

Regenerative Medicine Approaches for Osteoarthritis

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ABSTRACT

The progression of Osteoarthritis (**OA**) leads to the continuous degradation of articular cartilage, which in turn, eventually results in the loss of joint functionality. At the moment, OA has no cure. Tissue Engineering (**TE**) is one the most promising options to regenerate lost cartilage and try to prevent or postpone joint replacement surgery. Many different approaches to cartilage TE can be found in the literature, but up to now very few have attempted to reproduce cartilage characteristic zonal architecture. Next generation TE approaches need to address this. Further, TE studies should employ conditions similar to those in OA cartilage, i.e. an inflammatory, hypoxic environment and physical stimulation. Emerging techniques such as novel proteomic approaches and high throughput screening will surely lead to the identification of novel molecules with chondrogenic potential and improved characterization of the TE construct. In addition, there is still a need for a consensus score system that allows direct comparison among different approaches. Notwithstanding, there are several ongoing clinical trials being conducted to test the application of stem cells for cartilage regeneration, yet no randomized studies for OA have been performed yet. While some indicate promising results, there are still many challenges to be addressed to bring cartilage TE to the clinic.

Keywords: Osteoarthritis; Cartilage; Tissue engineering; Mesenchymal stromal cells; miRNA; Platelet rich plasma; High throughput screening

INTRODUCTION

Articular Cartilage Tissue

Articular cartilage is a highly organized tissue located at the ends of long bones in diarthrodial joints. It enables proper joint movement by providing a lubricated, load-bearing, and energy dissipation environment. It contains a single cell population, the chondrocyte. These cells are responsible for synthesizing and maintaining cartilage macromolecular Extracellular Matrix (ECM). This matrix is composed by charged molecules organized in complex fibrillar networks, which are also responsible for the transduction of chemical and mechanical signals from the surrounding tissue to the chondrocytes providing cartilage well known mechanical and lubrication properties. [1,2].

In human adult articular cartilage, the matrix composition, cell morphology, density and expression of specific markers is influenced by its zonal architecture: superficial, middle, deep, and calcified zones (Figure 1).

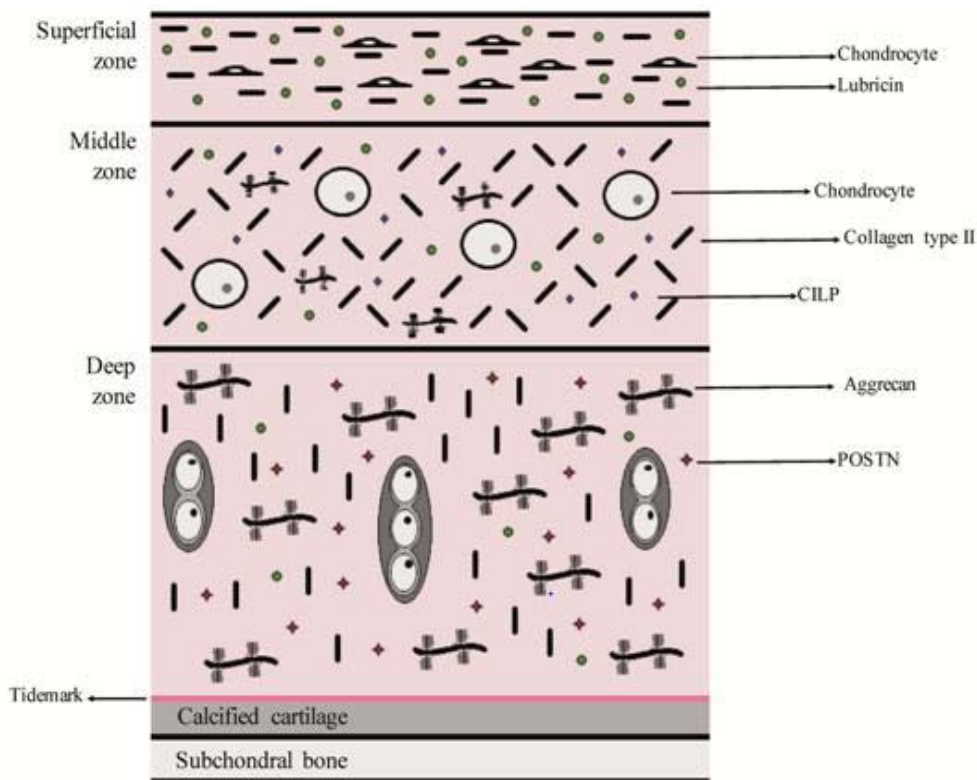


Figure 1: Articular joint, cartilage and bone. Cartilage different zones, and collagen and aggrecan distribution with zonal proteins are shown.

In the Superficial Zone (**SZ**), flattened chondrocytes can be found at high density, and are surrounded by an ECM rich in collagen fibrils, predominantly of type II, oriented parallel to the surface. SZ cells synthesize and secrete a specialized protein named lubricin or Proteoglycan 4 (**PRG4**) which plays a critical role in lubrication and wear protection. Although initially thought to be located uniquely in the cartilage superficial zone and in the synovial fluid, a few PRG4 positive cells can be found in cartilage Middle (**MZ**) and Deep Zones (**DZ**). Another potential marker for SZ chondrocytes is the production of clusterin that has been suggested to regulate chondrocyte apoptosis in OA tissue [3,4].

Moving to cartilage mid-zone, chondrocytes are round and synthesize a large amount of aggrecan. Here the ECM is rich in Hyaluronan (**HA**), dermatansulfate and type II collagen fibrils that become more randomly distributed. Cartilage Intermediate Layer Protein (**CILP**) is uniquely expressed in MZ and may be involved in mediating interactions among ECM components [5,6]. Finally, deep zone chondrocytes are spherical and larger; they present lower density and are organized in columns. Collagen fibrils become thicker and are distributed perpendicular to the joint. The lowest part of the DZ is partially calcified and a tidemark (as typically seen in a hematoxylin-eosin stain) represents the interface between non mineralized articular cartilage and calcified tissue. Periostin (**POSTN**) is expressed in DZ chondrocytes and interacts with integrin receptors to promote signal transduction. Additionally, type X collagen, a hypertrophy marker and indicator of endochondral ossification can also be found in the ECM of deep zone layers [7].

It is well known that adult articular cartilage has a very limited ability for self-repair as well as a low turnover rate, so any alteration of the ECM or degradation of its components, can lead to chondrocyte dedifferentiation, affecting cartilage homeostasis. These alterations make this tissue particularly vulnerable to degenerative processes and the development of pathological conditions such as osteoarthritis.

Rheumatic Diseases and Cartilage Damage

Osteoarthritis (**OA**) is the most prevalent rheumatic disease reaching up to 40% of people over the age of 70, and is expected to become the world's fourth leading cause of disability by 2020. The pain and physical disability that accompany this condition mean that a quarter of OA patients will be unable to perform major activities of daily life, severely affecting their life quality and expectancy [8]. The new standardized definition of the OA disease proposed by the OA Research Society International (**OARSI**) states that "Osteoarthritis is a disorder involving movable joints characterized by cell stress and extracellular matrix degradation initiated by micro- and macro-injury that activates maladaptive repair responses including pro-inflammatory pathways of innate immunity. The disease manifests first as a molecular derangement (abnormal joint tissue metabolism) followed by anatomic, and/or physiologic derangements (characterized by cartilage degradation, bone remodeling, osteophyte formation, joint inflammation and loss of normal joint function), that can culminate in illness" [9].

There are three main types of cartilage injury which can initiate a degenerative condition and lead to an OA disorder: superficial matrix disruption, partial and full thickness defects. Superficial matrix disruption arises from blunt trauma in which the ECM is damaged but viable chondrocytes can aggregate into clusters and are capable of synthesizing newmatrix. Partial thickness defects disrupt the cartilage surface but do not extend into the subchondral bone. These defects, unlike superficial matrix disruption, cannot be self-repaired. Lastly, full thickness defects arise from damage that penetrates deep into the subchondral bone. These result in the recruitment of cells from the bone-marrow inducing the formation of a fibrocartilaginous repair tissue [10].

Despite advances in our understanding of cartilage metabolism, OA progress and treatment strategies, only a few disease-modifying osteoarthritis drugs (**DMOAD**) have shown possible efficacy. Efficient strategies for the development of new modifying therapies with chondroprotective effects, halting disease progression or reparative effects on degenerative articular joints are crucial as so far no regulatory agency has approved a therapy that is a DMOAD [11]

There are a few existing surgical treatments focused on pre-OA cartilage defects but none has accomplished the formation of a neo-cartilage tissue that is fully integrated, hyaline-like in composition and presents a zonal architecture [12]. Eventually, the ultimate solution for many OA patients is joint replacement surgery. Further, fewer approaches have attempted to treat the osteoarthritic joint, facing the challenge to repair larger defects or resurfacing the entire OA surface [13]. As OA-affected population is estimated to be much higher than that affected by focal lesions, there is an urgent unmet need for scalable regenerative solutions that could face the raising human burden and economic cost of this disease.

CURRENT STRATEGIES FOR CARTILAGE REPAIR AND REGENERATION

Cell-based

Most of the tissue-engineered approaches that attempt to repair or regenerate articular cartilage are cell-based. A 20-year follow-up of Autologous Chondrocyte Implantation (**ACI**), introduced in 1994 [14], has demonstrated its efficacy and durability, although several limitations arise from the use of chondrocytes, such as reduced availability, low proliferative capacity and dedifferentiation *in vitro* [15].

Mesenchymal Stromal Cells (**MSCs**) have clear therapeutic potential for joint tissues as they constitute an abundant cell source, possess multipotent differentiation capacity and secrete a variety of cytokines and growth factors that confer them anti-fibrotic, anti-apoptotic, pro-angiogenic and immunosuppressive properties [16,17]. MSC-based therapy may act via different ways. MSCs are known to have immunoregulatory and anti-inflammatory properties, but they also prevent cartilage degradation through secretion of bioactive factors and can differentiate

into chondrocytes, thus contributing to cartilage repair. MSCs can be transitioned into chondroprogenitor-like cells, mimicking *in vivo* chondrogenesis, which represents a stage-specific sequence of events similar to the earlier skeletal development, through activated molecules. However, the requirements and conditions for effective induction of MSCs *in vitro* chondrogenesis and the production of a stable cartilaginous tissue are far from being understood [18].

Well-established chondrogenesis full inducers include three known members of the transforming growth factor family (TGF- β 1, 2 and 3) [19,20]. Other growth factors such as bone morphogenetic proteins (BMP2, 4 and 6) and the insulin-like growth factor (IGF1) have been regarded as promoters of chondrogenesis, when combined with TGF β , rather than inducers, as their action has not yet been confirmed [21]. Recent studies have also focused on the use of Parathyroid Hormone-related Protein (**PTHrP**) or matrix Gla Protein (**MGP**) which are thought to act as inhibitors of bone formation and cartilage calcification [22,23].

Human Induced Pluripotent Stem Cells (**iPSC**) have distinct abilities to self-renew indefinitely when cultured *in vitro* and to differentiate into any cell-type in the organism, including chondrocytes [24]. iPSC- derived MSC-like cells have emerged as a valuable cell source to be explored as an alternative to patient-derived MSC in cases where their chondrogenic potential may be compromised due to donor age or condition. In addition, iPSC provide a powerful and flexible platform for dissecting *in vitro* the molecular mechanisms that regulate chondrogenic differentiation under normal or pathological (i.e. using patient-specific iPSC) conditions [25].

Combining micro-RNAs Targeting with MSCs Technology

Increasing MSC therapeutic efficiency through the modulation of micro-RNAs (miRNAs) represents a novel therapeutic opportunity for OA treatment in which miRNA alterations could be corrected by either antagonizing or restoring miRNA function [26]. In one hand, modulation of miRNAs could enhance MSCs anti-inflammatory properties and their ability to suppress T-cell proliferation and on the other hand, miRNAs could be used to promote the chondrogenic differentiation potential of autologous MSCs for transplantation into articular cartilage defects [27].

Several studies have been performed reporting the expression profile of miRNAs in MSCs, generally by the comparison of healthy and pathological conditions [28]. Also, recent reviews have discussed the role of miRNAs in regulating chondrogenesis by targeting transcription factors and growth cytokines, and that could also participate in OA pathogenesis [28] (Figure 2).

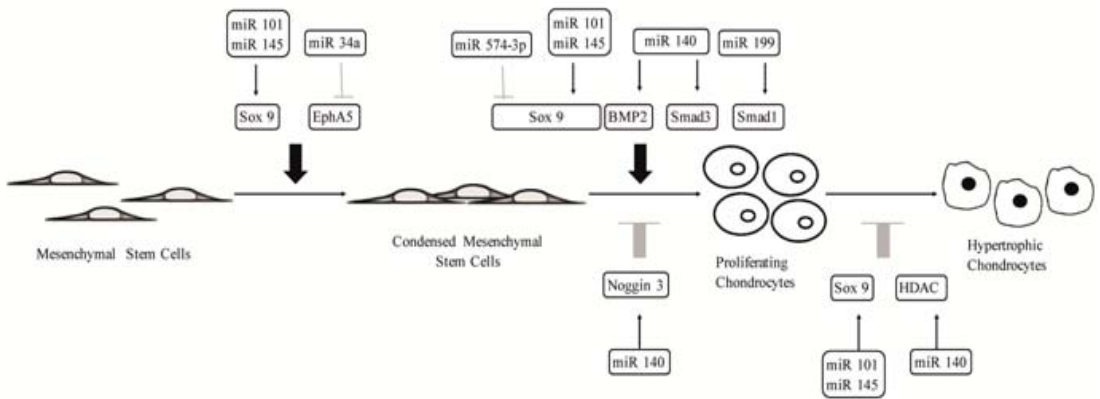


Figure 2: miRNA involvement in chondrogenesis and OA. Genes of interest: Sox9, EphA5 (Ephrin receptor A5 participate in development events), BMP2, Smad 3 and 1 (Transducers and transcriptional modulators), HDAC (Histone deacetylase) and Noggin 3 (binds and inactivates members of the transforming growth factor-beta superfamily signaling proteins).

One example is miR-34a, a negative modulator of chondrogenesis, particularly in chondroblast migration. Silencing miR-34a can prevent chondrocyte apoptosis, suggesting its involvement in cell apoptosis. In addition, miR-34a expression has been found up-regulated in human OA cartilage [29].

Other miRNAs have been frequently identified as involved in the regulation of chondrogenesis, such as miRNA-140, -199 and -574-3p [30]. Moreover, miR-101 participates in the interleukin (IL)-1 β -induced down regulation of collagen type II and aggrecan preventing IL-1 β -induced chondrocyte ECM degradation. Additionally, miR-145 expression is gradually reduced during transforming growth factor- β (TGF- β)-induced chondrogenic differentiation. SRY (sex determining region Y)-box 9 (**Sox9**) has also been identified as a miR-145 target in human chondrocytes. Thus, the attenuation of miR-145 expression can positively regulate Sox9 expression, resulting in the promotion of chondrogenic differentiation [31].

Some technical challenges should be solved before developing satisfactory MSCs-miRNA-based combined therapies for OA: miRNAs are highly soluble and have a short life so the fabrication of optimized controlled delivery systems is crucial; these systems could also allow the administration of several combined miRNA that could be more efficient than a single miRNA. The regulation of miRNAs might have carcinogenic effects, as miRNAs act as oncogenes, so side effects of their use must be considered before clinical use [32].

It is clear that deciphering the role of miRNA regulation in the chondrogenesis of MSCs may pave the way for the development of new treatments, but more evidence is needed since so far no data on the use of miRNAs in cartilage engineering or OA has been reported, and studies of miRNA-based tissue engineering are still rare.

Drug Screening

Despite the fact that considerable advances have been made in MSC-derived chondrogenesis, the signal transduction and molecular mechanisms involved in this process have not been fully elucidated. New technology such as High Throughput Screening (**HTS**) has emerged allowing the discovery of unexpected signaling pathways as well as the identification of novel compounds for the modulation of the MSCs chondrogenic differentiation process. HTS is a process that allows the screening of thousands of chemicals to identify potential compounds of interest for a specific application. Basically, it involves a primary screening for the selection of compounds (**HTS**) and a dose-response analysis and validation. After this a compound is usually considered as a lead and can then be further tested as potential drug candidate [33].

Although most HTS published studies have been performed in two-dimensional (**2D**) systems, for chondrogenesis studies it would be preferable to use three-dimensional (**3D**) systems that resemble mesenchymal condensation that occurs in the first steps of MSCs differentiation. Although this can be performed using conventional pellet-systems, the potential of novel super hydrophobic chips for this application should also be explored [34,35].

Recently, kartogenin and TD-198946 have been identified as stimulators of the chondrogenic process of bone marrow derived-MSCs [36,37]. Kartogenin acts via a novel biological pathway involving binding to filamin A and disrupting its interaction with the transcription factor Core-binding Factor β subunit (**CBF β**) which modulates Runx1. It was suggested that kartogenin may modulate endogenous stem cells to confer a regenerative/repair effect and/or a protective effect [36]. On the other hand, TD-198946 was the first small molecule compound to have both reparative and preventive effects in a mouse degenerative articular cartilage model. The authors propose that this compound exerts its effect by regulating Runx1 expression, a known inducer of chondrogenic differentiation and a suppressor of hypertrophy [37]. Both studies show the potential of HTS to identify new biological pathways and small molecules that could be used to promote the repair of degenerative articular joint.

Scaffold-Based

In recent years, natural and synthetic biomaterials with biomimetic clues have been used to create niches or microenvironments to control stem cell behaviour and differentiation towards cartilage formation trying to control cell-matrix interactions. Yet classical cartilage tissue engineering strategies do not mimic the structural organization or the zonal properties of articular cartilage and instead are focused on producing an homogenous tissue with bulk properties that resemble those of native cartilage [13].

Different authors have described how manipulating material composition, structure and/or degradability, using different combinations of synthetic Poly Ethylene Glycol (**PEG**), Poly (L-lactic acid) (**PLLA**) with natural origin materials (chitosan (**CHT**) or Chondroitin Sulphate (**CS**)) can

provide unique cues or create niches that influenced cell morphology, spreading and migration and chondrogenic differentiation [38,39]. Thus, appropriate control of the culture conditions and environmental signals through material compositions could provide guided differentiation of MSCs into the various zonal phenotypes of articular chondrocytes mimicking its organized architecture, as in normal articular cartilage [6].

Another challenge associated to cartilage regenerative strategies is the lack of optimal integration between the neo-tissue formed and native cartilage. Ideally, an adhesive should, at least temporarily, be part of the reconstructive scaffold and share properties of the host tissue. A multifunctional modified CS has been proposed by Elisseeff and co-workers, by chemically functionalizing both methacrylate and aldehyde groups of the CS molecule to form two functional arms that can chemically bridge biomaterials and tissue proteins present at the cartilage surface, via a Schiff's base linkage [40]. Finally, it is important to highlight the need to test tissue-engineered constructs in relevant experimental conditions that better resemble those of the osteoarthritic joint, such as an inflammatory environment, hypoxic conditions and under physical stimulation [18].

PLATELET-RICH PLASMA (PRP)-DERIVED SCAFFOLDS

PRP is, by definition, a volume of autologous plasma that contains a higher platelet count than peripheral blood. Platelets contain more than 800 proteins and molecules in their cytoplasmic granules, including cytokines, chemokines, membrane proteins, metabolites, messenger molecules, GF and other soluble proteins [41]. Platelet activation also leads to fibrinogen polymerization resulting in a fibrin clot. These two events are the basis for the therapeutic use of PRP as a TE material, that tries to reproduce natural wound healing and tissue reconstruction by providing a temporary scaffold and releasing bioactive molecules locally. PRP-derived TE products for cartilage regeneration are also unique in that they can be completely autologous. Figure 3 includes an overview of the different methods of PRP preparation.

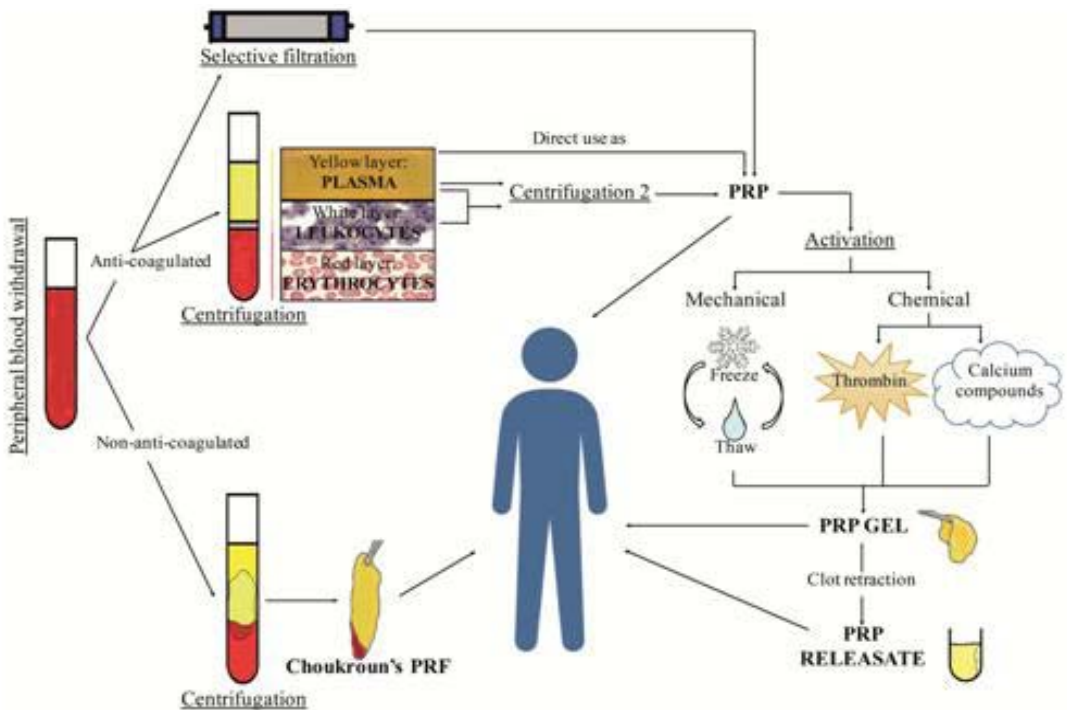


Figure 3: Overview of PRP preparation methodologies. Centrifugation of non-anti-coagulated blood results in Choukroun’s Platelet-Rich Fibrin (**PRF**). Anti-coagulated blood can be filtered or centrifuged (once or twice) to obtain leukocyte-depleted or leukocyte-rich PRP, which can, in turn, be applied directly or activated to obtain a gel or the releasate.

PRP application has been validated with *in vitro* assays, preclinical studies and clinical trials for oral and maxillofacial surgery, chronic ulcers, ophthalmology, dermatology and injuries and pathologies of tendon, muscle, cartilage and bone, among other fields [42,43]. As for OA, several preclinical and clinical trials have already tested PRP therapeutic potential, especially as an anti-inflammatory agent for infiltration procedures [44].

Notwithstanding, there is still a lack of clear experimental proof that PRP-derived fibrin hydrogels can provide suitable scaffolding for OA cartilage regeneration. In fact, PRP-derived fibrin hydrogels present a series of drawbacks, such as poor mechanical properties, difficult handling and random quick hydrolysis, which reduce their applicability. Yet, PRP *in situ* clotting would otherwise achieve an almost perfect integration with native cartilage, which, as mentioned above, is a known problem for preformed scaffolds. Therefore, attempts are being made to overcome PRP limitations and improve its stability and mechanical properties. Approaches such as cross linking PRP with genipin [45], ruthenium-catalyzed photo cross linking [46], addition of fibrinolysis inhibitors [47], or fibrinogen concentration adjustment [48] have been tested. Moreover, PRP can be combined with other biomaterials to provide the desired cartilage-like stiffness [49,50].

On the other hand, European Directive 2002/98/CE [51] that establish PRP quality and security manufacturing standards, as well as strict health requirements does not define PRP characteristics or platelet dose for different indications. As a result, numerous and diverse PRP preparation systems have been developed and, accordingly, PRP composition is widely variable, complicating comparisons between studies [52]. Consequently, concentrations of cytokines and plasma and platelet GF are disparate. All these parameters affect tissue biological response and hence PRP treatment efficacy.

CHARACTERIZATION OF *IN VITRO/EX VIVO* NEO FORMED CARTILAGE

As varied as the different TE approaches to regenerate cartilage are the assays and techniques used to characterise the neo formed cartilage (For a summary see Figure 4).

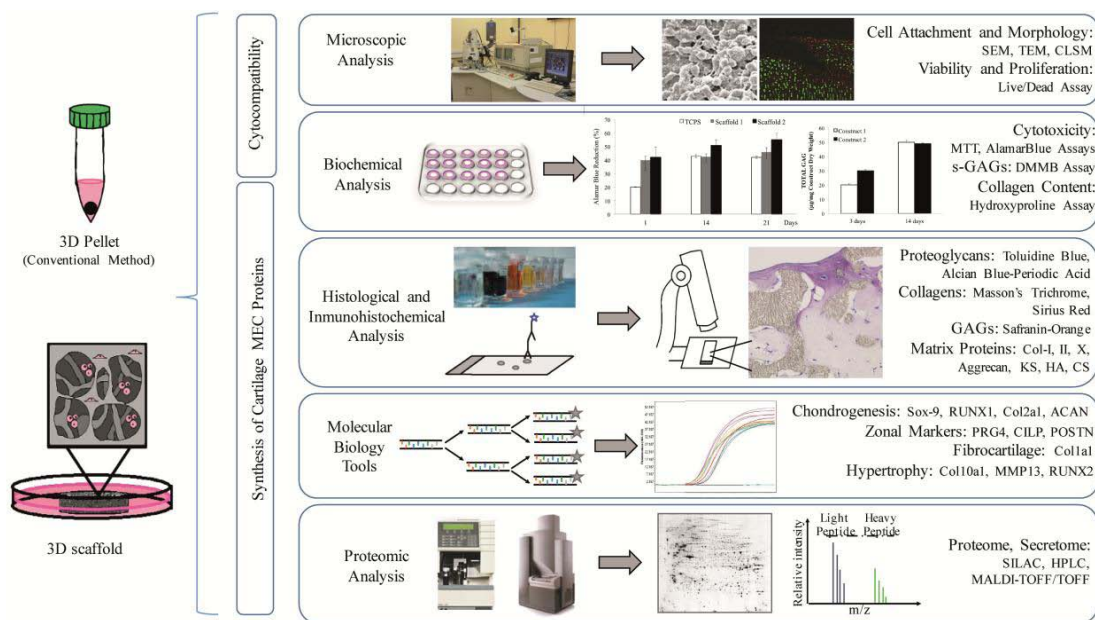


Figure 4: Summary of characterization techniques. SEM: scanning electron microscopy; CLSM: confocal laser scanning microscopy; TEM: transmission electron microscopy; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMMB: dimethyl methylene blue dye; SILAC: stable isotope labeling by amino acids in cell culture.

On a very initial base, scaffolds must be screened for their cytocompatibility with different cell types. Alamar blue dye assay can be easily used and allows a proliferation follow up during culture time. Microscopical analysis with fluorescent confocal laser scanning microscopy (CLSM) or with scanning electron microscopy (SEM) can be used to visualize cell proliferation and neocartilage morphology.

As mentioned, cartilage's most abundant proteins are collagen type II, Proteoglycans (**PGs**), such as Aggrecan (**Agg**) or versican, and Glycosaminoglycans (**GAGs**), such as Keratan Sulfate (**KS**), Chondroitin Sulfate (**CS**) and Hyaluronic Acid (**HA**). Therefore, most *in vitro/ex vivo* experimental cellular or TE models to repair cartilage aim to achieve a significant synthesis of these molecules. Histology and Immunohistochemistry (**IHC**), through the use of specific stains and antibodies, respectively, are the gold standard to detect them and evidence their synthesis [53]. At least one standardized grading scale has been developed for TE cartilage [54]. Proteoglycan and collagen type II synthesis have been commonly determined by radiolabelling with $^{35}\text{SO}_4$, L-[2,3- ^3H] proline and [^3H] hydroxyproline, which were respectively assumed to be proteoglycan, total protein and collagenous protein (hydroxyproline concentration is estimated to be 10% of collagen) [55,56]. Total GAGs content can be determined using the Dimethyl Methylene Blue dye (**DMMB**), which can be detected spectrophotometrically and through comparison to a calibration curve with CS standards [57].

More quantitative techniques, such as the Polymerase Chain Reaction (**PCR**) are now common place in virtually all cell laboratories. PCR and, in particular, Quantitative Real Time (**qRT**) PCR can detect small numbers of RNA fragments providing information about the relative, or even absolute, expression of the genes of interest in the experimental conditions. Molecular biology tools are quite established techniques to follow MSCs differentiation or chondrocyte phenotype maintenance in cartilage regeneration strategies. However, not all mRNA that is transcribed is translated into protein, and gene expression levels do not necessarily predict protein abundance because of alternative transcriptional and translational steps and the occurrence of protein degradation processes. Proteins also undergo posttranslational modifications before they can perform their intended functions. On the other hand, histology and IHC provide qualitative estimates of synthesized protein, but are not very reliable as quantification methods. They can be used to compare intra-experiment conditions, but not to quantify absolute abundance. Because of this, biochemical assays that quantify newly synthesized protein are also widely performed. Proteomic analyses are emerging as a relevant tool-set to follow protein interactions that are often essential to their biological activity and to elucidate the true picture of what is happening inside the newly formed tissue [58].

In summary, the best way to characterize the neoformed cartilage, regardless of the regeneration approach, would be to use a combination, as complete as possible, of the above mentioned techniques (Figure 4). An excellent example of a paper accomplishing this was published by Estes et al. in 2010[59].

CLINICAL OVERVIEW OF CELL THERAPY AND TE PRODUCTS FOR CARTILAGE

Several clinical trials (Phase 1-3) are being conducted to test the application of stem cells for regenerating cartilage. Most of them are focused in repairing cartilage defects or in treating

degenerative damage in knee, ankle, or hip OA. Still, in OA no randomized studies have been performed yet. Ongoing clinical trials on stem cell and biomaterials therapy can be found in clinicaltrials.gov resource from National Institutes of Health (NIH), which is frequently updated. Also the activities in Europe in the area of cellular and engineered tissue therapies are being regularly collected by the efforts of Martin and colleagues. In its sixth report they have identified musculoskeletal/rheumatological disorders as the main indication for these therapies (45%; 89% autologous) [60].

Different approaches being tested include: 1) Intra-articular injection of adult MSCs, either directly after collection or after expansion and culture during 2-4 weeks. Three sources of stem cells are used: bone marrow-, adipose-, and umbilical cord blood-derived MSCs. 2) MSCs with scaffolds for implantation. Different biomaterials have been described based on collagen I, collagen hydroxylapatite or decellularized human donor scaffolds.

Most of the reported studies indicate the presence of a hyaline-like cartilage repair tissue within the damaged area of cartilage defects. Still some challenges have been pointed out by different authors: quality and durability of the repair tissue, resistance to endochondral ossification, effective integration with the surrounding host tissue. In addition, other challenges regarding MSCs heterogeneity, the complexity of cell isolation, *in vitro* culture and transplantation are not solved. It is unclear the radiographic stage that would be optimal for MSC infusion although lesions of large size have been associated with poor outcome [61].

Nonetheless preliminary results intra-articular injection of MSC to treat OA joint and MSC combined with scaffolds to treat traumatic focal cartilage defects are promising [62].

CONCLUSION

Regenerative medicine approaches offer great promise to achieve regeneration of hyaline cartilage. The emergence of novel tools such as high throughput screening and proteomic techniques will improve our understanding of the molecular mechanisms, and their inducers, to accomplish stable chondrogenesis and matrix formation. Clinical trials are underway to test the safety and efficacy of some of these approaches before they can be translated to the patients.

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