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Cell sources for cartilage repair; Contribution of the mesenchymal perivascular niche

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1. ABSTRACT

Tissue and cell sources for cartilage repair are revised, including: 1) cartilage and subchondral bone (auto and allografts; single or multiple/mosaicplasty grafts), 2) cultured chondrocytes (autologous/ACI, characterized/CCI, matrix assisted/MAC, or allogenic), 3) adult mesenchymal stem cells (MSCs), 4) progenitor cells from perichondrium and periosteum, 5) embryonic and prenatal stem cells, 6) induced pluripotent stem cells, and 7) genetically modified cells. We consider the biological mechanisms that explain usage and possible complications, advantages and limitations, emerging technologies and possible modulations on extracellular matrix properties and on migration, proliferation, de-differentiation, re-differentiation, morphology, function and integration of the cells. The study of MSC role involve: a) identification, b) location (perivascular niche hypothesis, pericytes as progenitor cells), c) lineage (myoadipofibrogenic system: transit amplifying cells, fibroblast/myofibroblasts, chondrocytes, osteoblasts, odontoblasts, vascular smooth muscle cells and adipocytes), and d) use in cartilage repair, comprising: 1) MSCs recruited from neighbouring tissues (bone marrow stimulation, MSCs based "*in situ*" cartilage repair, microfracture) and 2) MSCs cultured and expanded from bone marrow, adipose tissue, synovial membrane or granulation tissue.

2. INTRODUCTION

Since spontaneous healing of the articular cartilage (an avascular tissue with very limited capacity for repair - 1) is practically non-existent, the treatment of cartilage damage (traumatic injuries or chondropathies) involves: 1) cartilage transplantation or 2) cell-based repair (stimulating new hyaline cartilage growth, in the main by tissue engineering procedures). The possible cell sources for cell-based cartilage repair (hyaline-like cartilage formation, providing durability and normal, pain-free articular function) include: a) chondrocytes, b) adult mesenchymal stem cells (MSCs), c) perichondrial and periosteal cells, d) embryonic and prenatal stem cells, e) induced pluripotent stem cells (IPS), and f) genetically modified cells. In any case, cartilage repair requires obtaining: a) the best possible tissue (hyaline-like cartilage formed by chondrocytes embedded within an extracellular matrix of collagens, non-collagenous proteins, and proteoglycans), which resists compression and shearing forces (the fibrocartilage mainly withstands tensile forces, while the hyaline cartilage resists compression forces), b) good tissue integration with the native cartilage, preventing additional cartilage deterioration, and c) smooth surfaces of the joint, allowing for movement of bones within the articulation, with the least possible friction. Our goal is to present potential tissue and cell sources in cartilage repair

and the biological mechanisms that explain usage and possible complications.

3. TISSUE BASED -REPAIR. CARTILAGE AND SUBCHONDRAL BONE TRANSPLANTATION

When the graft only consists of cartilage, the results are ineffective, since the cartilage has very limited capacity for repair due to its avascular nature and its matrix encapsulated chondrocytes, which are unable to initiate an effective repair and to recruit local sources of progenitor cells. To avoid these difficulties, the graft must contain cartilage and subchondral bone (a firm carrier that allows revascularization), and must be implanted in an osteochondral defect (to reach a zone of vascularization). Indeed, as the receptor subchondral bone is penetrated, the healing response allows for revascularization of the osseous part of the graft, its overlying cartilage remaining viable and well attached. Therefore, the source of tissues in this procedure is the subchondral bone tissue and the four articular cartilage zones (superficial, transitional, radial, and the interphase calcified cartilage zone). These zones contain their respective collagen networks (Type II collagen, as principal component, and smaller amounts of collagen III, VI, IX, X, XI, XII, and XIV) and proteoglycans (aggrecan, syndecans, glypican, decorin, byglican, fibromodulin, lumican, perlecan, and epiphycan). This procedure includes autograft or allograft transplantation of cartilage and subchondral bone. In osteochondral autograft transplantation, the graft is transferred from one part of the joint (non-weight bearing so as to prevent weakening the joint, which limits the size of the graft) to another (only small focal chondral defects because of the limited availability of autologous osteochondral graft). Single or multiple (mosaicplasty) grafts may be undertaken. Osteochondral allograft (cadaver donor, with more osteochondral tissue available) transplantation may be considered when the cartilage defect is extensive, prior procedures have failed, or in the older patient population. Survival of more than 80% of allografts at 3-10 years has been demonstrated (2, 3), although there are concerns about histocompatibility.

4. CELL-BASED CARTILAGE REPAIR (CBCR). CHONDROCYTES

Although cartilage has poor intrinsic capacity for regeneration, its cells can be cultured and expanded "*in vitro*". Indeed, the chondrocytes may be arthroscopically harvested from the healthy articular cartilage (e.g. from a non-weight-bearing area of the medial trochlear groove of the knee or the superior ridge of the femoral condyles), cultured, and expanded to obtain a sufficient amount (over a 3-6 week period), and applied to the cartilage defect during a second arthroscopic procedure (4). At the recipient site, re-implantation takes place in "bioactive chambers", covered by autologous periosteum (currently replaced by other materials because of periosteum hypertrophy) or tissue-engineered membranes (e.g. collagen or hyaluronic acid-based membranes) sealed with fibrin glue. The cells thrive in their bioactive chambers (either with a simple membrane or with a matrix structure) forming a new cartilage. Growth factors may stimulate the implanted cells to proliferate, re-differentiate, and form specific matrix cartilage. Second-generation tissues in cartilage repair include matrix-assisted chondrocyte implantation to create a cartilage-like tissue in 3-D culture systems (5).

In this way, the most commonly used cell source in cartilage repair is the implantation of "*ex vivo*" expanded chondrocytes (4, 6-9), principally the autologous chondrocyte implantation (ACI) and the characterized chondrocyte implantation (CCI). Allogenic chondrocyte implantation has occasionally been used.

ACI does not always form hyaline cartilage, but does form fibrocartilage or mixed hyaline/fibrocartilage (10, 11). In CCI, expanded chondrocytes expressing molecular markers predictive of the ability to form hyaline-like cartilage "*in vivo*" are used, optimizing the hyaline cartilage-like formation (better structural repair, compared with microfracture) (12-14), with clinical improvement (15, 16). Indeed, CCI is associated with less fibrous tissue, more chondrocyte-like cells, and a higher content of the physiological extracellular matrix components (collagen type II, aggrecan and hyaluronic acid, and in minor concentration collagens types VI, IX, XI, fibromodulin, decorin, biglycan, and cartilage oligomeric matrix protein) (15). Autologous chondrocytes may be harvested from other regions (non articular cartilage, such as costal and auricular) with lower morbility and greater capacity of proliferation and chondrogenic potential.

Since chondrocytes are immunoprivileged when surrounded with extracellular matrix, allogenic chondrocyte implantation (alginate-based scaffolds containing human mature allogenic chondrocytes) has been used with clinical and histological outcomes that are equal but not superior to those of other cartilage repair techniques (17).

The advantages and limitations of these procedures have been widely reviewed (5, 18, 19). A major limitation of all the methods previously outlined is due to the fact that chondrocytes "*in vitro*" lose their differentiated phenotype and their chondrogenic potential (dedifferentiation to a fibroblast-like phenotype during expansion). However, culture techniques to preserve the expression of the transcription factor Sox9, which maintains the chondrogenic lineage (20), have shown good results. In monolayer cultures, chondrocytes lose their phenotypes (they dedifferentiate, change their morphology and surface receptors, and develop a non-specific synthesis profile with different expression of the matrix components, such as presence of collagen type I, III, and IV, and reduction of collagen type II, aggrecan, cartilage olygomeric matrix protein, chondromodulin, chondroadherin, and factor Sox-9) (9, 21-23). Chondrocyte de-differentiation is not observed in 3-D cultures (24) and re-differentiation (re-expression of cartilage-specific genes) of the de-differentiated chondrocytes occurs in 3D matrices (24-26), including those cultured in alginate beads (25, 27) or when adding chondrocytes to a 3D collagen matrix (28). In this regard, emerging technologies and new generation issues in cartilage repair have been considered and

developed (5, 29), such as matrix-assisted autologous chondrocyte transplantation (MACT) in which autologous chondrocytes are implanted with their own pre-formed extracellular matrix after using biodegradable and biomechanically favourable biomaterials.

5. CBCR. ADULT MESENCHYMAL STEM CELLS

Adult mesenchymal stem/stromal cells (MSCs) may be used for cartilage repair. In this section we will briefly consider the following aspects of MSCs: a) MSCs, adult stem cells (ASCs), and transit amplifying cells (TACs), b) differentiation and functional role, c) criteria for identification, d) location, and e) use in cartilage repair.

5.1. MSCs, ASCs and TACs

The ASCs are able to self renew, to intervene in maintaining the structural and functional integrity of their original tissue, and to adopt functional phenotypes and expression profiles of cells from other tissues, expressing greater plasticity than traditionally attributed to them (30-52). TACs are committed progenitors among the ASCs and their terminally differentiated daughter cells and, with more rapid though limited proliferation, increase the number of differentiated cells produced by one ASC division. These cells intervene in the replacement of damaged or dead cells with new healthy cells using repair mechanisms. Repair includes two types of processes: regeneration and repair through granulation tissue. Regeneration occurs when dead, degenerated, or damaged cells are replaced by other cells of the same type (e.g. chondrocyte transplantation). In repair through granulation tissue (a provisional tissue), MSC lineage develops, proliferates, and differentiates together with angiogenesis and recruitment of macrophages. At first, the MSC lineage shares findings between ASCs and TACs. Finally, the transition is between TACs and terminally differentiated cells.

5.2. Differentiation and functional role of MSCs

MSCs may differentiate into a complex myoadipofibrogenic line, an apparently heterogenous population (a number of mesenchymal phenotypes) that shows various activities and may predominantly express some of them, such as extracellular matrix synthesis/fibrogenesis (e.g. fibroblasts/myofibroblasts, pericytes, chondrocytes, osteoblasts, and odontoblasts), contractility (e.g. vascular smooth muscle cells and pericytes), or lipid storage (adipocytes). Indeed, the adult mesenchymal stem cells may differentiate phenotypically into adipose, cartilage, bone, vascular smooth muscle, skeletal and cardiac muscle, and hematopoietic-supportive stromal cells (MSCs may even lead to hepatocytes and neural elements) (30, 33, 39, 46, 53-62). Furthermore, MSCs secrete large quantities of bioactive factors that are both immunomodulatory (inhibit lymphocyte surveillance of the injured tissue, thus preventing autoimmunity) and trophic (inhibit apoptosis and stimulate angiogenesis and the mitosis of tissue-specific and tissue-intrinsic progenitors) (63). Therefore, MSCs can be used in tissue engineering therapies and as inductive or instructive delivery vehicles (64).

5.3. Criteria for identification of MSCs

The criteria for identification of human MSCs include the following: a) adherence to plastic in standard culture conditions, b) expression of at least CD-73, CD-90, and CD-105, while CD-11b, CD-14, CD-19, CD-34, CD-45, and CD-79a are negative, and c) "*in vitro*" differentiation into chondroblasts, adipocytes, and osteoblasts (65).

5.4. Location of MSCs (perivascular/pericytic niche for MSCs)

MSCs have been isolated from numerous locations, such as bone marrow, periosteum and trabecular bone, muscle, adipose tissue, tendon, brain, liver, spleen, kidney, thymus, lung, pancreas, heart, ovary, dermis, synovium, and deciduous teeth (66-68).

Pericytes are considered by some authors as progenitor cells with great mesenchymal potential and as a source of undifferentiated mesenchymal cells (52, 69-78). In postnatal life, retaining considerable mesenchymal potentiality, pericytes isolated from different tissues may have the capacity to differentiate into other cell types (69-79), such as fibroblasts/myofibroblasts (75, 80-82), chondroblasts (83), osteoblasts (72, 75, 81, 84-88), odontoblasts (89, 90), preadipocytes (82, 91), vascular smooth muscle cells (the reverse conversion, SMCs to pericytes, as also pointed out - 69, 92-), myointimal cells (69, 74, 92-96), and phagocytes (97). Ultrastructural findings demonstrating transitional cell forms between pericytes, myofibroblasts, and arterial myointimal cells have been known since 1985 (95).

It has therefore been demonstrated "*in vivo*" that pericytes are the target of cartilage and bone growth or induction factors, which switch on the development pathway of pericytes to prechondroblasts and preosteoblasts (70,72). Subsequently, it was confirmed that vascular pericytes undergo osteogenic differentiation "*in vitro*" and "*in vivo*" (76, 98).

In this way, a perivascular (periendothelial) niche for MSCs has been suggested, based on pericyte plasticity and on the demonstration that MSCs and pericytes share the expression of some molecular markers. Interest in this hypothesis has increased in recent years (68, 77, 79, 90, 99-113) and MSC expression of pericyte markers is among the complementary studies that suggest the perivascular niche hypothesis (114). These studies point out the following: a) stem cells expressing STRO1 display positivity for alpha SM actin and CD146, and variable expression for the pericyte marker 3G-5 (90), b) co-expression of RgS5 (a marker for pericytes) and Notch3 (Notch signalling pathways regulate stem cell fate specification and express in perivascular cells) (115), c) immunohistochemical expression of Sca-1^+/Thy/1^+/ $CD31^-$ (67), d) co-expression of annexin A5 gene (a marker for perivascular cells expressed during early stages of vasculogenesis) and NG2, SM actin protein, PDGFR beta, FLK-1 kit, Sca 1, CD-34 (77, 103), e) the isolated perivascular cells have the capacity to differentiate into mesenchymal stem cell lineages (adipose and osteoblastic cells) and also display

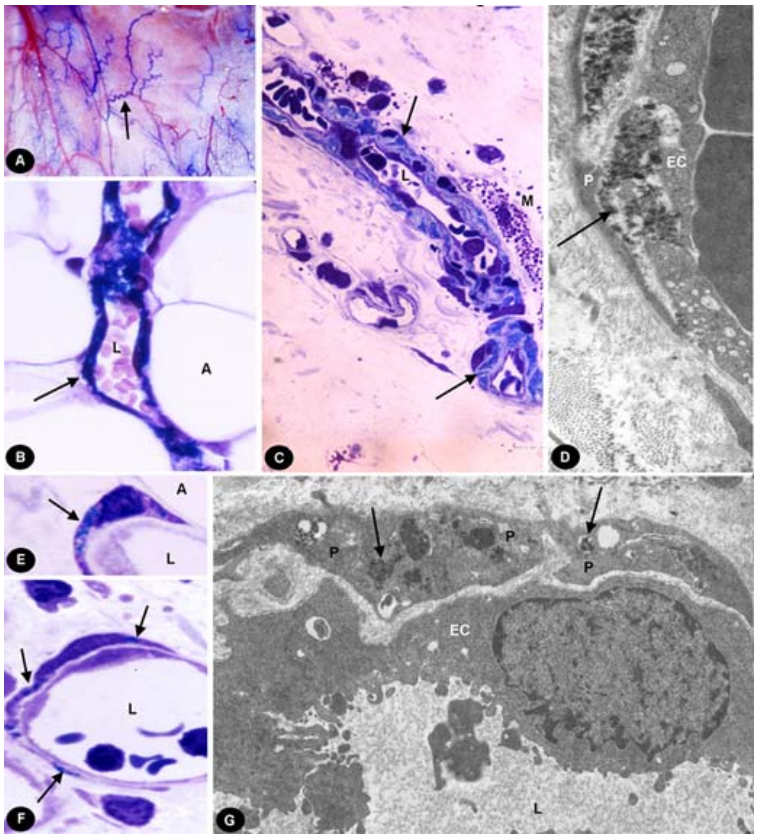

Figure 1. MB-labelled microvasculature. The labelled microvasculature is observed "*in vivo*" by stereoscopic microscopy, immediately after MB administration (Figure 1A, x 25) and, in processed tissues, by light (Figure 1B and 1C) and electron (Figure 1D) microscopy. The marker is observed as blue dots by light microscopy and as a dark deposit by electron microscopy. The marker impregnates the postcapillary venule walls (Figure 1A) and appears trapped (arrows) in the walls of postcapillary venules (Figures 1B, 1C and 1D). Note that the marker is not present outside the microvasculature. In Figure 1C activated mast cells are seen around a postcapillary venule. In tissues obtained 36 hours after MB administration, the marker is observed in the cytoplasm of pericytes (arrows) by light (Figure 1E and 1F) and electron (Figure 1G) microscopy. P: pericyte; EC: endothelial cell; A: adipocyte; L: vessel lumen; M: mast cell. Figure 1B -H&E, x 250. Figure 1C, 1E and 1F: Semithin sections, Toluidine blue, x 460, x 800, and x 800, respectively. Figures 1D and 1G: Ultrathin sections, Uranyl acetate and lead citrate, x 12000 and x 16000, respectively.

phagocytic activity (77, 103), f) self-renewal capacity and osteogenic and adipogenic potentiality have been demonstrated using cultures originated from glomerular mesangial cells (which are considered specialized pericytes) (68), g) human infrapatellar fat pad-derived stem cells expressing the pericyte marker 3G5 show enhanced chondrogenesis after expansion in fibroblast growth factor 2 (110), h) mesenchymal stem cells STRO-1, CD-146, and 3G5, exhibit a perivascular phenotype (116), i) demonstration of a linear correlation between the numbers of adipose stem cells (obtained from both highly and poorly vascularised sites of equine adipose tissue) and vascular density (107, 117), and f) freshly isolated stromal vascular fraction cells, expressing CD34, separated from $CD31^+$, $CD144^+$ EC, co-express mesenchymal (CD10, CD13 and CD90), pericytic (chondroitinsulphate proteoglycan, CD140a and CD 140b), and smooth muscle (alpha actin, caldesmon and calponin) markers (114).

To check the possible relationship between pericytes and MSCs, we selectively labelled "*in vivo*" the postcapillary and capillary mural cells of the rat inguinal pad adipose tissue with an exogenous marker (Monastral Blue) (Figure 1). Subsequently, the pericyte cell lineage was followed to determine whether the marker was present in the expanded adherent cell population of MSCs (characterized by adherence to plastic in culture and expression of markers of MSCs.) (Figure 2). The fact that adipose-derived mesenchymal stromal cells, obtained from this labelled tissue, continue showing the exogenous marker confirms the perivascular (periendothelial) niche hypothesis for the MSCs in this tissue. After rat "*in vivo*" implantation some of the cells with the marker in their cytoplasms have characteristics of pericytes and myofibroblasts (Figure 3). Previously, we had shown that under certain conditions, labelled pericytes could be a source of new chondrocytes and osteoblasts (see below).

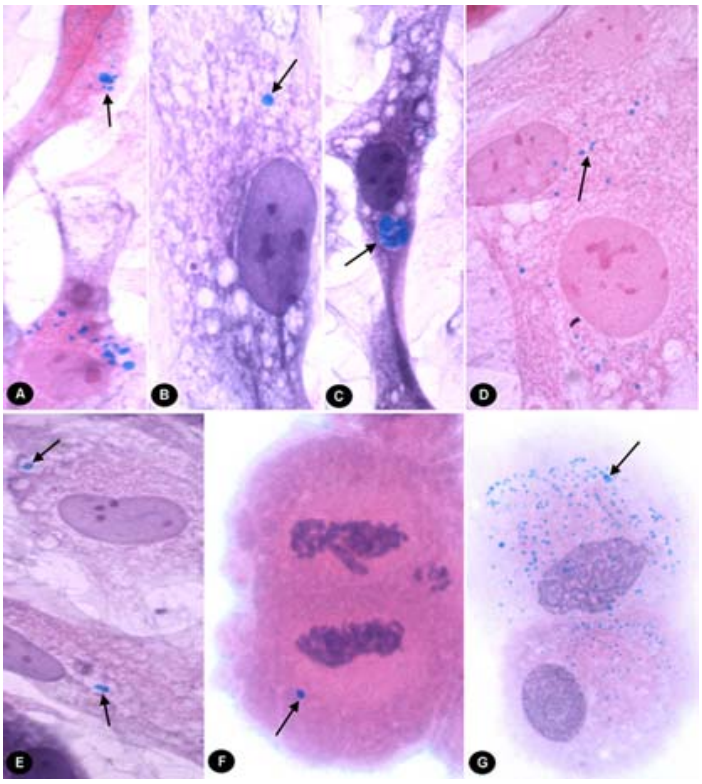

Figure 2. Presence of the marker MB (arrows) in ASCs in cultures at 2 days (Figure 2A, 2B and 2C) and 10 days (Figures 2D, 2E, 2F and 2G). Note the variable quantity of the marker in the cytoplasm of ASCs. In Figure 2F, during an ASC mitoses, the marker is observed in one of the daughter cells. Figure 2G shows part of a sample of expanded and lifted ASCs used for "*in vivo*" implantation. H&E, x 800.

Given the above, MSCs (pericytes, a subset of pericytes, or pericyte-like cells) reside in most post-natal tissues and organs, and their specific and physical location is the abluminal side of the endothelial cells, constituting a three-dimensional microenvironment, including endothelial cells, extracellular matrix and basal membrane components, as well as transmigrating cells (perivascular/pericytic niche for MSCs). In resting tissues, complex regulatory mechanisms, such as intimate association and bidirectional interactions between pericytes and endothelial cells (ECs) (118, 119) and microenvironment influences, contribute to maintenance of vascular stability and therefore to a quiescent stage of the perivascular mesenchymal stem cell niche (review in 120). During postnatal life, the regulatory mechanisms that facilitate vessel instability, such as neovascularization (as occurs in repair through granulation tissue), could also activate the perivascular mesenchymal stem cell niche (113). Indeed, in these conditions, the relation between the cells is modified, and the ECs and pericytes change from an associated, quiescent and stable state to another dissociated, mobile and proliferative state in a modulated substrate (leakage of proteins and disintegration of vascular basal membrane and extracellular matrix). In this activated state, the resident mesenchymal stem cells and transit amplifying cells proliferate and migrate toward the interstitium wherein, depending on the appropriate stimuli, they differentiate into certain cells of the complex myoadipofibrogenic line (myoadipofibrogenic system) (Figure 4).

5.5. Use of MSCs in cartilage repair

In cartilage repair, MSCs may be involved in the following: a) when a granulation tissue is originated next to the injured site (bone marrow stimulation) and b) after being obtained, cultivated, and differentiated.

5.5.1. MSC recruitment from neighbouring tissues (bone marrow stimulation. MSC-based "*in situ*" cartilage repair. MSC action after originating a granulation tissue next to the injured site)

Depending on interpretation and techniques, different terms are used to describe this process (based on the penetration of the subchondral bone plate at the bottom of the cartilage defect -121). These terms include subchondral bone perforation, osteochondral lesion, bone marrow stimulation, drilling (122), abrasion (123), and microfracture (124). The steps in this procedure (125) are the following: a) MSC recruitment to the site of damage, b) cell adhesion and proliferation, and c) differentiation towards osteoblasts and chondrocytes, with corresponding matrix production, integration with neighbouring subchondral bone and cartilage tissues, and adaptation to biomechanical loading and tissue homeostasis.

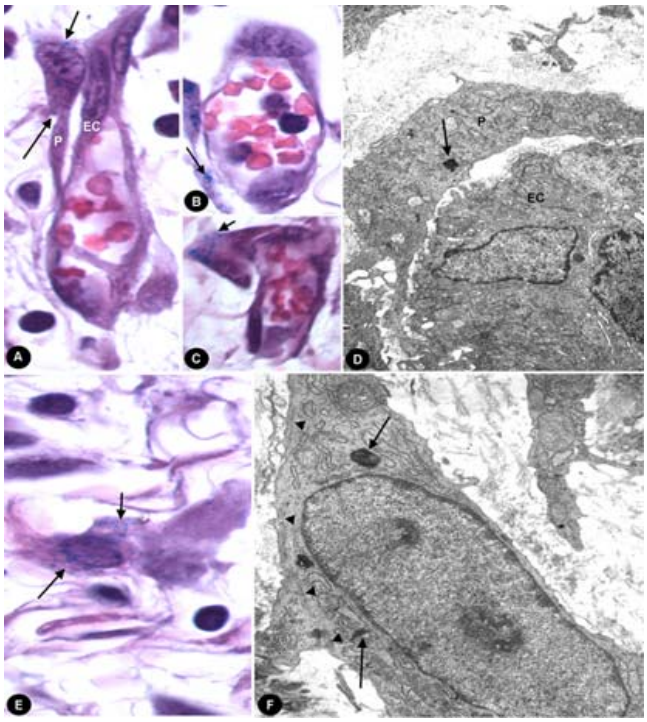

Figure 3. Labelled cells, eight days after labeled-ASC implantation. Some of the cells with the marker in their cytoplasms (arrows) have characteristics of pericytes (incompletely surrounding the endothelial cells, with which they establish focal contacts), as shown by light (Figure 3A, 3B and 3C) and electron (Figure 3D) microscopy. Labelled myofibroblasts are also present in the interstitium (Figure 3D and 3E), showing the usual ultrastructural characteristics (Figure 3F): presence of a prominent rough endoplasmic reticulum and characteristic microfilaments with dense bodies (arrowheads). P: pericyte-like cell; EC: endothelial cell. Figs 3A, 3B, 3C, H&E, x 800. Fig 3D and 3E, ultrathin sections. Uranyl acetate and lead citrate, x 14000 and x 18000, respectively.

Although the bone marrow is stimulated through this process, the principal action is to reach a zone of vascularization, creating a new blood supply and a healing response. The bone marrow microvasculature networks have been hypothesized as one possible niche for MSCs (76), coinciding with the concept that niches are highly vascularized sites (90, 111, 126). Indeed, in the bone marrow microvasculature there is a continuous layer of subendothelial pericytes (127), which acquires a reticular morphology (reticular cells) in the venous side. Therefore, the marrow pericytes may be the same entity as the bone marrow stromal cells, since they share features such as: a) similar location of pericytes and stromal cells, b) expression of similar markers, such as SMA, PDGFR beta, EGFR, and CD146, and c) similar response to growth factors (99). Generally, the authors consider that events triggered in the healing response after subchondral perforation consist of bleeding from the subchondral bone spaces, which yields a blood clot, stimulating recruitment, proliferation, and chondrogenic differentiation of MSCs and different precursor cell types from the bone marrow, from bone, and from adipose and vascular tissue (125, 128-130). In our opinion, the healing response includes granulation tissue formation. Thus, the resulting provisional tissue (granulation tissue) is similar to that which appears in other vascularized regions. Indeed, the regions with the capacity to repair through granulation tissue have a common characteristic: the presence, in or near, of an active preexisting pericytic microvasculature, where the repair phenomena develop (113). Therefore, the repair sequence includes stages of granulation tissue formation: hematoma (fibrin-deposition binding of platelets), macrophage recruitment, angiogenesis (neovascularization), recruitment and proliferation of multipotent mesenchymal stromal cells, re-absorption of the fibrin clot, and development of a vascularized scar-like tissue (113, 131). Both the origin of the participating cells and the growth factors and cytokines that intervene in the granulation tissue have been previously reviewed (113, 120). Subsequently (between days 10 and 14), new bone and overlying new cartilage are formed (in their appropriate location within the repair tissue). In the latter, the involution of the neovessels, the differentiation of the MSCs and their TACs into cartilage, and the regulation of the metabolism and homeostasis of cartilage are influenced by the local environment (mechanical and biological factors, including TGF-β family, FGFs, and Wnt - 132).

Some authors report good to excellent results in animals (in the absence of any specific treatment - 128) and in 60-80% of patients treated by these procedures (131, 133). However, spontaneous differentiation and remodelling mainly result in a fibrocartilaginous repair tissue, which may be subjected to excessive deformation

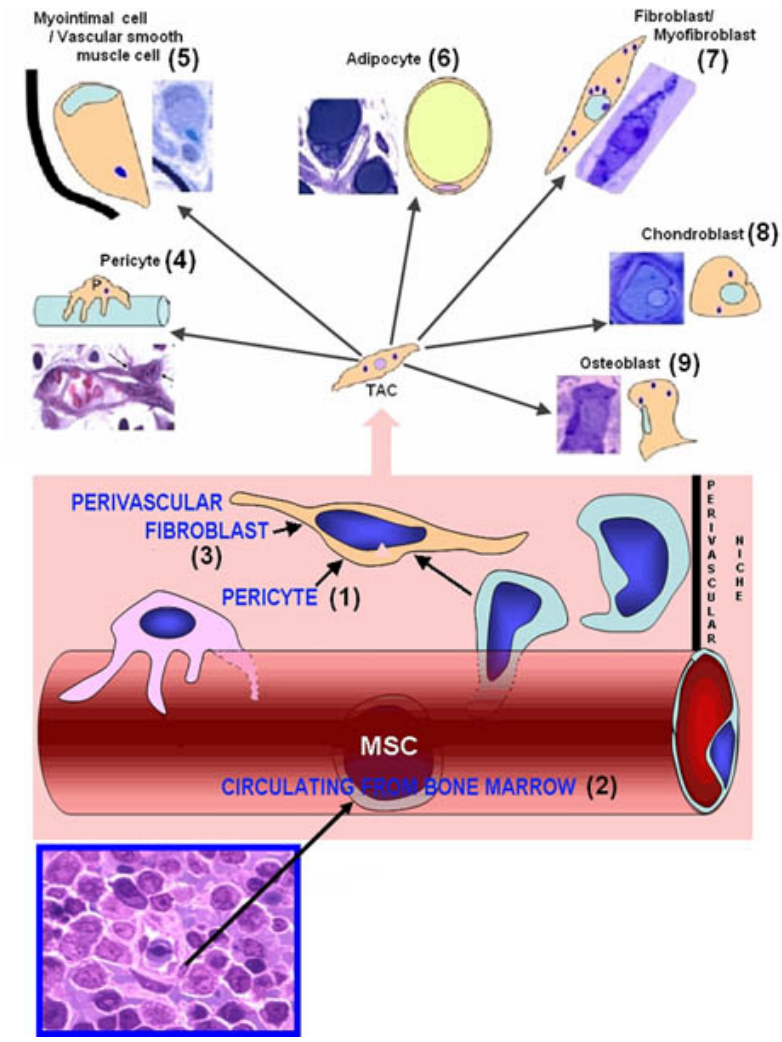

Figure 4. Perivascular niche hypothesis for MSCs and the myoadipofibrogenic system. The perivascular niche hypothesis for MSCs involve a heterogeneous population of mural cells in the pericytic microvasculature, including pericytes (1), subsets of pericytes, recruited bone marrow progenitors (2) and delimiting (perivascular) fibroblasts (3), together extracellular matrix and other transmigrating cells. This niche is the substrate of regulatory mechanisms, such as mesenchymal cell proliferation and differentiation control. When the complex regulatory mechanisms are modified, these cells dissociate and migrate, behave as transit amplifying cells (TAC), and may differentiate into the cells of the myoadipofibrogenic system. Thus, with appropriate stimulation, marked pericytes and pericyte-like cells differentiate "*in vivo*" in other pericytes (4), myointimal/vascular smooth muscle cells (5), adipocytes (6), fibroblast/myofibroblats (7), chondrocytes (8) or osteoblasts (9).

with mechanical failure and degeneration (after 20-48 weeks - 128, 130). Furthermore, the new collagen does not project into or intermingle with the native cartilage, thus hampering the integration and adherence of the newly generated cartilage. Indeed, the results after microfracture in the knee and their comparison with ACI demonstrate problems regarding the durability of the repair tissue in major defects and in defects located in areas other than the femoral condyles. Covers that trap the cells in the initial stages of granulation tissue formation (preventing escape of cells and anabolic cell mediators from the site of repair, since fibrin deposition contains the highest percentage of migrating mesenchymal stem cells) have been developed (e.g. collagen matrix) (121, 134). Therefore, this procedure protects and stabilizes the blood clot and may enhance the chondrogenic differentiation of the MSCs (autologous matrix-induced chondrogenesis - AMIC). Likewise, scaffolds (e.g. poli (DL) lactide-coglycoide or alginate-gelatin biopolymer hydrogel), cell-free or seeded with autologous chondrocytes, with osteochondral regenerative potential, have been developed experimentally, with restoration of hyaline cartilage and bone (135-139), mainly in those seeded. Currently, a porous nano-composite multi-layered biomaterial has been used and evaluated with promising preliminary results. No differences in healing were found between seeded (with autologous chondrocytes) and empty scaffolds (140).

Thickening of the subchondral bone, formation of subchondral cysts, and presence of intralesional

osteophytes have been demonstrated in patients treated with microfracture (133, 141). Likewise, the increased failure rate of autologous chondrocyte implantation after previous treatment with marrow stimulation techniques has been described (three times more likely to fail than patients who had undergone marrow stimulation), limiting future treatment options (142).

5.5.2. Use of cultured and expanded MSCs

5.5.2.1. Tissue selected for obtaining MSCs

The most commonly used source of MSCs is the bone marrow. Indeed, MSCs are located in the complex system of the bone marrow stroma (bone marrow stromal cells) (See above). Bone marrow aspirate contains very few MSCs, which can be isolated by means of Stro-1+ antibody recognition (143, 144). These cells have the capacity to differentiate into mesenchymal lineage cells and, with appropriate environmental conditions, also into cells of different embryonic origin, such as cells with visceral mesoderm, neuroectoderm, and endoderm characteristics; in other words, with high capacity of transdifferentiation and plasticity (48, 54, 145-147).

The MSCs in adipose tissue have an important potential for use in tissue engineering, since adipose tissue is an abundant and easily procured source, enabling extraction of a voluminous quantity. Moreover, the frequency of these MSCs, after removing adipocytes, is far greater than in bone marrow. Indeed, adipose tissue contains MSCs and committed adipogenic and vascular cells (101, 148-158), with the capacity to differentiate into adipose (101, 151, 154, 159), cartilage (160-162), bone (101, 151, 163-165), endothelial (166), hematopoietic (167), skeletal (156, 168, 169) and cardiac (170-172) muscle, hepatic (173), pancreatic endocrine (174) and neuronal (175-177) cells. In this way, the biology, multilineage differentiation ability, growth kinetics, gene transduction efficiency, and cell senescence of multipotent adipose-derived stem cells are similar, although not identical to bone marrow MSCs, sharing the expression of Stro-1, CD90, CD44, SH3, and CD105.

MSCs have been enzymatically released from the human synovial membrane, and their ability to proliferate and to differentiate into the chondrocyte, osteocyte, and adipocyte lineage has been demonstrated (178).

Granulation tissue is rich in MSCs and TACs (120) and may be used as a source of these cells to repair and regenerate injured tissues (e.g. cultured and propagated cells obtained experimentally from granulation tissue that forms around perforated polivinyl tubes placed in the subcutaneous space) (179)

5.5.2.2. Culture of MSCs (growth/expansion and differentiation)

Although the MSCs isolated from various tissues and involved in cartilage repair are a therapeutic promise, their expansion and differentiation requires coordination and the maintenance of the regular chondrogenic capacity (a problem not fully resolved - paradox between "*in vitro*" promise and "*in vivo*" efficacy - 9, 19, 180). In this way, 3-D supports, such as collagen, alginate, fibrin, and biopolymers are required for the MSCs to undergo chondrogenesis (181-187). Bosnakovski and cols. 2006 (187), demonstrated that differentiation (expression levels of the chondrocyte specific genes Sox9, collagen type II, aggrecan, and cartilage olygomeric matrix protein) was more prominent in cells cultured in collagen type II hydrogel and that it increased in a time dependent manner. In this way, to induce and maintain chondrogenesis, transforming growth factors (TGF) b1 and b3, fibroblast growth factor, bone morphogenic proteins (BMPs)-2, -6, and -9, and insulin-like growth factors, may be used (188-190) (e.g. the combined use of bioreabsorbable scaffolds along with gels and incorporated growth factors for localized delivery therapies). Likewise, physical factors can also participate in the regulation of MSC differentiation (191). Among these are the mechanical properties of the supports, since rigid or soft scaffolds may be used. Thus, rigid scaffolds are more suitable for cartilage tissue engineering, while soft scaffolds facilitate adipose differentiation (192, 193). Thus, to demonstrate the importance of mechanical factors in cartilage tissue repair, we implanted perforated rigid tubes in the rat soft tissue, generating a peritubular granulation tissue, which progresses through the holes reaching tube light. The granulation tissue evolved into connective (Figure 5A) and adipose tissues, except in the intratubular zone near the tube wall close to the holes (in the angle formed between the inner surfaces of the hole and the tube wall), where, during contraction, the granulation tissue presses onto the rigid material, the cells differentiating into chondrocytes (Figure 5B, 5C, 5D, and 5E). Therefore, mechanostimulation along with appropriate biomolecules (e.g. growth factors) can promote chondrocyte differentiation.

6. CBCR. PROGENITOR CELLS FROM PERICHONDRIUM AND PERIOSTEUM

The perichondrium and periosteum share the same origin, regulatory mechanisms, and some morphological and functional characteristics. The perichondrium may differentiate into the periosteum (194, 195). Both perichondrium and periosteum produce multiple positive and negative factors regulating the differentiation of the underlying skeletal elements (196, 197) (e.g. regulating gene expression in the underlying chondrocytes - 198). Both structures have two distinct morphologic layers, an outer fibrous layer and the cambium or inner cellular layer. The inner cellular layer contains fibroblasts and chondroprogenitor / osteoprogenitor cells (Multipotent periosteum cells) (199-201). Therefore, the periosteum can promote new cartilage and its chondrogenic potential decreases with age (200).

In this way, the use of periosteum as a cell source for graft engineering in bone and articular cartilage repair is a therapeutic possibility. For instance, a rapid curing alginate gel system has shown periosteum-derived cartilage tissue (202) and the action of FGF-2 enhancing TGF- beta 1-induced periosteal chondrogenesis (203).

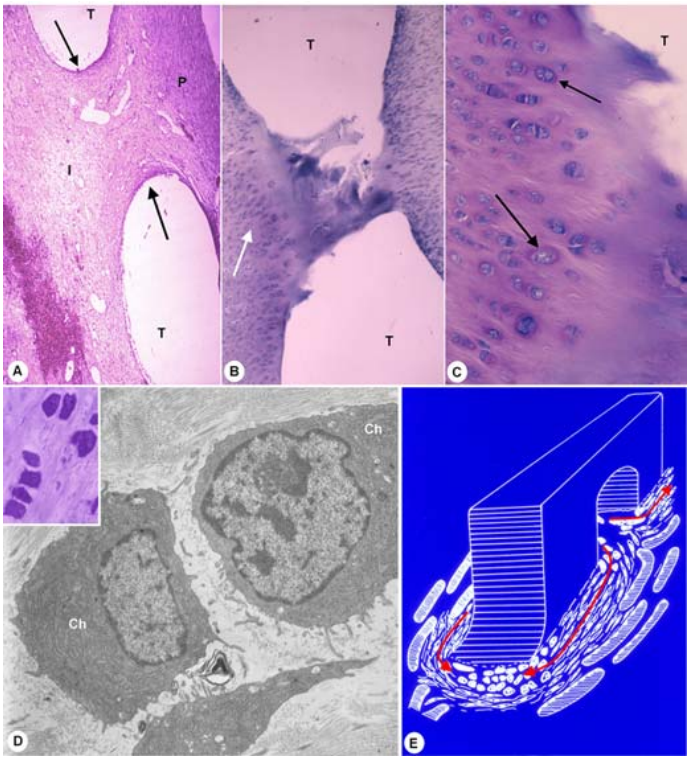

Figure 5. Tube implantation. Figure 4A: Partial vision in a histological section of a perforated rigid tube implanted in the rat soft tissue. A newly formed connective tissue (developed from a provisional granulation tissue) is observed in the periphery (P), in a parietal hole (H), and in an intraluminal region (I) of the tube. The empty spaces (T) correspond to the tube wall material dissolved during inclusion of the sample. Figure 4B and 4C: The arrows point to modified areas in which neocartilage develop, after 14 days of tube implantation. Figure 4D: Transmission electron photomicrograph of neochondrocytes (Ch). In the insert: neochondrocytes in a semithin section. Figure 4E: Diagram showing how during contraction (arrows), the granulation tissue presses onto the rigid material in the angles formed between the surfaces of the hole and the tube wall, with differentiation of the cells into chondrocytes. Figure 4A H&E, x30; 4B and 4C, Toluidine blue, x60 and x160; 4D ultrathin section, Uranyl acetate and lead citrate, x 14000; Insert semithin section, Toluidine blue, x220.

The study of perichondrial chondrogenesis "*in vivo*" and "*in vitro*" revealed that perichondrocytes of the inner layer of the perichondrium were relatively differentiated cells with the potential to develop cartilage (204, 205). Subsequently, the growth of two types of cartilage after implantation of free autogeneic perichondrial grafts has been demonstrated (83). The location and characteristics of both types of cartilage suggested that one of the types came from perichondrocytes of the inner perichondrial layer, whereas the other type originated from the undifferentiated perivascular mesenchymal cells. When the perichondrium or the periosteum were activated, and the pericytes of the local postcapillary venules were labelled with an exogenous marker, the process of cartilage and bone formation from chondrogenic and osteoprogenitor cells already present in the perichondrium and the periosteum was augmented by proliferation and differentiation of the labelled pericytes, which contributed a supplementary population of newly formed chondrocytes and osteoblasts (showing intracellular particles of the marker - Figure 6) (70, 72. Therefore, perichondrium and periosteum not only provide chondrogenic and osteoprogenitor cells but act as inducers of proliferation and differentiation of cells with mesenchymal capacity.

7. CBCR. EMBRYONIC AND PRENATAL STEM CELLS

Embryonic stem cells (ESCs) comprise the zygote, the descendents of the first two divisions, and those from the inner cell mass of blastocytes. The zygote (fertilized oocyte) and the descendents of the first two divisions are considered to be totipotent, able to give rise to the embryo, placenta, and supporting tissues. Those from the inner cell mass of blastocytes have been attributed with a pluripotent potential and therefore with the capacity to generate all or most cell lineages derived from the three embryonic germ layers: ectoderm (skin and neural lineages), mesoderm (blood, fat, cartilage, bone, and muscle), and endoderm (digestive and respiratory systems) (206, 207). During development, ESCs divide and originate distinct subpopulations, including non-self-regenerating progenitors that undergo terminal differentiation. In this way, ESCs isolated from human embryos (208) could have potential to be used in cartilage repair. However, ASCs, which may contribute to the differentiated adult lineages native to other tissues and organs, are more useful than ESCs in regenerative medicine and tissue engineering, since ASCs have no ethical problems and are not predisposed for teratoma formation.

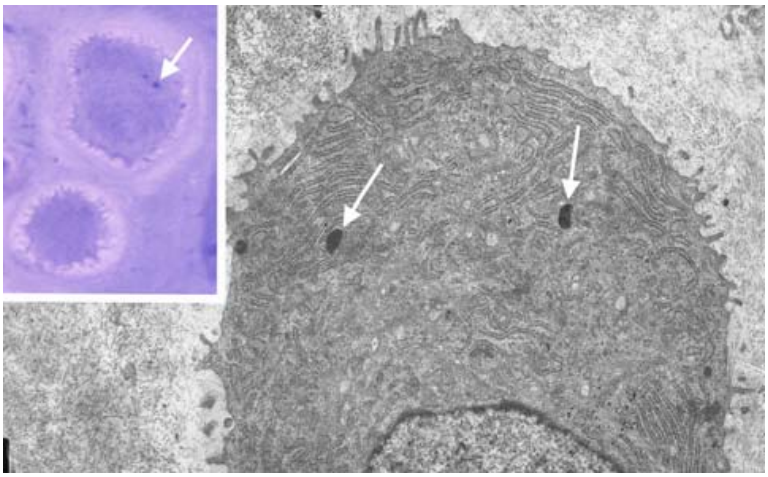

Figure 6. Transmission electron micrograph of a neochondrocyte showing particles (dense material) of the marker Monastral Blue B (arrows) in its cytoplasm after perichondrial implantation and pericyte labelling in the host tissue. Uranyl acetate and lead citrate, x14000. In the insert, a chondrocyte with blue particles (arrow) of the marker is shown in semithin section. Toluidine blue, x800. (Therefore, cartilage formation from chondrogenic cells present in the perichondrium was augmented by proliferation and differentiation of the labelled pericytes).

Prenatal stem cells expressing MSC-related markers have been isolated from primitive tissue of the umbilical cord (Wharton's jelly) (human umbilical cord perivascular cells - HUCPV cells) (209, 210), cord blood (211-216), , umbilical cord vein (217, 218), and amniotic fluid (amniotic fluid stem cells - AFSCs) (219). Although experiments have mainly demonstrated differentiation towards osteogenic lineage, the capacity to differentiate into chondrogenic lineage has also been observed (210, 214, 215), including expression of collagen II (210)..

8. CBCR. INDUCED PLURIPOTENT STEM CELLS (IPS CELLS)

Induced pluripotent stem cells (IPS cells) can be derived from somatic cells by introducing a small number of genes in these differentiated cells ("reprogramming"), expanded in culture, and differentiated for transplantation in cell therapy, without immunological rejection concerns. Therefore, IPS cells may lead to advances in regenerative medicine (220, 221). Indeed, for example, IPS cells can be generated from adult human fibroblasts (221, 223).

9. CBCR. GENETICALLY MODIFIED CELLS

Genetically modified cells have promising potential in cartilage repair (221). Indeed, cells expressing bone formation cytokines, including over-expression of BMPs, have been developed (223, 224). Thus, genetically modified cells could act as cartilage-inducing components.

10. CONCLUSION

We have reviewed one of the basic aspects in cartilage repair: its tissue and cell sources, considering advantages and limitations, as well as the biological mechanisms that explain usage, particularly to achieve the structure and durability of natural hyaline-like articular cartilage. Although this review concentrates on tissue and cell sources, in the sections where the cell-based cartilage repair was treated, we have briefly referred to other basic aspects, reviewed by several authors, highlighting interest in this area (See 9**,** 15, 125, 229, 132, 221, 225, 226, 227, 230) whose study covers: a) scaffolds that facilitate environment for chondrogenesis, including natural (collagen, fibrin, alginate, hyaluronan, agarose, chitosan) or synthetic materials that provide a biodegradable matrix with biochemical properties, supporting neomatrix deposition by chondrocytes, b) several signalling pathways and transcription factors (e.g.: Wnt, transforming growth factor β/bone morphogenetic protein signalling, PDGF, IGF-1, EGF, HGF), which act in migration (PDGF, IGF-1, EGF, HGF, TGF β), proliferation (EGF, PDG,F TGF β), and differentiation (dexamethasone, TGF β). Some may be locally introduced, modulating cell differentiation into cartilage, c) procedures to prevent escape of cells and anabolic cell mediators from the site of repair, d) strategies for cartilage integration, e) inhibition of cartilage degeneration and inflammation (TNF-x and IL-1 application), and f) gene transfer for optimization of cell chondrogenic capacity.

Further understanding of the cellular sources and their behaviour during proliferation and differentiation into cartilage open a promising new path in joint tissue engineering.

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