Studia Universitatis "Vasile Goldiş", Seria Ştiinţele Vieţii
Vol. 21, supp. 1, 2011, pp. 35-42

CELLULAR METHODS FOR *IN VITRO* ENGINEERING OF HUMAN AUTOLOGOUS CARTILAGE. A REVIEW

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ABSTRACT. Articular cartilage repair remains a challenge for regenerative medicine. This review summarizes our research findings that have been undertaken, with a particular emphasis on those techniques that have been introduced into our laboratory, via *in vitro* and preclinical studies.
Key words: osteoarthritis, chondrocytes, flow-cytometry, tissue engineering, regenerative medicine

A challenge in tissue engineering is the *in vitro* generation of human cartilage. Self-repair of human hyaline cartilage does not occur. Therefore, cartilage injuries initiate a progressive degradation that eventually results in osteoarthritis (Buckwalter J.A. and Lane N.E., 1997; Saxon L. *et al*., 1999).

Osteoarthritis is defined as an age-related disorder generally affecting articular cartilage. In adult vertebrates, articular cartilage is devoid of nerves, blood vessels or lymphatics and contains only one cell type, the chondrocyte. These resident cells are highly specialized and solely responsible for the maintenance and turnover of extracellular matrix macromolecules including type II collagen, aggregating proteoglycans and non-collagenous proteins (Muir H, 1995).

The biochemical performance of cartilage depends on the biochemical and biophysical properties of extracellular matrix macromolecules and thus on the normal metabolic activity and homeostatic status of chondrocytes (DeLise A.M. and Archer C.W., 1999). Consequently, the survival of chondrocytes is essential for normal cartilage function.The ageing of whole organisms is linked to cellular senescence and parallels apoptosis as a cellular response to stress. Replicative senescence occurs when cells stop dividing. Senescent cells remain viable but show alterations in phenotype and senescence associated β-galactosidase (SA-β-Gal) expression. In 2002, Price *et al.* (Price J.S. *et al.,* 2000; Radisic M. *et al,* 2003) presented the first demonstration of SA- β-Gal-positive cells in cartilage *in vivo* and showed that these cells occur predominantly associated with the osteoarthritic lesion. Martin and Buckwalter (Martin J.A. and Buckwalter J.A, 2002; Li Y. *et al.,* 2004] suggested that progressive chondrocyte senescence marked by expression of the senescence associated enzyme β-galactosidase, generate erosion of chondrocyte telomere length and mitochondrial degeneration due to oxidative damage cause the age related loss of chondrocyte function. This hypothesis was confirmed by Yudoh *et al.* (Yudoh K. *et al.,* 2005; Li Y. *et al.,* 2004) who showed that the oxidative stress induces telomere genomic instability, replicative senescence and dysfunction of chondrocytes and consequently, might be responsible for the development of osteoarthritis.

The results we obtained (Takacs-Buia L. *et al.,* 2008) and others (Hamilton D.W. *et al.,* 2005) show that human osteoarthritic (OA) chondrocytes showed a significant proportion of senescent cells (i.e. SA-β-Gal positive staining cells) as compared to "normal" chondrocytes. In this regard, staining for senescence-associated β-galactosidase is shown in Fig.1.

A number of recent published findings have suggested a strong association between apoptosis, age-related diseases and aging. From the phenotypic point of view, aging can be defined as the age-related progressive decline of physiological functions. Cellular senescence is a progression of events which cells move from an actively dividing to a non-dividing state, yet remaining metabolically active. In conjunction with the loss of the ability to divide, changes occur in the morphology, shape and physical appearance of the cells. Although the cells may remain viable for a long time, at the end of the process cell death usually occurs. There are two distinct patterns of cell death: necrosis and apoptosis. Massive cell injury, often accompanied by inflammation, can lead to necrosis. In contrast to the accidental death of cells, apoptosis is an active process with specific morphological changes which are characterized by chromatin condensation, nuclear DNA fragmentation, cell shrinkage, plasma membrane blebbing and membrane-enclosed cell fragments which are apoptosis bodies. Apoptotic cells are phagocytised by phagocytes thereby avoiding inflammation (Price J.S. *et al.,* 2002; Kerr J.F.R*., et al*., 1972).

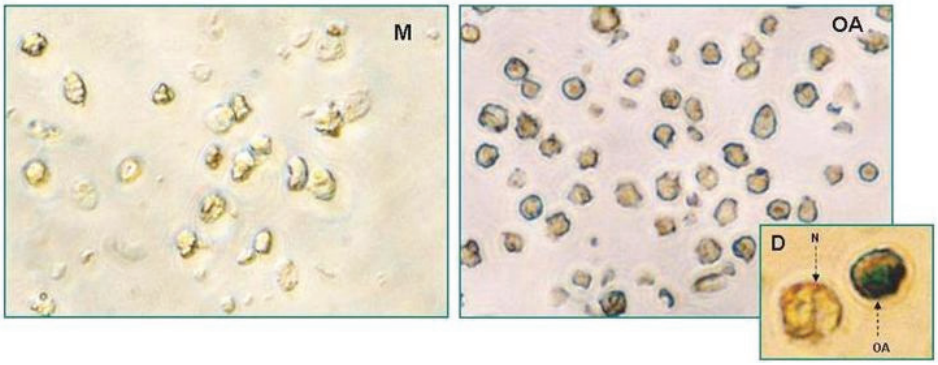

Fig.1. Microscopic analysis of human "normal" (M) and osteoarthritic (OA) chondrocytes stained with the chromogenic substrat (X-Gal) for senescence-associated β-galactosidase activity determination.

Chondrocyte number descrease with age. This has been correlated with increased frequency of cartilage fibrillation in the human femoral head (Martin J.A. and Buckwalter J.A., 2002; Vignon E., *et al.,* 1976). With reference to the relation between apoptosis and cartilage degeneration in osteoarthritis, a number of recent studies have shown that apoptotic cell death takes place at an increased rate in osteoarthritic cartilage (Yudoh K., *et al.,* 2005; Takacs-Buia L. *et al.,* 2008; Aigner T. and Kim H.A., 2002; Aigner T. *et al.,* 2004). In cartilage there are no macrophages and the fate of apoptotic bodies is uncertain. In those conditions, secondary necrosis would inevitably result with the disadvantage of uncontrolled release of lysosomal enzymes from apoptotic bodies causing serious damages to the extracellular matrix, including calcification, unless the cell had developed alternative mechanisms of destruction (Kerr J.F.R. *et al.,* 1972; Roach H.I. *et al.,* 2004). However, not all developmental cell deaths have the distinct morphological features of the "classical" apoptosis identified by Kerr *et al.* (DeLise A.M. *et al.,* 1999; Kerr J.F.R. *et al.,* 1972). There is a variety of cell types, including neuronal cells (Vignon E. *et al.,* 1976; Aigner T. and Kim H.A., 2002; Fukuda T. *et al.,* 1999; Sperandio *et al.,* 2000), erythrocytes (Aigner T. *et al.,* 2004; Bratosin D. *et al.,* 2008) and chondrocytes (Roach H.I. *et al.,* 2004; Fukuda T. et al., 1999; Roach H.I. and Clarke N.M.P.,1999; Roach H.I. and Clarke N.M.P., 2001) which demonstrate the existence of more than one pathway for programmed cell death (Sperandio *et al.,* 2000; Bratosin D. *et al.,* 2008; Leist M. and Jaattela M., 2001; Bursch W. *et al*., 2001).

To understand the mechanisms underlying the process of cell death in cartilage destruction, we investigated by flow cytometry, cellular viability (Cell viability calcein-AM assay) and apoptosis (Light scattering properties of chondrocytes analysis, study of chondrocytes death by Annexin-V-FITC and propidium iodide double - labelling, caspase-3 activity determination) of human chondrocytes isolated from normal and osteoarthritis cartilage (Takacs-Buia L. *et al.,* 2008).

The results we obtained show that apoptotic phenomenon appears to be consistent and relevant and could contribute to cartilage degeneration as previously described by others (Fig.2).

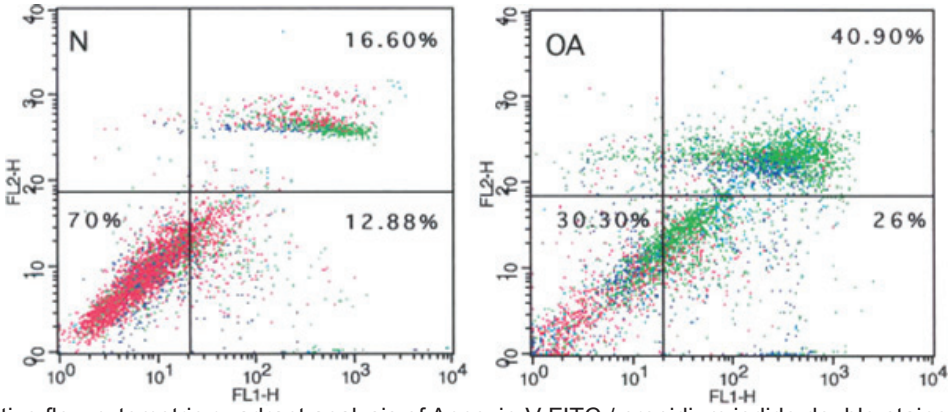

Fig. 2. Comparative flow cytometric quadrant analysis of Annexin-V-FITC / propidium iodide double-stained of human "normal" (N) and osteoarthritic chondrocytes(OA). Abscissae: log scale green fluorescence intensity of Annexine-V-FITC (FL-1). Ordinates: log scale red fluorescence intensity of propidium iodide (FL-2). Low left quadrant: viable cells (Annexin-V and propidium iodide negative cells); low right quadrant: apoptotic cells (Annexin-V positive and propidium iodide negative cells); upper right quadrant: dead cells (Annexin-V and propidium iodide positive cells). % refers to the cell percentage of each population. Number of counted cells: 10,000. Results presented are from one representative experiment of three performed.

On the basis of results accumulated up to now on the mechanism of chondrocyte cell death, it appears that a combination of factors are probably responsible for accelerated senescence of chondrocytes, leading to chondrocyte apoptosis and ultimately resulting in degradation of cartilage. These results represent in the future a potential therapeutic target in articular diseases to limit chondrocytic cell death and to prevent the development and progression of osteoarthritis.

The ability to manipulate and reconstitute tissue structure and function in vitro has tremendous clinical implications and is likely to have a key role in cell therapies in coming years. Developments of therapeutic strategies for cartilage repair have increasingly focused on the promising technology of cell therapy based on the use of autologous chondrocytes or of other cell types to regenerate articular cartilage in situ. Autologous Cell Implantation (ACI) is a currently practiced cell-based therapy to repair cartilage defects. Several strategies have been explored to expand the number of chondrocytes *ex vivo*. Autologous chondrocytes are recovered from the patients and amplified in tissue culture prior to re-implantation. However, these methods are unable to provide sufficient quantity of chondrocytes with unaltered phenotype because chondrocytes propagated in monolayer culture lose their original characteristics by assuming a fibroblastoid morphology and shift from production of collagen.

Tissue engineering is part of a new wave of developments in biomedicine and a base for new treatments for musculoskeletal diseases. This has emerged through a combination of many developments in biology, material science, engineering, manufacturing and medicine. The engineered tissue constructs attach, migrate, and integrate with native tissues, thereby meeting important requirements for tissue reconstruction and/or regeneration. Chondrocyte is a unique cell type in articular cartilage tissue and is essential for cartilage formation and functionality.

The regeneration of functional hyaline cartilage, using expanded chondrocytes and biodegradable polymers, is an important research area in the field of cell-based therapies and tissue engineering that may provide a solution for cartilage reconstruction. Tissue engineering plays an increasingly important role in the functional repair of diseased and missing cartilage that complements reconstructive and orthopedic surgery (Roach H.I. and Clarke N.M.P., 1999; Roach H.I. and Clarke N.M.P., 2001; Randolph M.A. *et al*., 2003; Koch R.J. and Gorti G.K., 2002). A common remedial approach is to harvest cells from a small tissue biopsy from the donor, expand them in vitro, and seed the population into biodegradable and porous scaffolds, producing an enhanced semi-artificial structure for cell distribution and population in culture. The cell-cultivated construct is then implanted in vivo for extracellular matrix production and, eventually, for cartilage regeneration. It has been asserted that a high-quality cell/scaffold construct requires high spatial uniformity of seeded cells, high scaffold cellularity to enhance the tissue development rate, and sufficient nutrient and oxygen supplies to maintain cell viability (Leist M. and Jaattela M., 2001; Zhao F. and Ma T., 2005; Almarza A.J. and Athanasiou K.A., 2005). In producing high-quality cell-assisted implantable constructs, cell density may significantly affect cartilage and bone formation (Bursch W. *et al.,* 2003; Almarza A.J. and Athanasiou K.A., 2005). Primary isolated chondrocytes from a small biopsy specimen, which may itself be diseased, hinder the in vitro expansion of a clinically useful number of chondrocytes and they have the tendency to de-differentiate in *ex vivo* culture. Unfortunately, long periods of in vitro culture may induce cellular dedifferentiation, limiting the utility of the procedure for patients requiring tissue therapy under expedited terms and yet increasing the possibility of *in vitro* contamination leading to infection *in vivo* (Randolph M.A. *et al.,* 2003; Vujak-Novakovic G. and Radisic M., 2004; Radisic M. *et al.,* 2003). To prevent elongated cell-culture requirements and improve cell uniformity, have been investigated a number of methods to increase cell-seeding density (Randolph M.A., 2003; Koch R.J. and Gorti G.K., 2001; Vujak-Novakovic G. and Radisic M., 2004). A cell-seeding device, utilizing the synergistic effects of vacuum, centrifugal force, and fluid flow, has been used with porous scaffolds (Li Y. *et al.,* 2001). Magnetic nanoparticles have been used to guide fibroblast cells even through commercially available scaffolds; the magnetite nanoparticles were directed with a magnetic field to induce ‘‘mag-seeding,’’ subsequently increasing cell density and seeding efficiency (Liu X. and Ma P.X., 2004).

A multitude of studies have been done using different ways to culture chondrocytes. Recently, we compared two methods for obtaining and cultivation of human osteoarthritic chondrocytes, by Carell method and by classical method (Fig.3).

Several strategies have been explored to expand the number of chondrocytes *ex vivo.* However, these methods are unable to provide sufficient quantity of chondrocytes with unaltered phenotype because chondrocytes propagated in monolayer culture lose their original characteristics by assuming a fibroblastoid morphology and shift from production of collagen. To maintain the original phenotype in monolayer culture and to expand cell proliferation as well as maintaining synthesis of cellular products, primary human chondrocytes isolated by enzymatic digestion were cultured in a patented DMEM medium supplemented with Ac- Gly-Gly-OH dipeptide (French Patent 0707113 of 10/10/ 2007; European PatentWO 2009/080914 A2; US Patent Application 20100256234 Published on October 7, 2010- ‘‘Method of stimulating proliferation of differentiated cells belonging to chondrocytes line’’).

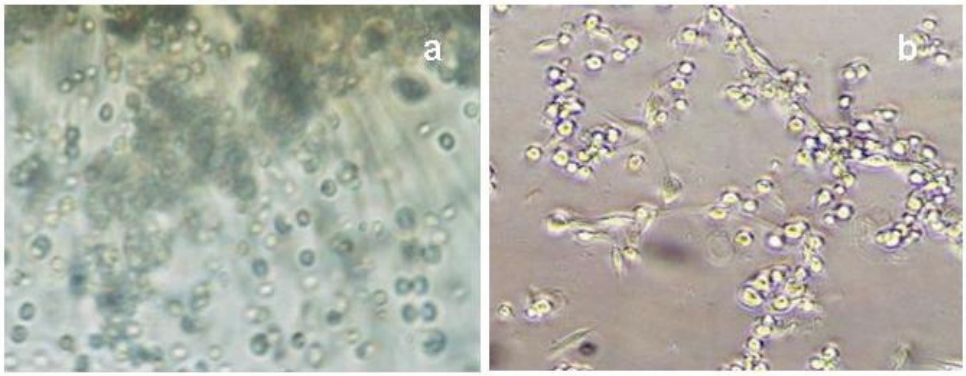

Fig. 3. Comparative microscopic analyses of human osteoarthritic chondrocytes after 6 days of culture by Carell method (a) and by conventional cell-seeding method (b).

The results we obtained provide that proliferation and viability of chondrocytes cultured in presence of DMEM medium containing Ac-Gly-Gly-OH were higher and thus can be used in the culture of chondrocytes devoted to reconstructive clinical procedures (Fig 4).

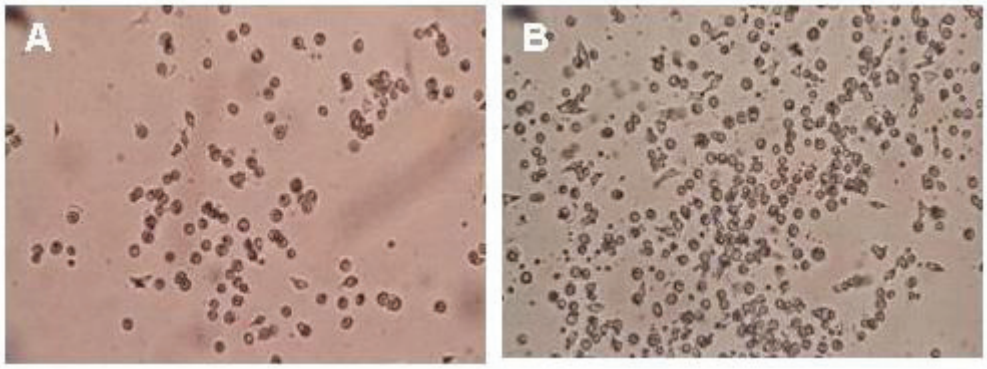

Fig. 4. Cell-seeding density to provide favorable cell growth. Chondrocytes (cells of OA cartilage) cultivated for autotransplant .in DMEM medium (A) and cultivated according to the method patented by us (B). It can easily be seen the massive cell multiplication.

Uniform cell distribution and proliferation in engineered scaffolds are also critical issues in regenerative medicine. The primary goal of research in cell seeding and cultivation is to promote cell population and uniformity in scaffolds before they are implanted in vivo. Production of high and uniform cell density is limitated, because due to the complexity of scaffold structure, the migratory abilities of the cells in the structure during seeding are insuficient, as also the tendency of cell movement to the periphery and out of the periphery and out of the implantable materials in (DeLise A.M. *et al.,* 1999; Martin J.A. and Buckwalter J.A., 2002; Hamilton D.W. *et al.,* 2005; Soletti L. *et al*., 2006). Consequently, prolonged in vitro cell culture has been required to meet effectual cell density and distribution profiles prior to implantation (Bursch W. *et al.,* 2001; Koch R.J. and Gorti G.K., 2002; Almarza A.J. and Athanasiou K.A., 2005; Shimizu K. et al., 2006; Ushida T. *et al*., 2002; Guo J.F. *et al.,* 1989). Seeding cells on scaffolds in bioreactors to enhance cell density and uniformity has also been investigated (Leist M. and Jaattela M. 2001; Koch R.J. and Gorti G.K., 2002; Hamilton D.W. *et al.,* 2005; Ushida T. *et al*., 2002; Paige K.T. *et al.,* 1995; Aydelotte M.B. *et al.,* 1998). For generating cartilage in vitro that have not only the morphology but also the physiological behaviour of chondrocytes, it is necessary to culture the cells in a three-dimensional arrangement. It is known that a three-dimensional arrangement leads to a specific cell shape and environmental conditions determining gene expression and behavior of cells.

A more simplified method was pursued to increase seeded cell density using protein gels to temporarily engage a well-distributed cell mixture with porous scaffolds (Yang K.G. *et al.,* 2006; Grandolfo M. *et al.,* 1993). Various gels prepared as cell-encapsulating scaffolds have been investigated for high cell-density seeding. In Fig.5 we present a simple gelation method using alginate gel, a natural polymer, as gelling material to incorporate a high cell density in 3D porous scaffolds during cell seeding and restrain the cells in the scaffolds from escaping during the subsequent cell culture, aimed to achieve ultimate high cell density and uniformity in porous structures (Buia-Takacs L. *et al*., 2010).

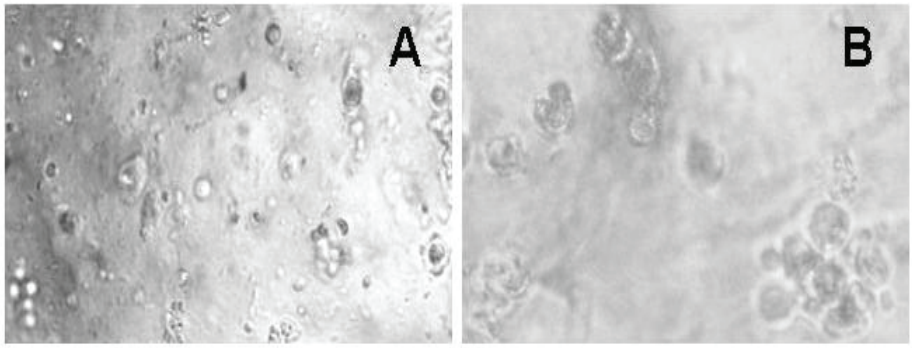

Fig. 5. Analysis by phase inverted optical microscope (20X) of human osteoarthritis chondrocytes cultured by microencapsulation in alginate gel after 3 (A) and 7 days (B).

Alginate gels have been studied for cartilage tissue engineering applications as a matrix for cellular encapsulation and culture (Ushida T. *et al.,* 2002; Soletti L. *et al.,* 2006). The method involves no complex equipment, and the scaffold system is entirely constructed from natural polymers that have proven their biocompatibility and are favored for a wide spectrum of tissue engineering applications (Yang K.G. *et al*., 2006).

Our research compared morphological changes, cellular viability and apoptosis of human chondrocytes cultured in alginate gel and conventional cell-seeding methods by applying flow cytometric methods provide the usefulness of the gel in the culture of chondrocytes for reconstructive clinical procedures (Buia-Takacs L. *et al.* 2010).

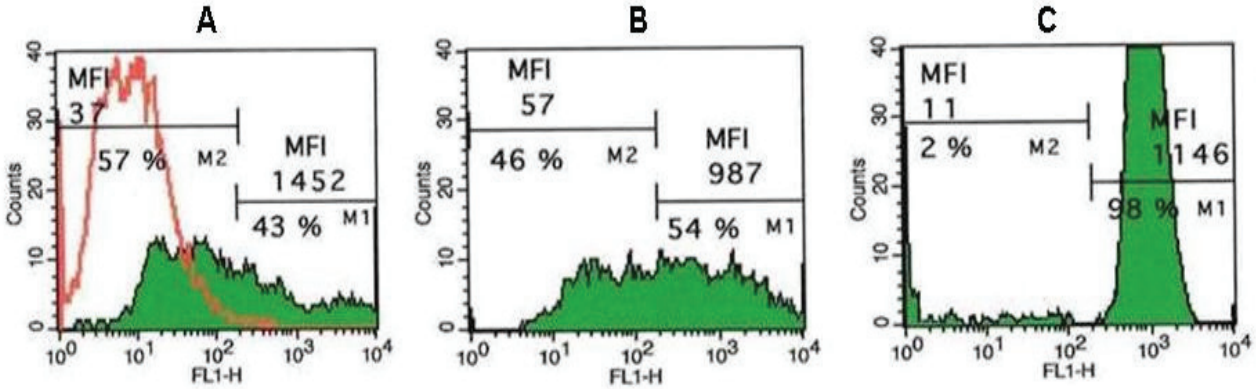

Fig. 5. Comparative flow cytometric histogram analysis of human osteoarthritic chondrocytes viability by cell esterase activity measurement using calcein-AM. A: Osteoarthritic chondrocytes before seeding B: after 7 days of monostrat layer culture and C: after 7 days of culture in alginate gel. Abscissae: log scale green fluorescence intensity of calcein (FL1). Ordinates: relative cell number. M1: region of fluorescent cells with intact membranes (living cells) and M2: region of nonfluorescent cells with damaged cell membranes (dead cells). Numbering refers to the cell percentage of each population. Number of counted cells: 10,000. Results presented are from one representative experiment of three performed.

Our data, in the context of other published findings, underline the advantages of culturing chondrocytes with alginate microspheres. In fact, chondrocytes cultured using this method represent a single phenotypic population with a tight cellular uniformity as shown by the FSC/SSC analyses and show a very high viability (over 90%). These findings recommend the implementation of this biotechnology in tissue engineering.

The results we obtained also emphasize the advantages of using flow cytometry to quantitatively evaluate various methods of growing chondrocytes in tissue culture. This analysis method was very useful in characterizing cell viability, which may represent the starting point for using this method to screen for growth factors and better culture conditions.

CONCLUSIONS

Because the capacity of articular cartilage for repair is limited, defects in this cartilage are a major clinical problem. To regenerate articular cartilage by cell transplantation, it is essential that cells proliferate without losing their capacity for differentiation.

ACKNOWLEGEMENTS:

This work was supported by Structural Funds POSCCE-A2-O2.1.2: OPERATIONAL PROGRAMME "INCREASE OF ECONOMIC COMPETITIVENESS" Priority axis 2–Research, Technological Development and Innovation for Competitiveness, Operation 2.1.2: Complex research projects fostering the participation of high-level international experts, Nr. Project SMIS-CSNR: 12449, Financing contract nr. 204/20.07.2010, „Biotechnological center for cell therapy and regenerative medicine based on stem cells and apoptosis modulators"-BIOREGMED. Authors are also grateful with many thanks to Dr. Francis Goudaliez, Director of MacoPharma C°, Tourcoing, France, who provided technical support (FACScan flow cytometer).

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