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Is the Articular Cartilage Regeneration Approachable Through Mesenchymal Stem Cells Therapies?

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1. Introduction

Today great hope is set on regenerative medicine in all medical fields. Leland Kaiser introduced the term “Regenerative Medicine” in 1992. He forecasted that a “new branch of medicine will develop that attempts to change the course of chronic diseases and in many instances will regenerate tired and failing organ systems” (Kaiser, 1992). Since then, scientists all over the world try to develop cell-based approaches to regenerate damaged tissues, or even substitute whole organs (Ehnert et al., 2009).

Degenerative disease of articular cartilage (AC), generically known as osteoarthritis (OA), is an irreversible evolution process towards terminal articular failure. Due to its high prevalence on population and its socioeconomic impact, this condition is of great concern, and this way more resources and effort are dedicated to the research on its development. Cartilage tissue engineering seeks to combine cells, biomaterial scaffolds, and bioactive signals to create functional tissue replacements to treat cartilage injuries or osteoarthritis (Song et al., 2004).

Cartilage degenerative disease, OA, is the end stage of several conditions such as trauma, inflammatory diseases, overweight etc. The fatalistic theory that states that it is impossible to recover the cartilage once it has been damaged leads to the assumption that the progression to any form of OA is unavoidable (Fig. 1). The annual incidence of young adults suffering any cartilage injury in UK is 10000 and this figure is continuously increasing (NICE, 2008).

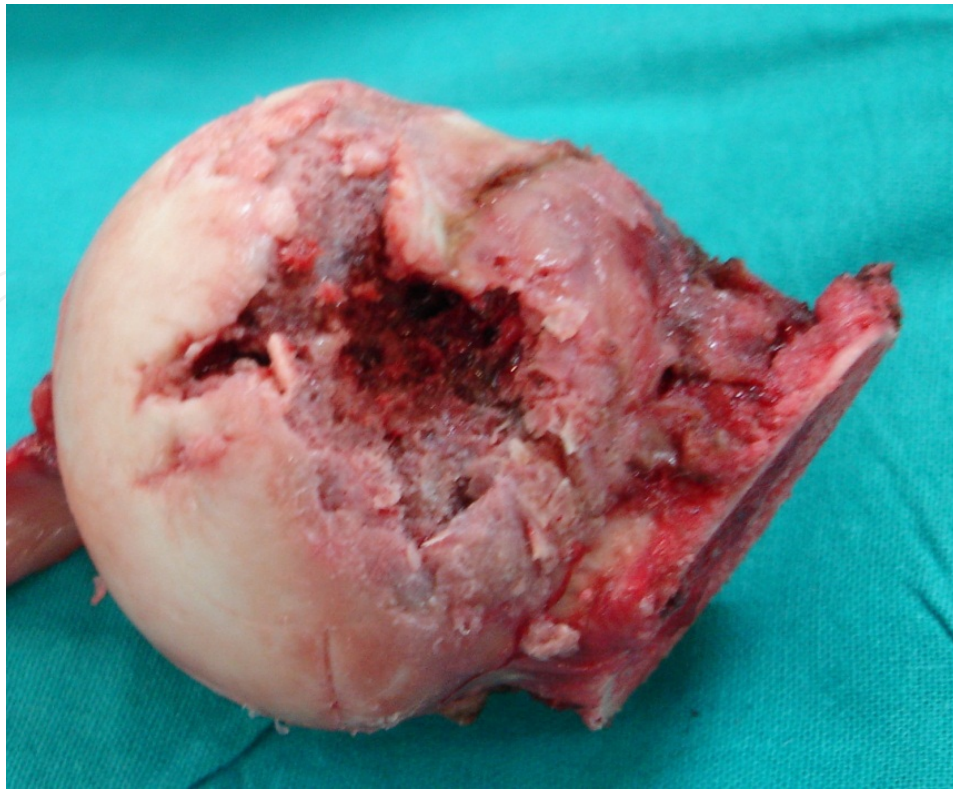


Figure 1. Osteochondral injury in a femoral head from a surgical intervention in our hospital

The new patient does not accept a reduction in his demands and quality of life because of the OA as the culmination of an articular injury during sport activity. On the other hand, a higher risk for developing degenerative disease and obesity, mainly knee arthritis, has been correlated. The symptomatic cohort of pain, swelling, range of motion diminution and loss of quality life can only be partially recovered by a total joint replacement (Fig. 2).

Unfortunately, joint replacement is neither a procedure free of complication nor a forever-realistic solution. It is expected that by the year 2030 the number of total hip or knee replacement implanted annually will be respectively 1.8 and 7 times the current figures (Kurtz et al., 2007). It is doubted that such an economic impact could be ever be afforded. Finding an alternative option to manage these lesions will be a challenge and this must be closer to achieve an almost AC tissue able to bear the requirements of an active long period of life.

AC is a quite simple structure with scarce cellularity within an extracellular matrix (ECM). However, in spite of this simplicity, its structure and performance is very complex.

The ability of the AC to heal these injuries on its own is almost none. The lack of blood supply is the main handicap this tissue has at the time of healing. Without vessels the preliminary inflammatory step of the healing process is not possible. To start with, the dead tissue needs to be removed and the flood of new cells from the vascular stream is essential for this.



Figure 2. Treatment of a OA by total joint replacement performed in our hospital

Furthermore, the cell mediators will not appear as the granulocytes are not present and the degranulation of these is its source. Secondly, the chondrocytes are well-differentiated cells with a limited capacity to produce ECM. This is sufficient to cover the necessities of a still cartilage turnover but not to cope with the healing of a traumatic defect.

In any case, if the trauma produces a lesion that breaches the subchondral plate, it allows access to the vascular network and the final produced tissue is made of collagen type I, more resistant to tension, instead of type II, more resistant to compression.

1.1. How can this be sorted out?

Several attempts have been made in order to repair the traumatic defect and to achieve a regeneration of the original injured cartilage.

A first group of interventions can be described as “marrow stimulating techniques”. Drilling the defect beyond the subchondral plate is its essential and allowing a repair promoted by the bleeding from the subchondral bone creating a “super clot” its rationale (Mithoefer et al., 2005). The star cells here are the mesenchymal stem cells (MSCs) emerging with the hematoma and its subsequent proliferation and differentiation. Unfortunately, the final result is the promotion of a fibrous tissue not durable in time (Steret et al., 2004). Pridie et al. promoted this concept in 1959. Abrasion arthroplasty (Steret et al., 2004), microfracture (Steadman et al., 2003), or Autologous Matrix Induced Chondrogenesis (Gille et al., 2010) have been more recently developed with the same rationale.

In essence, previous treatments have attempted to promote the healing of the damaged reminding nature. That is to say, allowing the flood of blood from the inner areas of the subjacent bone. Unfortunately, this will promote a scar tissue that in the long run will be lost and the joint degenerative process will be stated (Kreuz et al., 2006).

In a second group of interventions, it is proposed the articular defect to be covered by a “replacement technique”. In these, the defect is reshaped to a standard cylindrical way and substituted with a plug of osteochondral graft harvested from a non-weight bearing donor site. Using this technique two handicaps can be appreciated: the limited available graft and the morbidity of the donor site. Additionally, the differences in characteristics between the donor and the receiver areas may impede a complete integration. This may be the reason why the clinical results have not been in accordance with the initial enthusiasm (Mishima et al., 2008). Both, auto- and fresh allograft (Gross et al., 2002) have been attempted with the name of mosaicplasty. With the aim of avoiding the donor site morbidity, synthetic reabsorbable scaffolds have been used to fill up the osteochondral defect. A “toast and butter” cylinder, engineered mimicking components, bone and cartilage. In contrast with the graft, this scaffold will have osteoconductive properties instead and eventually resorb in 12 months. The pseudo-cartilage now created will be poorly incorporated and the biomechanics will fail (Yasen et al., 2012).

The third group of proposed interventions are cellular-based. The aim in this alternative option is producing a regeneration of the cartilage mediated by the own patient chondrocytes. This is a two-stage procedure. During the preliminary intervention a biopsy from the cartilage is obtained and then the chondrocytes are isolated and cultured till the number of cells is about fifty folds. For the second intervention, *ex vivo* expanded chondrocytes are implanted in the damaged area. This procedure has evolved with the aim of getting a watertight seal environment in order to receive the chondrocytes and avoid the leakage. First, it was attempted a sheet of periosteum, then a collagen gel and a collagen membrane has been developed. But, are the delivered chondrocytes, the MSCs coming from the subchondral bone or the cells evading from the layer of periosteum the source for promoting the repaired tissue?. In spite of the rationale of this techniques, the autologous chondrocytes implant has not finally reached the expected results, the final tissue obtained was fibrous instead of AC (Tins et al., 2005) and hence, the OA is once more the undesirable expected long-term result (Hunziker, 2002; Temenoff et al., 2000).

2. Therapeutic interventions without active biologics

2.1. Bone marrow stimulation

First approaches to heal cartilage by *in situ* regeneration date back to 1959. Pridie technique was directed to BM cells recruitment to be used in cartilage defects by drilling small holes into the subchondral BM space underlying the damaged cartilage regions. It was improved later on by reducing the size of the perforations and being then called microfracture technique which is now a frequently performed and well-studied procedure (Steadman et al., 1999). This technique is based on the mechanism of mesengensis or capacity of the non-differentiated mesenchymal cells in choosing a determined phenotype as a response to inducing or GFs. A non-differentiated cell from the BM can be promoted to different cell types such as osteoblasts, with a later maturing to osteocytes, chondroblasts and chondrocytes, but also to endothelial cells, mesothelial cells, fibroblasts or adipocytes. It is a cell signalling process of local cytokines on local cells. In order to achieve all this, the surgical technique is based on drilling the subchondral plate to get bleeding and a superclot that will become a scaffold and supply cells and proteins, starting this way the physiological cascade of the chondrogenic cell differentiation. Other alternative techniques of BM stimulation to regenerate cartilage would be abrasion chondroplasty and in case the articular surface remained untouched, the retrograde stimulation technique. Cartilage defects are repaired only with fibrous tissue or fibrocartilage when using these methods, probably because the number of chondroprogenitors recruited from the BM is too small to promote the hyaline cartilage repair and results are often followed by degeneration of the repair tissue. This was used as an explanation for the observations of other studies that good short term results may be followed by deterioration starting about 18 months after surgery.

Clinical observations and theoretical considerations pointed towards several possible limitations of marrow stimulation techniques. The non-adhesive properties of the cartilage surface and the softness and shrinking of the superclot can lead to only partial defect filling and facilitate an early loss of repair tissue from the cartilage lesion. To avoid this, the treatment has been recently advanced into a matrix-supported technique in which the performed defect was stabilized in an additional way with a biomaterial. The microfractured lesion is covered with a collagen type I/III scaffold and it is called autologous matrix induced chondrogenesis (AMIC) (Kramer et al., 2006; Steinwachs et al., 2008). This technique has been developed to allow the treatment of larger defects by microfracturing and it is used as alternative treatment to autologous chondrocytes transplantation (ACT).

2.2. Autologous osteochondral transplantation: Mosaicplasty

Autologous osteochondral mosaicplasty, sometimes known as osteoarticular transfer system, OATS, is an effective method for the resurfacing of osteochondral defects of the knee. The technique consists in transplantation of many osteochondral autologous plugs obtained from the periphery of the femoral condyle articular surface, which supports less weight and transferring them to create a durable resurfaced area in the defect (Fig. 3). The procedure shows some advantages regarding other repair techniques, such as the viable hyaline cartilage

transplantation, a relatively short rehabilitation period and the possibility of carrying out the procedure in one only operation.

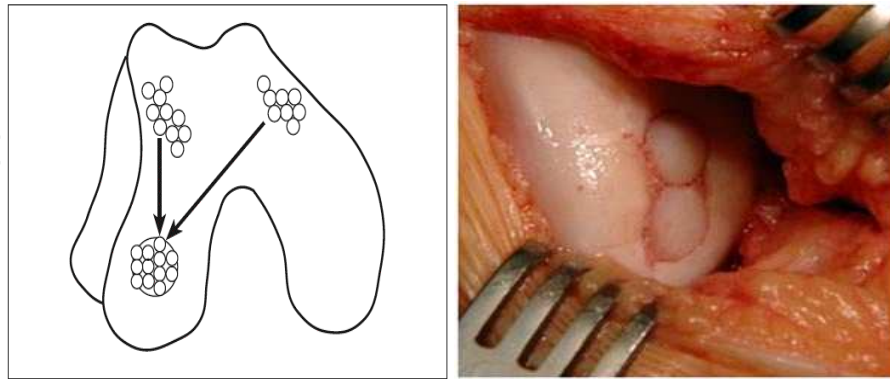


Figure 3. In mosaicplasty cylindrical osteochondral plugs are harvested from nonload-bearing sites in the affected joint and pressed into place within the osteochondral defect, creating an autograft “mosaic” to fill the lesion

However, the OATS limitations are the donor-site morbidity and a limited availability of grafts that can be obtained from the femoropatellar joint or the area adjacent to the intercondylar fossa. Other possible limitations are differences in bearing, thickness and mechanical properties between the donor’s and the receiver’s cartilages, as well as the graft sinking into the surface due to the support of weight after surgery. Besides, the lack of filling and the possible dead space between cylindrical grafts can limit the repair quality and integrity. Lane et al. transplanted autologous osteochondral grafts into sheep knee joints and reported the lack of integration of the cartilage, which determined the persistence of gaps through the full thickness in all the specimens (Mishima et al., 2008).

2.3. Alogenic osteochondral transplantation

Osteochondral allograft transplantation is a procedure for cartilage resurfacing which involves the transplantation into the defect a cadaveric graft composed of viable, intact AC and its underlying subchondral bone. It is a well known resource, especially for tumour surgery. The defect size, its location and its depth are crucial factors for the suitability of the donor graft. Advantages of using osteochondral allografts are the possibility of achieving a precise architecture of the surface, the immediate transplantation of viable hyaline cartilage in a one-time procedure, the possibility to repair large defects, even half-condyles and the donor-site lack of morbidity. Gross et al. have reported results from fresh allografts in 123 patients with good clinical results in 95% of the patients after five years (Gross et al., 2002). There are different possible allografts. Fresh osteochondral allografts are generally used because both freezing and cryopreservation have proved to reduce the chondrocytes viability. Traditionally grafts have been obtained, kept in lactated Ringer’s solution at 4 °C and then transplanted in a week. Another alternative for allografts conservation and implantation is cryopreservation, which involves freezing at a controlled speed of specimens within a nutrients rich medium, a cryoprotector agent (glycerol or dymethyl sulfoxide), to minimize the cells freezing and keep

their viability; finally, there is the possibility of fresh-frozen allografts, with the advantages of lower immunogenic capacity and less transmission of diseases but with lower chondrocyte viability.

2.4. Soft tissues transplantation

Two main theories support the practicing of covering the cartilage defects with soft tissues, such as perichondrium or periosteum. On one hand, the defect has to be covered mechanically and on the other, we know about the presence of pluripotential stem cells in the perichondrium and the periosteum cambium layer. The different factors able to promote these cells differentiation into active chondrocytes still remain unknown.

3. Therapeutic interventions with active biologics

3.1. Autologous chondrocytes implant

The clinical use of the autologous chondrocytes implant (ACI) technique was first reported by Brittberg et al. in 1994, following animal studies which had shown its effectiveness (Grande et al., 1989). In this method, chondrocytes are obtained from a biopsy taken from a non-weight bearing part of the patients cartilage, and are expanded *in vitro*, followed by the injection of a suspension of chondrocytes into cartilage defects, covered with autologous periosteal flap (Fig. 4). This technique premises are based on the capacity of adhesiveness of the cells to certain surfaces, they spread on them and proliferate producing their specific ECM. Although clinical results of the original ACI looked promising (Minas, 2001; Peterson et al., 2000), this procedure has some potential disadvantages, such as leakage of transplanted cells, invasive surgical method, hypertrophy of periosteum (Haddo et al., 2005; Kreuz et al., 2009) and loss of chondrogenic phenotype of expanded chondrocytes in monolayer culture (Benya & Shaffer, 1982). Second generation ACI, named membrane autologous chondrocyte implantation (MACI), has a similar procedure, but a collagen type I and III membrane instead of periosteum. This technique was introduced to improve the ACI problems, and biomaterials such as collagen type I gel (Ochi et al., 2002), hyaluronan-based scaffold (Manfredini et al., 2007) and collagen type I/III membrane (Bartlett et al., 2005) were applied to secure cells in the defect area, to restore chondrogenic phenotype by way of three dimensional cultures (Gigante et al., 2007) and to replace the periosteum as defect coverage. This is the way MACI technique is created *a posteriori*, by implanting autologous chondrocytes in three dimensional matrices of collagen types I and III, or hyaluronic acid.

At present, only two prospective studies comparing the original and second generation ACI are available (Bartlett et al., 2005; Manfredini et al., 2007) and both studies show no significant differences in the short term clinical results. As for the first generation ACI, the newly regenerated cartilage often consists of fibrous tissue (Horas et al., 2003; Tins et al., 2005), possibly due to the limited number of chondrocytes and their low proliferation potential. Bone overgrowth that causes thinning of the regenerated cartilage and the violation of the tidemark are also of concern. Moreover, this method still sacrifices healthy cartilage. Thus, these aspects

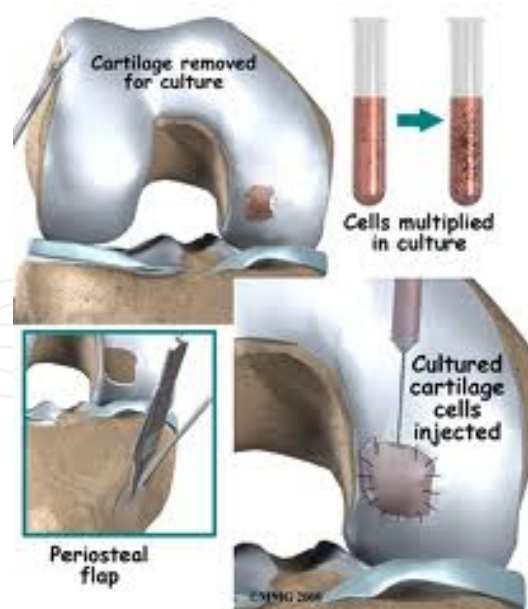


Figure 4. In autologous chondrocytes implantation (ACI) a chondral biopsy is taken from a donor site at the time of clinical examination to be treated with enzymes in the laboratory to obtain chondrocytes cultures that are re-injected under the periosteal flap

limit ACI in the treatment of large defects and may increase the long-term risk of osteoarthritis development.

3.2. Are Mesenchymal Stem Cells what we need?

Stem cells are of particular interest in Regenerative Medicine. They inhere several unique characteristics that distinguish them from other cell types. Besides autograft transplantation and ACI, current therapeutic concepts of cartilage defects include the recruitment of MSCs. Tissue engineering (TE) based on cell and genetic therapy offers some of the most promising strategies of tissue repair, including AC repair. It is the science able to create alive tissue to replace, repair or strengthen ill tissue. Thus, the TE refers to a wide variety of techniques.

The process for using MSCs to produce cartilage tissue comprises four elements: cells, inductor factors, scaffold to deliver the cells and vascular supply to the host area.

From the previous experiences we have learned that we need:

- To minimize the risk and inconveniences of the donor site. In this case, MSCs are easily available from bone marrow, synovial membrane, adipose tissue, etc. So then, we can get a variable number of cells from a different tissue, partially avoiding the donor site secondary complication (Winter et al., 2003). In ACI, this extra procedure adds a risk for infection, inflammatory changes in the joint, pain and rises up the final cost of the treatment (Hunziker, 2002)
- To achieve a minimum number of cells. MSCs have a high proliferation and differentiation potential. In any case, MSCs coming from different tissue have an uneven chondrogenic

differentiation capacity and it must be related to the special cytokines, growth factor and induction molecules composition of the medium (Claros et al., 2012; Hennig et al., 2007). To induce the differentiation of predifferentiated cells, MSCs, is a more controlled way of driving the process to a cartilage final effector. One of the cons of chondrocyte transplantation is the dedifferentiation process that these cells suffer when they are treated *in vitro* and the limited ability to redifferentiate them (Benz et al., 2002). On the contrary, MSCs are very stable and they do not suffer this dedifferentiation (Najadnik et al., 2010) process and have a high differentiation capacity.

Up to now, MSCs have been used to treat osteochondral defects in two different ways. We have the option of implanting the MSCs once expanded. It is of concern in this case the lack of control on the growth after transplantation and how much exhausted the cells will result after the expansion and how it will influence on the aging of the new induced tissue. The clinical results after implantation of these expanded MSCs have been analysed and were substantially the same compared with those obtained after any marrow stimulating techniques (Wakitani et al., 2002; López-Puerta, 2013). A second option is to implant the MSCs after the induced differentiation to a chondrocyte phenotype. In these circumstances, to assure the stability of the achieved phenotype is of concern as this lineage is able to go on with the complete chondral process and ends in producing final stages of hypertrophy and calcification (Peltari et al., 2006; Andrades et al., 2010; López-Puerta, 2013). In any case, the final challenge will be to produce a stable cellular lineage that produces an AC tissue that works under compression forces without losing its initial characteristics as time goes by.

Beside the characteristics of MSCs exposed before, these cells have self-renewal potential as well as multilineage differentiation potential (Becerra et al., 2011), including chondrogenesis (Johnstone et al., 1998; Pittenger et al., 1999; Prockop 1997; Sacchetti et al., 2007). MSCs chondrogenesis was first reported by Ashton et al. (1980) and the first ones to describe a defined medium for *in vitro* chondrogenesis of MSCs were Johnstone et al. (1998), who used micromass culture with TGF- β and dexamethasone. Sekiya et al. (2001, 2005) reported that addition to bone BMPs enhanced chondrogenesis under the conditions employed by Johnstone et al. (1998). Nowadays, the micromass culture is widely used to evaluate chondrogenic potential of MSCs *in vitro*. However, this *in vitro* chondrogenesis does not mimic cartilage formation during development. During micromass culture, MSCs increase expressions of both collagen type II (chondrocytes marker) and X (hypertrophic chondrocytes marker) (Barry et al., 2001; Ichinose et al., 2005). Other cytokines such as IGF (Pei et al., 2008) and parathyroid hormone-related peptide (PTHrP) had been tried for better differentiation cocktails, but it is still difficult to obtain *in vitro* MSC-based cartilage formation comparative to native cartilage tissue.

To initiate any regeneration based on MSCs activity, the cells first have to be recruited to the site of damage (Fig. 5). Second step is adhesion to a local matrix followed by activation and extensive proliferation to provide the necessary high numbers of chondroprogenitor cells to build up new tissue. In stem 3, the cells need to switch from expansion to chondrogenic matrix production by induction of chondrogenesis to build up the shock absorbance and gliding characteristics for proper tissue function. The seamless integration with neighbouring cartilage and bone tissue depends on successful crosstalk between new and old tissue, and an instruc-

tional capability to guide neighbouring cells. For durable cartilage regeneration, the tissue eventually needs to regenerate a tidemark, adapt to biomechanical loading and build up a balanced tissue homeostasis. We are going to summarize these steps including talking points taken from our annual meetings in NACRE (New Approaches for Cartilage Regeneration; CIBER-BBN, Spain) consortium, in order to propose strategies for the Regenerative Medicine of AC (Becerra et al., 2010; Andrades et al., in press)

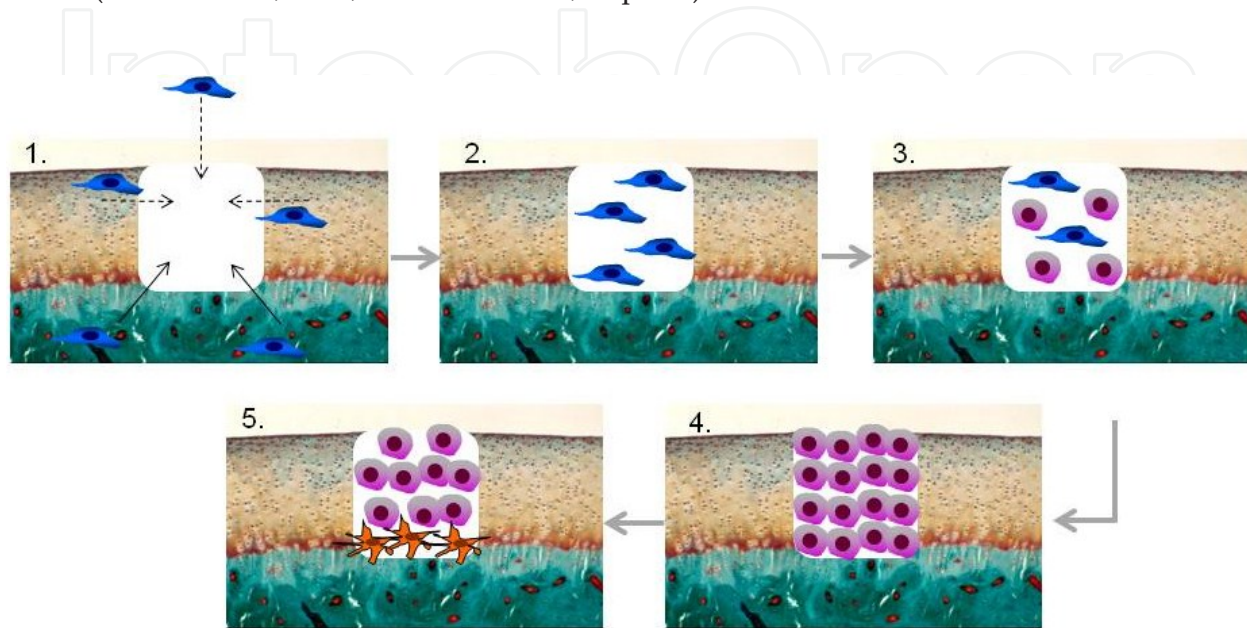


Figure 5. Principal mechanisms for *in vivo* cartilage regeneration. 1) MSCs recruitment from the surrounding tissues such as synovial fluid/membrane, neighbors cartilage, or subchondral bone; 2) MSCs retention by local adhesion, and proliferation; 3) MSCs are induced to chondrogenic differentiation by local factors; 4) chondroblasts and chondrocytes arranged according to the cartilage zones pattern and biomechanical loading; 5) integration of repair tissue interaction with cartilage and subchondral bone

3.2.1. Recruitment of MSCs

Cell migration is a prerequisite for development from conception to adulthood and plays a major role in regeneration of all tissues. Even articular chondrocytes, which are encased in a dense matrix throughout their life, can show cell motility when transferred *in vitro*. A number of studies demonstrated that chondrocytes migrate under the action of different stimuli on or within planar and 3D matrices. Attracting factors include bone morphogenetic proteins (BMPs) (Frenkel et al., 1996), insulin-like growth factor-one (IGF-1), transforming growth factor-beta (TGF- β), fibronectin (Andrades et al., 2003; Chang et al., 2003), platelet-derived growth factor (PDGF) (Fujita et al., 2004), fibroblast growth factor (FGF) (Hidaka et al., 2006), fibrin and collagen type I (Kirilak et al., 2006).

But, what are the best factors to attract more MSCs to a cartilage lesion? Recent work has established that MSCs are not largely distinct from chondrocytes regarding the panel of factors capable to attract the cells *in vitro*. All tested factors were further effective in stimulating the migration of MSCs but not fibroblasts (Ozaki et al, 2007). Factors contained in a natural blood clot are highly chemoattractive for MSCs; augmentation of a clot-stabilizing matrix with a

potent chemoattractive factor like PDGF may be an attractive way to further enhance cell numbers early after microfracturing.

In this sense, a quick release kinetics within hours and days from the biomaterial is desired for this first step of healing. Unfortunately, no 3D *in vitro* model in a biomatrix has yet been applied to test chemoattraction of human MSCs under more natural conditions and no adequate animal model has been used to dissect the factor requirement of progenitor cell recruitment from bone marrow into cartilage defects. Most likely, the presence of synovial fluid and the joint loading initiated pumping mechanism will strongly affect attraction and retention of MSCs. A search for superior factor combinations to enhance and speed up MSC attraction, and the best retention matrix to keep cells local, thus, is an important topic for upcoming studies.

3.2.2. Multiplication of MSCs

Proliferation of MSCs is the second important step to rapidly enhance the cell numbers in the repair tissue. As the replicative lifespan of MSCs is, however, not unlimited and telomerase activity is absent or low (Parsch et al., 2004) this can only ground on the sufficient attraction of initial MSCs numbers to the defect. During embryonal development proliferative chondroprogenitors are densely packed and condensed to an area in which cartilage tissue is forming. The high cell numbers may be needed to deposit the vast amount of ECM characterizing this tissue. Low cellularity of AC is rather a late phenomenon during tissue formation and may have developed in adaptation to its biomechanical competence and the extremely slow turnover of its ECM. Proteoglycan turnover in cartilage is up to 25 years and collagen half-life was estimated to range from several decades up to 400 years (Eyre et al., 2006). Based on the assumption that embryonal traits should best be recapitulated during tissue regeneration, early defect filling tissue should contain densely packed proliferating chondroprogenitors. This would mean that rather hundreds of millions of new cells are needed per cm³ during this step in the defect to allow for optimal chondrogenesis and rapid ECM production. At a later phase, cell numbers could decline to a density of 5-10 million cells per gram tissue, when homeostasis is the left over task for the cells in the fully regenerated tissue.

These points towards an outstanding need for highly efficient transient induction of proliferation, a typical feature ascribed to adult stem cells. Growth requirements of human MSCs are distinct from those of other species (Kuznetsov et al., 1996) and many factors have been identified as potent mitogens, being PDGF-BB, EGF and TGF- β have been regarded as the most important amongst them (Kuznetsov et al., 1997). As they induced the migration of MSCs and have at the same time the potency to enhance their proliferation, the migration and proliferation steps of MSCs can take place simultaneously *in vivo*. However, whilst nutrients and oxygen are almost unlimited in tissue culture, a rapid supply of cells with O₂ and nutrients may be more restricted in a cartilage defect and depend on the distance to and conditions found within the subchondral bone. Histology of early human cartilage repair tissue demonstrates that indeed cartilage differentiation initiates in contact with subchondral bone (Steck et al., 2009) whilst upper regions remain fibrous for quite a long time and may need to mature over years (Brun et al., 2008). Furthermore, earliest chondrogenesis is often seen in areas where active remodeling of the subchondral bone plate occurs and, thus, enhanced nutrition and a

higher anabolic rate of the cells can take place. Beside strong mitogenic factors used for augmentation of a clot-stabilizing biomaterial, the access to optimal nutrition in the course of tissue remodeling may indeed be a limiting aspect of cartilage regeneration techniques. Enhanced remodeling of microfractured compared with unopened subchondral bone areas is likely, but quantitative and localization-dependent studies have so far not been reported. The stimuli mentioned here could be obtained by direct administration of recombining growth factors in the culture media or via transfer of the respective genes. Thus, the possibility of considering genetic therapy as an applicable measure for the treatment of cartilaginous lesions arises.

4. The recapitulation of AC morphogenesis through MSCs differentiation

In spite of therapeutic strategies mentioned, which offer the patient a temporary relief of symptoms, they do not resolved, in the medium or long term, the disease that affects the joint. In most cases, fibrocartilage is generated that does not provide the necessary structural integrity and this often results in the subsequent replacement of the damaged joint (Mahmoudifar and Doran, 2012).

The main problem with these strategies is that only tries to restore the biomechanical characteristics of tissue but not its physiology. That is why the goal of current research in AC regeneration with MSCs is moving to methods that attempt to recapitulate the morphogenesis of the tissue. The challenge is to develop strategies that will cover the widest possible range of intervening factors including: the dynamics of genes and proteins that control and participate in the chondrogenic process, their spatiotemporal patterns of expression, variations in culture conditions, biomaterials functionalized with effector molecules inducers of chondrogenesis and the inclusion of differentiation enhancers using genetic engineering and others.

4.1. Novel genes and protein: The path to recapitulating AC morphogenesis

As previously mentioned, the AC's ECM is composed mainly of collagen type II fibers and proteoglycans with strong negative charges, aggrecan being the most abundant of them (Han et al, 2011). That is why the detection of both proteins and their corresponding genes has been classically used as markers of chondrogenesis in numerous studies *in vitro* and *in vivo*. However, most modern regenerative strategies should aim at obtaining a more complete and organized ECM that achieves the greatest similarity with the ECM on the original AC. With this objective, have been identified dozens of genes that play a fundamental role in the formation and maintenance of AC, which are potential targets for research in the design of regenerative strategies (Quintana et al., 2009; Bobick et al., 2009; Mahmoudifar and Doran, 2012).

4.1.1. *Proteoglycan 4 (PRG4): Biological function and its relationship to OA*

A good example is the proteoglycan 4 (PRG4), also known as "superficial zone protein" (SZP) and "lubricin". It is a proteoglycan specifically synthesized by chondrocytes located at the

surface of AC and by synoviocytes. Their functions are the lubrication of articular joints, elastic absorption and energy dissipation of synovial fluid (Jay et al., 2007).

It has been shown that mutant mice *Prg4*^{-/-} have normal joints at birth and during the newborn period but older mice exhibit accumulation of proteinaceous deposits on the cartilage surface, disappearance of the surface zone of flattened chondrocytes, synoviocyte hyperplasia, calcification of structures flanking the ankle joints and, consequently, a total failure of the joint (Coles et al., 2010). These data are consistent with OA degeneration, and have also been observed in veterinary cases of sheep with early OA which has been shown a downregulation of the expression of *PRG4* (Young et al., 2006). In a human clinical example, a case study reported in 2004 a 10 year old boy with Camptodactyly-Arthropathy-Coxa vara-Pericarditis syndrome (CACP) which arises as a result of truncating mutations of this gene. Clinical manifestations of CACP include congenital or early-onset camptodactyly, noninflammatory arthropathy with synovial hyperplasia and progressive coxa vara deformity, all symptoms related with the cartilage's physiology (Choi et al., 2004).

All these studies show that PRG4 is closely related to morphogenesis, maintenance and functioning of the AC as well as defects in its expression are related to the development of OA. That is why in recent years has aroused interest in the field of *in vitro* studies as a potential aim in developing AC regenerative strategies.

4.1.2. PRG4: An *in vitro*, *in vivo* and clinical marker of chondrogenesis

Several studies show that this protein participates in the chondrogenic process and that its expression can be induced on *in vitro* chondrogenesis experiments. Recently, has been found an increase in *PRG4* gene expression in cells derived from infrapatellar fat pad (IFP) in response to treatment with TGF- β 1 and BMP-7, demonstrating that the *in vitro* induction of this gene expression is possible. Furthermore, it served to demonstrate the suitability of the source cell used (Lee et al., 2008). However, the same research group in a similar characteristics experiment but using human embryonic stem cells (hESC) was able to induce chondrogenesis but failed inducing PRG4 expression, concluding that the induction conditions should be optimized. PRG4 use as a marker allowed the discrimination of both experiments (Nakagawa et al., 2009). It has also been observed that in rat skeletal muscle-derived mesenchymal stem/progenitor cells (MDMSCs) treated with TGF- β 1 and BMP-7, PRG4 increased in a time-dependent manner on days 3, 7 and 10. As early as day 3, there was a three-fold increase of the PRG4 detected by ELISA analysis. This was confirmed by immunochemical localization of PRG4 as early as day 3 after treatment with TGF- β 1. Even, the mRNA expression of PRG4 was enhanced by the two factors, along or in combination. This work demonstrates that is possible induce *in vitro* not only the increased of PRG4 gene expression, but also the accumulation of the protein in the medium (Andrades et al., 2012).

In clinical setting, there are also studies related with PRG4. Patients with OA tend to form small cartilaginous deposits in the exposed subchondral bone. The histological study of aggregates of patients undergoing total knee replacement, reveals that these aggregates were fibrocartilaginous, positive staining for glucosaminoglycan Safranin-O and type II collagen expression but, more interestingly, of PRG4. In those aggregates embedded in the bone, the

staining was positive for the entire surface while in which protruded to the surface, the PRG4 was detected only in the edge surface as would be observed in normal cartilage. This *in vivo* observation is an excellent example of the cell's genetic response to the environment. Osteochondroprogenitors contact with the synovial fluid and physicochemical stimuli inherent to the articular surface is able to modify the spatial distribution patterns of PRG4 expression and thus, the tissue architecture. These results are an invitation to test culture conditions that attempt to emulate not only the biochemical environment, but also mimic the biophysical characteristics of the physiological niche (Zhang et al., 2007) (Fig. 6).

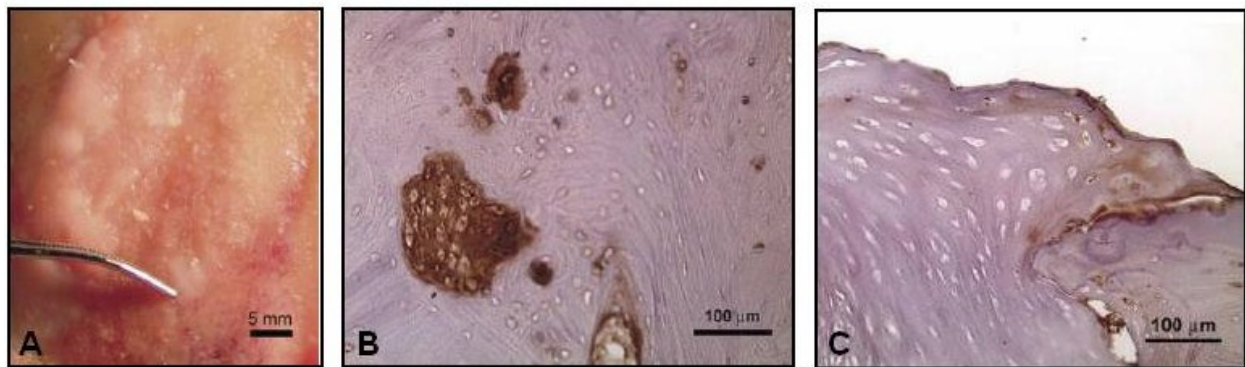


Figure 6. A) Probe pointing to a white spot on the exposed bone surface of an osteoarthritic femoral condyle obtained at the time of total knee arthroplasty. B) Immunohistochemistry for PRG4 to subsurface chondrocyte aggregate in subchondral bone staining fully for PRG4; and C) fibrocartilaginous deposit protruding through the joint surface containing PRG4 in a zone just below the surface. *From the same authors, published by Journal of orthopaedic research: official publication of the Orthopaedic Research Society 25(7): 873–883. Copyright 2007*

It has also been demonstrated the possibility of reversing the decline in the expression of PRG4 in cartilage chondrocytes culture from OA patients. In this study, cartilage explants were obtained from healthy and OA patients and performed monolayer cultures and encapsulated in poly (ethylene glycol) diacrylate scaffold (PEG-DA). The OA cartilage explants have weaker immunolabeling of PRG4 than healthy cartilage explants. However, the difference was reduced significantly between these 2 samples when were cultured in PEG-DA hydrogels. OA chondrocytes regain the ability to express PRG4 at levels virtually identical to those obtained in chondrocytes from normal cartilage demonstrating the importance of culture conditions in the chondrogenic induction (Musumeci et al., 2011).

All these evidences indicate that the inclusion of proteins as PRG4 in experimental designs offers advantages such as:

1. The inclusion of more highly specific markers that allow a better understanding of chondrogenic potential of cell sources, bioactive scaffolds and treatments applied.
2. Implant design that better mimic the characteristics of the AC and try to emulate not only the composition but also tissue architecture.

3. The ability to understand more deeply the dynamics of diseases that affect the AC like OA, in order to raise regenerative strategies that offer to patients, medium and long term solutions.

4.2. Genetic engineering: Enhance of chondrogenic potential beyond biochemical signals and functionalized scaffolds

4.2.1. SOX9: The key regulator of the chondrogenic process

Given the low regenerative capacity of the AC, it is vital find ways to increase the chondrogenic potential of bioimplants designed. Today, not only can enhance the biochemical environment biomaterials and implants, but genetic engineering can increase the potential of MSCs used, changing their gene expression patterns from "inside". For this, it is important to consider transcription factors and their intracellular signaling cascades. Perhaps the most studied of these factors is SOX9, considered the key regulator of the chondrogenic process (Bi et al., 1999). The expression of SOX9 is upregulated by members of the FGFs, TGF- β s and BMPs, all of them widely used chondroinducers. In turn, SOX9 is responsible for regulating SOX5, SOX6 and activating the expression of collagens type II, VI, IX and XI, the proteoglycans Aggrecan, Byglican and Perlecan and important binding proteins as COMP (Quintana et al., 2009) (Fig. 7).

On the other hand, during skeletogenesis, SOX9 is responsible for the osteochondroprogenitors differentiation into chondroblasts and not into osteoblasts to direct and indirect repression of RUNX2 (the main regulator of osteogenic differentiation) favoring the endochondral ossification (Zhou et al., 2006).

4.2.2. Transfection of SOX9 as an activator of chondrogenesis

The application of these regulatory genes on regenerative design strategies could be useful to increase both specificity and efficiency of the bioimplants. This opens the possibility of using not only functionalized biocompatible scaffolds, but also cells previously treated to have a higher chondrogenic potential. In a recent study, human MSCs are transfected with a nonviral vector plasmid complexed with SOX9 cDNA in order to induce chondrogenesis. Micromass culture and transplantation into nude mice of control and transfected cells were made. Both procedures showed increased levels of mRNA for COL2A1, Aggrecan and COMP; increased GAG content; alcian blue staining positive and detection of type II collagen and Aggrecan by immunofluorescence, all of the cells transfected with respect to control. Similar results were achieved using a viral vector transfection of SOX9 (Fig. 8). Additionally, this group demonstrated that transfection of the gene, in addition to inducing chondrogenesis, reduces the levels of markers of hypertrophy, osteogenesis and adipogenesis, thereby inhibiting the possibility that the human MSCs to differentiate into these mesenchymal lineages (Venkatesan et al., 2012).

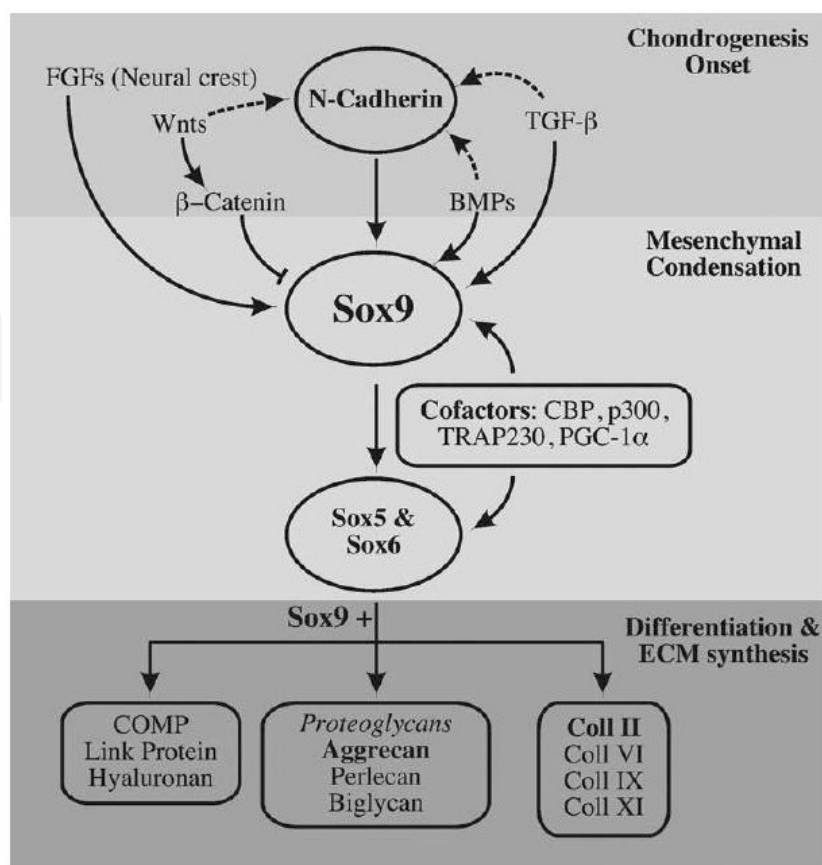


Figure 7. Upstream and downstream regulation of Sox9. From the same authors, published by Tissue engineering. Part B, Reviews 15(1): 29–41. Copyright 2009

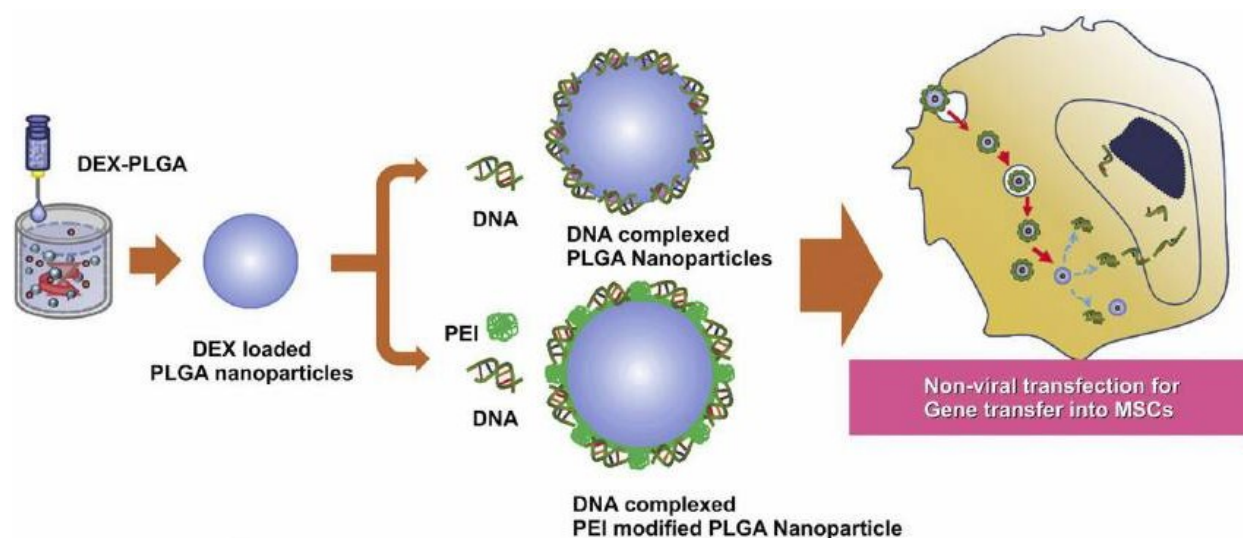


Figure 8. Schematic diagram of SOX9 gene transfection using a modified and non-modified biodegradable nanoparticles, an example of Non-viral transfection. During hMSCs transfection, nanoparticles interact with the negatively charged lipid bilayers and are influxed into endosomes and destabilized, resulting in the release of the transfected genes into the cytosol. From the same authors, published by Biomaterials 32(1): 268–278. Copyright 2011

The possibility of genetically engineering the different types of MSCs used in cartilage regeneration is a promising tool for increasing chondrogenic capacity and, consequently, improving future regeneration bioimplants. A deepest and detailed knowledge of gene regulation involved in the process and their possible clinical utilities will lead the way of successful recapitulation of AC morphogenesis through MSCs differentiation.

5. Commercial and industrial translations in tissue engineering

Applications for TE and biomaterials were originally limited to prosthetic devices and surgical manipulation of tissues but now include development of biomaterial scaffolds, bone/cartilage engineering, tissue-engineered blood vessels and wound healing, among other fields.

As an industry, it could significantly contribute to economic growth if products are successfully commercialized. However, to date, relatively few products have reached the market owing to a variety of barriers, including a lack of funding and regulatory hurdles. Policy interventions, including increased translational government funding, adaptation of policies, and regulatory clarity, would likely improve the general outcomes for the regenerative medicine industry.

The technical challenges of TE are, of course, intellectually and scientifically interesting and can add substantial and previously unattainable knowledge to our understanding of biological systems (Mansbridge et al., 2006). TE models of biological systems can even provide insight into pathologic processes. However, perhaps the major attraction of academic researchers and industrial organizations to this field is the potential of the technology to be readily converted to clinical applications. For this to happen, the technology almost always will be transferred from an academic environment to an industrial organization that will lead the comprehensive translational studies and convert scientific observations into a manufactured product.

As a technology, TE has been shown to be feasible *in vitro* and *in vivo*, but the true demonstration of the potential value of the technology is in its clinical applications. Although the field is still in its infancy, there are already tissue-engineered products on the market, addressing previously unmet clinical needs in wound care and in orthopedics and demonstrating that the attractiveness and motivation of the field is justified. Perhaps one of the next major challenges is demonstration that the technology can lead to commercially feasible products, with manageable investment, product development costs, and time to market and, finally, a revenue generation that justifies the expense. The close connection between new technology, clinically effective treatment, and commercially feasible product is obvious and is no better demonstrated than in TE. All three of these areas, each complex in itself, must be aligned and achieved before TE can be regarded as successful.

5.1. Product development pathway

To appreciate the challenge of developing a tissue-engineered product, it is useful to first understand in general terms the various processes that must be completed (Fig. 9). The

development of a product through to approval, manufacture, and marketing is complex, and most companies (within and outside health care) use a staged process to ensure efficient and effective product development. The general scheme that applies for health care products (devices, biologics, or drugs) is outlined in the figure. These stages encompass all the activities that are required to develop a product through to the market. This integrated product development process can be customized to be appropriate for the development of products addressing repair and regeneration.

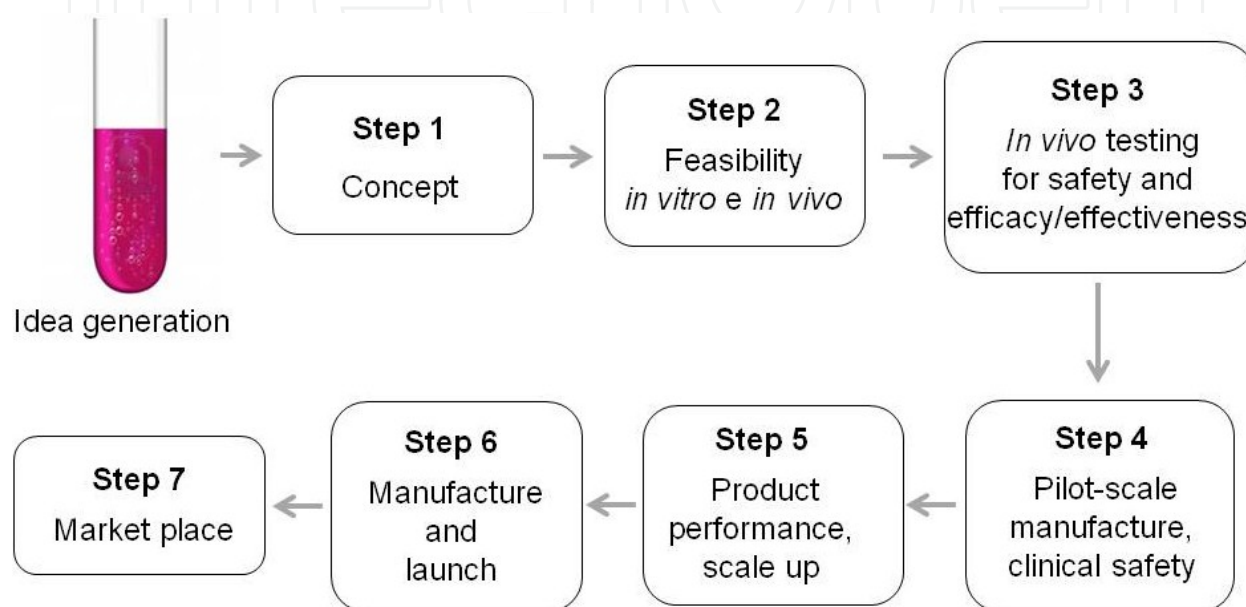


Figure 9. The general product development pathway used to develop tissue-engineered products

Patient protection for an individual product is a critical feature of product development. Developing new patents is costly, the outcome is uncertain, and it must occur near the beginning of product development. The limited time for patent protection (usually 10 years from initial submission) requires that the product development pathway be followed in an efficient manner; otherwise, patent protection will be lost by the time profitability arrives.

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