can also be employed to form spheroids, by seeding cells into a device where liquids are controlled and manipulated at a scale of a few microliters. Liquid perfusion within microfluidic devices improves the distribution of growth factors inside the spheroids (57-58), and this dynamic (albeit relatively complex) methodology offers high resolution, sensitivity, low cost and scalability, as well as reducing the time required for viability and other assays (59). Microfluidic systems such as 'organ-on-a-chip' have already been used to form 3D spheroids from several cell lineages (60-61).

Spheroids can also be formed using micromolded non-adhesive hydrogels, from agarose or polyacrylamide (Figure 2). Cells are settled at the bottom of a hydrogel containing a varying number of molded resections, and cell-cell interactions induce the formation of a single spheroid in each resection. This method can be scaled up to form spheroids with homogenous shape, size and cell composition by using automated platforms such as epMotion™ 5070 (Eppendorf), currently in use by our research group. In addition, the hydrogel method allows cells to be registered as they self-assemble, and it is easy to change the culture medium and to add drugs, antibodies or growth factors to the system, allowing long-term spheroid culture and treatment (8,62,63).

5.2. Spheroids for bone engineering

Spheroids cultured for three days in osteogenic medium have more calcium nodules than cells cultured in monolayers for three weeks under the same conditions. Thus, spheroids have an accelerated osteogenic differentiation *in vitro* when compared with cell monolayers (6). Before the formation of bone nodules, MSC, ASC and osteoblast precursors undergo a complex differentiation process, in which cells change their morphology from a fibroblastoid to a cuboidal shape and start to produce an extracellular

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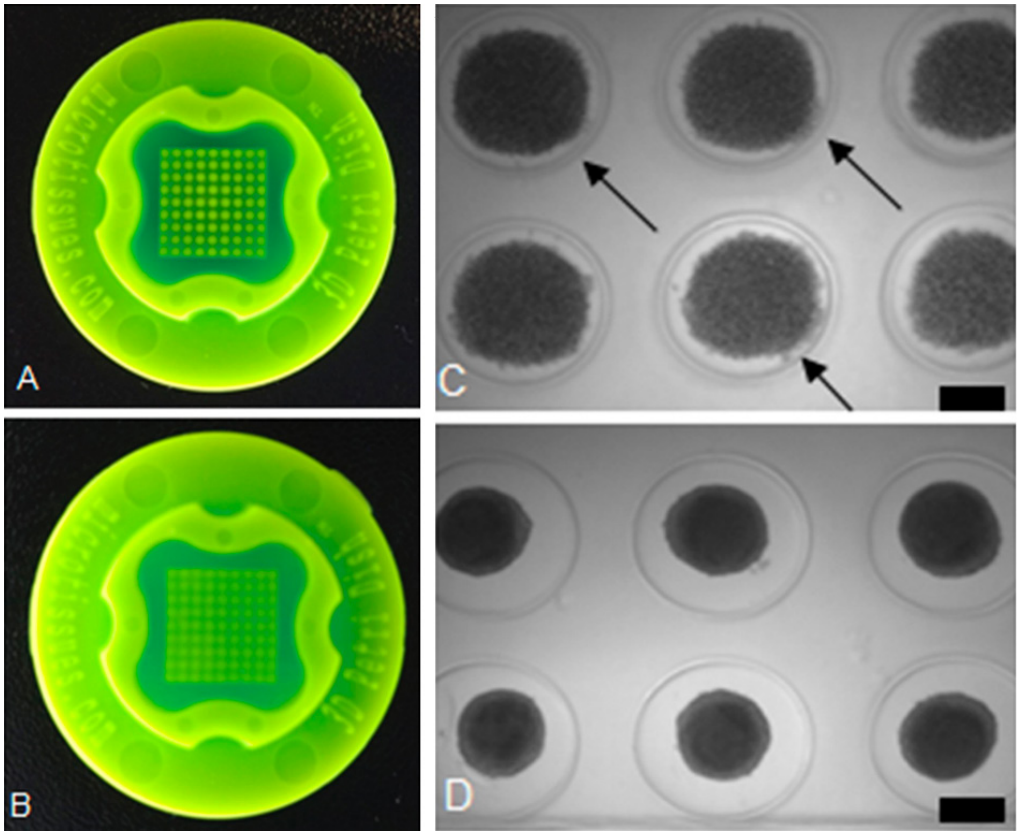

Figure 2. Spheroids fabricated through the micromolded non-adhesive hydrogel technique. Macroscopic image of the MicroTissues 3D Petri Dish® silicone mold (SigmaAldrich, St. Louis, MO, USA) (A). Silicone mold after the addition of agarose to produce a micromolded non-adhesive hydrogel (B). The Hydrogel with resections is transferred to culture plates and ASC are seeded in each resection (C). Note the resections filled with cells (arrows). After 24h, spheroids formation is complete (D). Scale bar: 200 µm.

matrix typical of bone, with collagen type I and several bone-specific proteins such as the primary mineralization nucleators osteopontin and osteoclalcin (64). Several studies have demonstrated that the scaffold-free 3D culture of osteogenic induced cell aggregates such as spheroids increases osteogenesis - both *in vitro* and *in vivo* - compared with 2D cultures, as it improves intercellular as well as extracellular matrix interactions and tissue-specific properties.

Kale and collaborators (2000) (65) pioneered the use of spheroids for osteogenesis *in vitro*. These authors used calvarial and bone marrow-derived osteogenic cells to produce osteogenic induced spheroids, using serum-free TGF-β1 treatment, and showed that the assembly of cells into bone spheroids upregulates several bone-related proteins, such as type I collagen and osteonectin, and increases ALP secretion, in a period of 3–7 days. Importantly, the spheroids formed *in vitro* had a micro-crystalline bone-like structure. This work demonstrated that the *ex vivo* bone formation using spheroids is able to provide crucial information on the timing of osteogenesis. After this pioneering work, numerous studies have confirmed and extended the potential of spheroids for bone tissue engineering (Figure 3).

Hildebrandt and co-workers (2011) (66) established a highly efficient protocol for the generation of MSC spherical aggregates with size control, based on non-adhesive 96-well culture plates. Using this method, the authors demonstrated that MSC spheroids can differentiate towards the osteogenic pathway (66). Baraniak and McDevitti (2012) (67) showed that when murine bone marrow MSC spheroids (produced by the external force technique) were induced to the osteogenic pathway, they had more robust extracellular matrix mineralization than cells in monolayers. Similarly, Vidyasekar and collaborators (2016) (68) showed that bone marrow MSC spheroids (grown on PLA microspheres) had higher potential for osteogenic lineage after differentiation induction, as determined by increased mineralization, compared with monolayer cultures. Spheroids maintained in growth medium remained viable (67,68), and Baraniak and McDevitti (2012) (67) observed no differentiation in the absence of induction factors.

Our research group works with human ASC to produce spheroids using the micromolded non-adhesive hydrogel technique (69). We are currently establishing in our laboratory a model for osteogenesis. After 24 hours of cell seeding into the hydrogel, the

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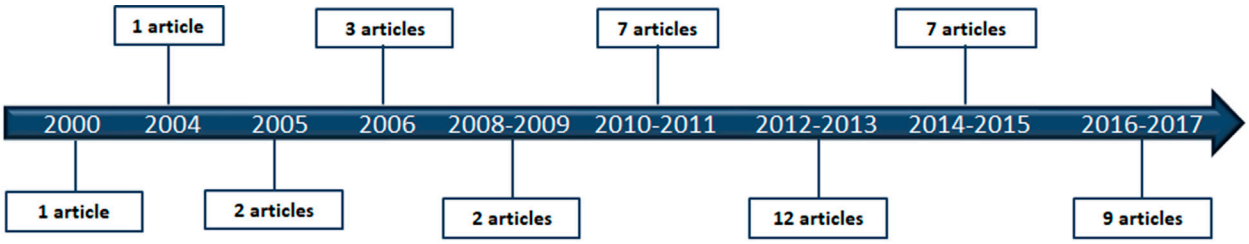

Figure 3. Timeline of numbers of articles published with spheroids as a model for osteogenesis. The search was performed on the *PubMed* database, by entering the words 'spheroids' and 'osteogenesis'. From the total of 57 articles retrieved by the search, only the studies performed with spheroids as a model for osteogenesis *in vitro* and/or *in vivo* were indicated for each year in the timeline. These articles were selected by abstract analysis. The search was conducted on December 09, 2017 at 9:23 p.m.

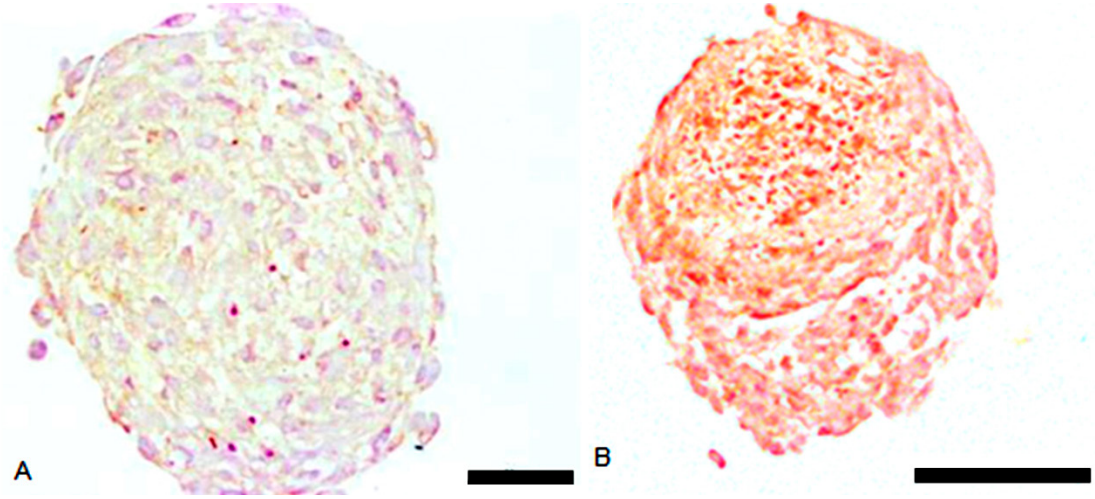

Figure 4. Alizarin red stainning showed calcium deposits in spheroids of ASC induced to the osteogenic pathway. Briefly, spheroids were fixed in 4% paraformaldehyde, dehydreted in an ethanol series, clarified in xylol and embebedded in paraffin. Samples were cut in a Cut 5062 microtome (Slee Medical) and histological sections were stainned with Alizarin red (solvents, paraffin and Alizarin red were from Sigma). Control spheroids without calcium deposits (A). Scale bar: 50 µm. Induced spheroids with calcium deposits (B). Scale bar: 100 µm.

spheroids formed in the hydrogel resections are treated for 3 weeks with an osteogenic medium containing β-glicerophosphate, dexamethasone and human recombinant BMP-7. Our preliminary result indicate that spheroids treated with the osteogenic medium differentiate into bone, since we were able to detect calcium phosphate deposits only in the induced spheroids (Figure 4).

Spheroids have been used for *in vivo* bone formation with promising results. Using a rat model of bone regeneration, Suenaga and collaborators (2015) (70) showed that implants of spheroids of human bone marrow derived MSC (formed by rotation culture) show signs of new bone formation – such as osteoclastin and osteopontin accumulation – as visualized by imaging methods. Furthermore, Raman spectroscopy revealed similarities between the spectral properties of the repaired bone and those of the native calvarial bone. In another model of rat bone formation, Yamaguchi and collaborators (2014) (9) reported that spheroids of MSC fabricated using the low-binding plate technique provided increased calvarial defect regeneration compared with MSC cultured in monolayers, as assessed by micro-computed tomography and histological assays. In addition, quantitative PCR (RT-PCR) analysis revealed that spheroids had higher levels of osteogenic markers (osterix, Runx2, osteopontin and bone sialoprotein) than 2D cultures, *in vitro*, and more calcium deposition was detected in spheroids of MSC. Shen and collaborators (2013) (11) were the first to investigate the potential of spheroids of human ASC for osteogenesis *in vivo*, compared with monolayers, showing that spheroids of this particular stem cell type had increase matrix mineralization, both *in vitro* and *in vivo*, similarly to that described for MSC from other sources.

The combination of spheroids and biomaterials has not been widely explored in bone tissue engineering. Biomaterials may be used to guide spheroid properties (such as viability, proliferation and differentiation). Ho and collaborators (2016) (71) showed that osteogenic induced implants of spheroids of MSC encapsulated in Arg-Gly-Asp (RGD)-modified alginate hydrogels or nonfouling unmodified alginate showed mineralized tissue 8 weeks after subcutaneous

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implantation, in a model of immunodeficient mice. The spheroids in RGD-modified alginate had higher mineralization *in vivo*, and differentiated to the osteogenic lineage *in vitro*, as attested by incresed ALP activity, osteocalcin expression and calcium deposition. Murphy and collaborators (2014) (72) reported that osteogenic induced spheroids of MSC encapsulated in fibrin gels had increased viability and secreted significantly more VEGF *in vitro* than the dissociated MSC in the same material. However, fibrin gels with spheroids and those with dissociated cells had similar levels of osteogenic differentiation markers, such as calcium deposits and ALP activity.

5.3. Spheroids as templates for endochondral ossification

Although the use of osteogenic induced spheroids showed some positive *in vivo* results for bone regeneration, improved graft integration could be achieved by using an endochondral ossification template, because of its inherent ability to form vascularized bone (17). Vascularization is improved with the use of spheroids of MSC and ASC as potent initiators of blood vessel formation *in vivo* (70,11,73,74). Therefore, the use of spheroids as *in vitro* templates for endochondral ossification, known as developmental engineering (1), has the potential to effectively address the treatment of bone diseases such as osteoarthritis and osteoporosis.

The synthesis of growth factors in spheroids is elevated and can induce osteogenesis. In 3D cultures, TGF-β1, TGF-β3, BMP-6 and insulin growth factor (IGF) are commonly added to induce endochondral ossification. Bioreactors are currently being tested to optimize this approach, by creating a better microenvironment for bone generation through the improved distribution of growth factor gradients and nutrients (75).

Muraglia and collaborators (2003) (76) showed that spheroids of bone marrow MSC (made by pellet culture) produced a mineralized tissue around hyaline cartilage, recapitulating endochondral ossification *in vitro*, when cultures were maintained in chondrogenic medium for 4 weeks and then for 1-3 weeks in osteogenic medium. This study showed that the culture of ASC spheroids for the treatment of osteochondral defects can be optimized by first differentiating these cells to the chondrogenic lineage, and then changing the culture medium to osteogenic factors (76). Spheroids have also been tested in studies of endochondral ossification *in vivo* aimed at avoiding the use of biomaterials for tissue regeneration in osteochondral defects (74,10,77). Yoon and collaborators (2012) (10) showed that *in vivo* cartilage formation improved when spheroid of ASC induced to the chondrogenic lineage (and produced using spinner flasks) were transplanted into the subcutaneous space of athymic mice, compared with the transplantation of non-aggregated cells previously cultivated as monolayers. The *in vitro* chondrogenic differentiation of ASC was also improved in spheroids compared with cells in 2D cultures, probably due to the activation of hypoxia-related cascades and to increased cell–cell interactions in spheroids of ASC. This study showed that spheroid of ASC can be used effectively for *in vivo* cartilage formation, after large-scale chondrogenic differentiation *in vitro*.

Recently, Murata and collaborators (2015) (74) reported positive results for the regeneration of articular cartilage and subchondral bone using a 3D construct made of spheroids of porcine ASC placed into a cylindrical mold. Under specific conditions, the cells differentiated into osteogenic, chondrogenic and adipogenic lineages, as shown by tissue-specific gene expression and extracellular matrix composition. The 3D construct was implanted in a site of osteochondral defect, in the femoral trochlear groove. Histopathology evaluation of the implant revealed active endochondral ossification underneath a thicker-than-normal fibrocartilage at 6 months after implantation. Twelve months after implantation, the fibrocartilage had decreased in thickness, matching the surrounding normal cartilage, and the implant contained subchondral bone. These results suggested that implantation of spheroids of porcine ASC into osteochondral defect sites induces regeneration of the original structure of cartilage and subchondral bone, over a 12-month period.

Ishihara and collaborators (2014) (77) also reported the regeneration of both cartilage and subchondral bone in rabbit knees using a novel technique, the scaffold-free autologous construct, consisting spheroids of MSC loaded into a molding chamber to produce a columnar structure of fused spheroids. Although both bone and cartilage were regenerated up to one year after implantation, and cartilage thickness remained constant, the authors observed overgrowth of the surface, and subchondral bone regeneration was extremely limited.

While these reports showed positive results for the use of spheroids in developmental engineering *in vivo*, additional studies are necessary to improve the techniques employed, and long-term analysis (for more than a 12 months) *in vivo* should be performed. Furthermore, the translation of laboratory research into clinical trials demands adaptation and standardization of methodologies for spheroid biofabrication (49). Spheroids could also be used as a model to study the molecular mechanisms involved in endochondral pathologies such as osteoarthritis, to guide the development - *in vitro* and *in vivo* - of new regenerative medicine strategies (Figure 5).

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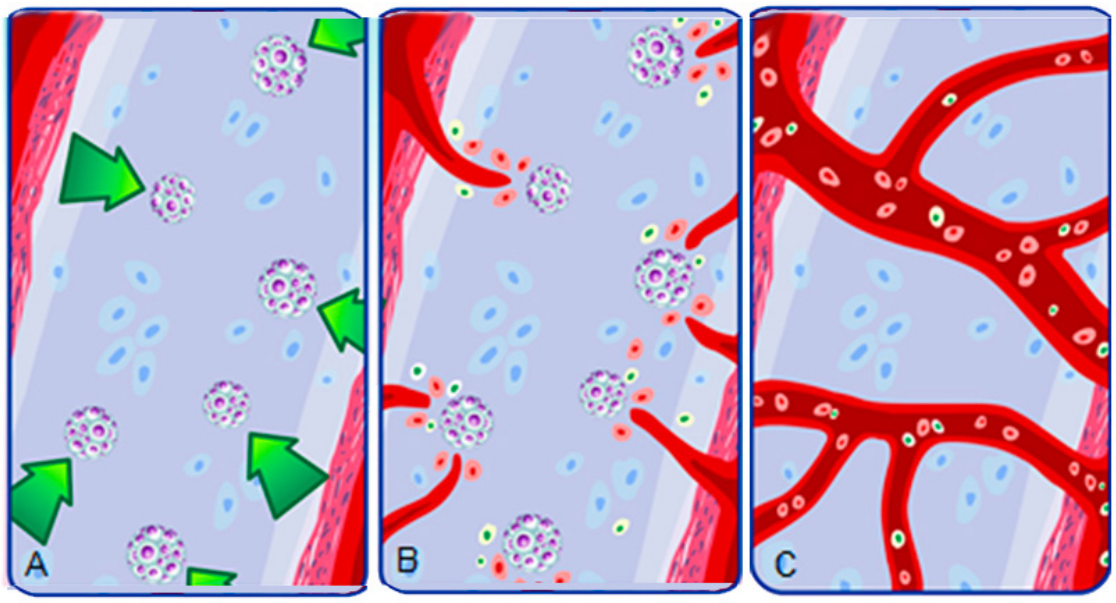

Figure 5. Spheroids as an *in vivo* model of endochondral ossification for efficient bone engineering. Spheroids pre-induced to the chondrogenic pathway are delivered at the implantation site (A). Spheroids attract blood vessels and progenitor cells *in vivo* (B), and the endochondral ossification process is initiated (C).

6. 3D BIOPRINTING OF SPHEROIDS INTO TISSUES AND ORGANS

The generation of complex human tissues and organs by bottom-up tissue engineering requires scalable techniques to fabricate spheroids of homogeneous size, and the development of integrated and automated robotics tools or organ biofabrication lines, to incorporate spheroids into constructs. For example, micromolded non-adhesive hydrogels allow the generation of uniformly sized spheroids that can be incorporated into constructs using a robotic dispenser, as mentioned above (78). The resulting spheroids, biofabricated in large scale, must be subjected to a quality control protocol. We propose that this protocol should include as key quality control elements an analysis of bone biomarkers in the spheroid supernatant, as well as mechanical testing (Figure 6). Our research group have performed cyclic compression assays (using a MicroSquisher, from CellScale) to measure the physical resistance of spheroids of ASC previously induced to bone and cartilage, and we also analyzed by proteomics, the spheroid of ASC secretome, which contained proteins associated with osteogenesis, such as osteonectin, tenascin C and osteoglycin (manuscript in preparation).

Tri-dimensional bioprinting is a novel additive manufacturing technology currently used by many groups to produce complex engineered tissues from modular components or 'building blocks'. This relatively new technology has attracted increasing attention due to its immense potential for scalable tissue construct production. Spheroids could be used as building blocks to assemble functional tissues or organs by 3D bioprinting, if incorporated into a 'bioink'. In this model, spheroids are expected to accelerate tissue formation and maturation through the fusion mechanism. Alternatively, cells could be printed in a hydrogel that would act as the 'paper' in the bioprinting process, contributing for the assembly of spheroid building blocks (79,80) (Figure 7).

The fusion of homogeneous spheroids using a bioprinter, which involves the presence of adhesion molecules, could be done by dispensing spheroids continuously inside one cylindrical non-adhesive support, where they would be spontaneously arranged. In this context, ASC or MSC could be used to produce spheroids of cartilage, bone or adipose tissue, when an appropriate inductive medium is employed and, after their fusion using a bioprinter, the constructs formed could be used in a variety of pre-clinical and clinical studies. The ability of spheroids to fuse to each other may vary depending on the level of spheroid maturation, possibly due to matrix production and rearrangement, or mineralization. For example, spheroids from human cartilage progenitor cells (CPC) cultured for 21 days undergo spontaneous chondrogenic differentiation, but have reduced capacity to fuse compared with CPC spheroids cultured for 2 days, when no differentiation has occurred (81).

Many challenges need to be addressed for the successful production, in the future, of tissue and organ constructs by 3D bioprinting in a cost-

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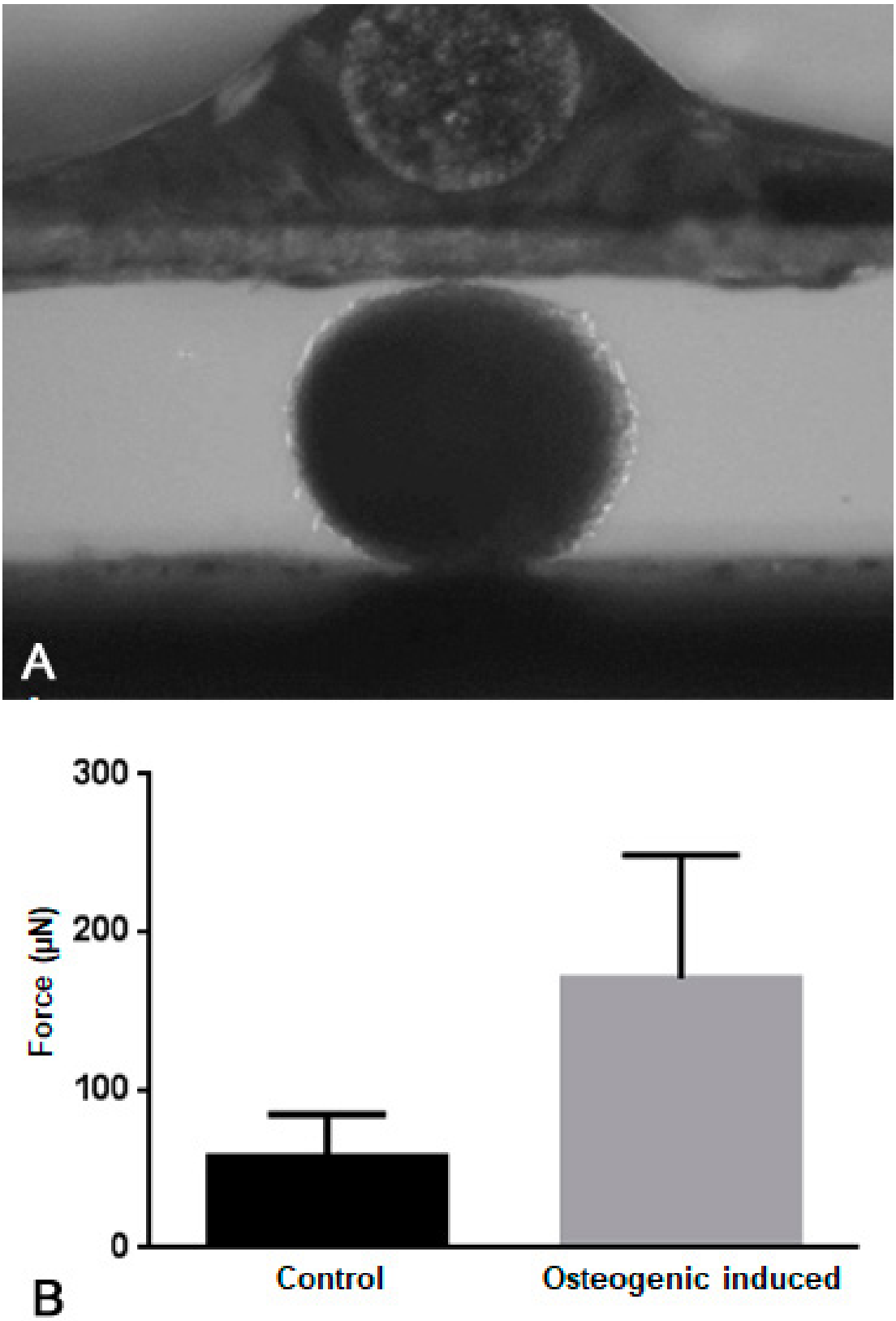

Figure 6. Mechanical resistance of spheroids of ASC induced to the osteogenic pathway. Note an induced spheroid of ASC between the two plates of the Microsquisher mechanical test system (CellScale), during a compression assay (A). Force measurement (in μN) of spherois left untreated (control) or induced to the osteogenic pathway (B).

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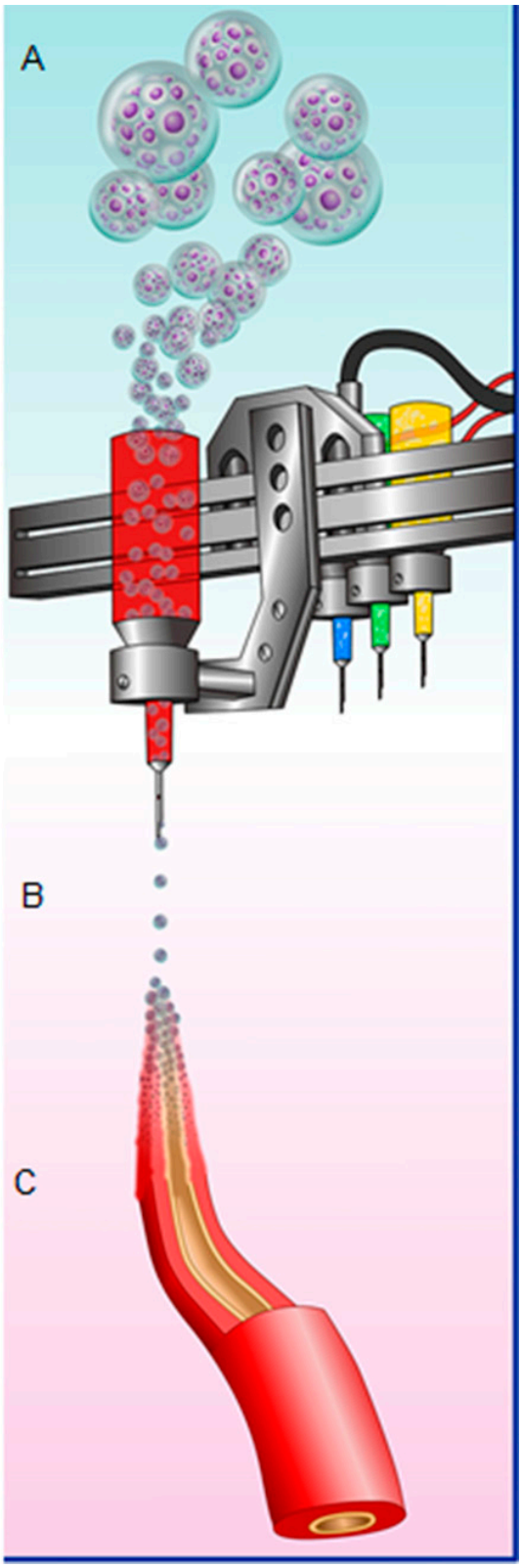

Figure 7. Spheroids as building blocks for 3D bioprinting. Spheroids are first encapsulated into a hydrogel (A), and then dispensed close to each other by a 3D bioprinter (B). Then, spheroids fuse to one another and form a more complex tissue structure, in a pre-defined shape (C).

effective and scalable manner. The establishment of biofabrication research centers for organ printing requires sophisticated hardware and software - including cell sorters, spheroid biofabrication equipment, bioprinters, rapid prototyping machines and bioreactors - as well as strong intellectual expertise (particularly in mathematical modeling) to support such as complex task (35).

7. CONCLUSION: SUMMARY AND PERSPECTIVES

The inadequate healing of fractures and the complications associated with autografts and allografts severely limit bone tissue regeneration, and highlight the impotance of developing new tissue engineering approaches to promote bone regeneration (82). While classical top-down tissue engineering improved considerably our understanding of bone mechano-physiology (83), it has many disadvantages, including poor cell viability, non-homogeneous cell seeding (26), and difficulties in controlling the mechanical and physical properties of the scaffold (36). Bottom-up tissue engineering - which relies on the biofabrication of microscale tissue building blocks with high cell densities and elevated metabolic activity - has the potential to overcome some of the drawbacks of the top-down approaches.

Bottom-up approaches using spheroids produced by different methods has become an increasingly popular choice in bone and cartilage tissue engineering, and involves the *in vitro* production of templates for bone and cartilage tissues, for subsequent implantation *in vivo*. A new perspective in this field is the use of spheroids as a model for endochondral ossification, also known as developmental engineering, which has the potential to improve the treatment of frequent bone diseases such as osteoarthritis and osteoporosis. Pre-clinical studies using spheroids showed *in vivo* regeneration of osteochondral defects, since spheroids can produce high concentrations of pro-angiogenic factors, promoting vascularization. However, additional studies are necessary to improve the techniques for spheroid use in bone tissue regeneration, and to provide long-term regeneration analysis.

Finally, spheroids can be used as building blocks for 3D bioprinting of tissue and organ constructs with clinically relevant dimensions (78). Spheroids of ASC or MSC can support the production of autologous bone with high complexity *in vitro*, and with physical and mechanical properties similar to those of native tissues. However, the engineering of complex tissues by 3D bioprinting is still in its infancy, and further technological developments are required to allow 3D bioprinting using spheroids of ASC to become a reality in tissue engineering and bone therapy.

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Abbreviations: MSC: mesenchymal stem cells; Col2a1: collagen type II alpha-1 chain; FGF: fibroblast growth factor; TGF-β: transforming growth factor β; BMP: bone morphogenetic protein; ASC: adipose stem cells; ALP: alkaline phosphatase; β-TCP: beta-tricalcium phosphate; PCL: polycaprolactone; PLA: polylactic acid; PGA: polyglycolic acid; VEGF: vascular endothelial growth factor; bFGF: basic fibroblast growth factor; PIPAAm: N-isopropylacrylamide; RGD: Arg-Gly-Asp; IGF: insulin growth factor; CPC: human cartilage progenitor cells

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