10

Cartilage Tissue Engineering Using Mesenchymal Stem Cells and 3D Chitosan Scaffolds – *In vitro* and *in vivo* Assays

Natália Martins Breyner, Alessandra Arcoverde Zonari, Juliana Lott Carvalho, Viviane Silva Gomide, Dawidson Gomes and Alfredo Miranda Góes

Universidade Federal de Minas Gerais (Federal University of Minas Gerais), Institute of Biologic Science, Department of Biochemistry and Immunology, Brazil

1. Introduction

Cartilage tissue has only one cell type, the chondrocyte, wich is immerse in extracellular matrix composed mainly by collagen type II. Because of such properties, cartilage tissue doens't heal spontaneously after a lesion, which with time becomes progressive and chronic. Cartilage lesions may be caused by automobile and sport accidents, as well as by normal wear due to age, and usually generate severe pain and difficulty of mobility in patients. Therefore, cartilage disease is a common type of lesion to which everyone is susceptible and represents a very important public health problem in the world (Willians et al, 2006).

Initial therapies to treat cartilage lesions included replacement surgery with artificial or natural organs and tissue grafts. Artificial and natural organ transplants and tissue grafts, on the other hand, are able to fully replace organs or tissues, but require continuous and permanent immune therapy to reduce immunological response to graft and to increase the longevity of transplanted tissue. Therefore, although major progresses were done in the field of cartilage tissue regenerative medicine during the years, current therapies still present limitations. Moreover, no adequate cartilage substitute has been developed. Thus, most of the severe injuries related to cartilage are still unrecoverable or not adequately treated. Therefore, these methods are helpful but need modification to develop better novel or alternative therapies (Ikada et al, 2006 and Tabata et al, 2009).

In such context emerges tissue engineering, which has been defined by Langer and Vacanti as: ''an interdisciplinary field of research that applies the principles of engineering and the life sciences towards the development of biological substitutes that restore, maintain, or improve tissue function" (Salgado et al, 2004).

Tissue Engineering or Bioengineering is based on three elements: (i) cells; (ii) scaffolds and (iii) signalling molecules. These elements integrate themselves and promote the new tissue development (Langer and Vacanti, 1993; Ikada et al, 2006 and Chiang et al, 2009). In order to mimic tissue structure, tissue engineering also requires 3 dimensional cell cultures, which, in contrast to traditional bidimensional cell culture, has only been developed recently. Nowadays, it is beyond dispute that this cell culture strategy presents many advantages,

including continuous exchange of nutrients and oxygen, metabolite removal and mechanical and chemical stimuli. All these factors allow and facilitate cell differentiation and proliferation (Ikada et al, 2006 and Tabata et al, 2009).
Used as scaffolds to 3D cultures, biomaterials studied in tissue engineering can be derived from natural or synthetic sources and may belong to one of three classes: metals, ceramics, or polymers. Once transplanted, biomaterials can be reabsorbed in vivo and replaced by new tissue (Ikada et al, 2006 and Tabata et al, 2009).

1.1 Cells

Tissue engineering strategy demands high numbers of cells, therefore, ideal cell sources for tissue engineering application must be easily isolated, expandable to higher passages, be non-immunogenic and have a protein expression pattern similar to the tissue regenerated (Salgado et al, 2004).
Chondrocytes derived from autologous tissue constitutes the most obvious choice to be used in tissue engineering, for their absence of immunogenicity and possibility of limited expansion in vitro. However this methodology suffers from many limitations, such as the generation of a second site of cartilage lesion, as well as the limited amount of cells obtained at the end of the procedure.
As an alternative, stem cells present a great therapeutic potential due to their capacity of differentiation to many cell lineages. These cells are able to self-renew and proliferate for long periods *in vitro* (Zuk et al, 2002 and Mountford et al, 2008).
Stem cells are divided into two great classes: adult and embryonic stem cells and also divided based on their differentiation potential. even though they may also be described based on their differentiation potential. According to this latter classification, the zygote and the cells produced by its first two divisions are considered totipotent, or capable of generating any cell of the embryo as well as the trophoblast. Continuing the embryo development, at the fifth day the embryo is constituted of two cell types, which compose the trophectoderm and the inner cell mass. Cells from the inner cell mass (ICM) are also called embryonic stem cells and are classified as pluripotent, for their capacity of generating the three embryo germ lines. ICM cells are not totipotent because they lack the capacity to generate extra-embryonary tissues. Later in the development, present in fully differentiated tissues, there are multipotent stem cells, which present more limited differentiation potential, being restricted to generate cells from the same embryonary origin as the tissue where they are found. However, according to the literature, multipotent stem cells may present a broader differentiation capacity then initially expected (Friedenstein et al, 1966; Owen et al, 1988; Zuk et al, 2002 and Conrad et al, 2004).
In 2007 yet a another type of stem cell was generate in vitro, the induced Pluripotency Stem Cell (iPSC). This new type of cells is produced by reprogramming adult cells, such as fibroblasts to a pluripotent state similar to that observed in embryonic stem (ES) cells, by retroviral transduction of some genes (Nanog, Oct4, Sox2, c-Myc, Klf 4 and Lin 28). The forced expression of such genes was capable of giving differentiated adult cells pluripotent differentiation capacity akin to the embryonic stem cell. This technique was termed cellular reprogramming (Takahashi et al, 2006; Yu et al, 2008 and Yamanaka et al, 2009).
It is important to aknowledge each stem cell type properties, for all stem cell types present inherent advantages and disadvantages, depending on their application.
Among adult stem cells there are:

- *Mesenchymal stem cells* (MSCs), which take part of the mesenchyme of varied tissues such as the bone marrow, adipose tissue, brain, dental pulp and skin, and are capable of differentiating into many cell lineages. MSCs present great potentials to the treatment of several diseases due to their low immunogenicity, immunomodulatory properties, the possibility of autologous transplantation, easy isolation and *in vitro* proliferation possibility.
- The bone marrow was the first source of MSCs described in the literature, and still remains the more thoroughly studied stem cell type. Also present in the bone marrow, there are:
- *Hematopoietic stem* cells (HSCs), which differentiate into all the hematopoietic and lymphoid cells from the blood. Therefore, HSCs are studied due to their roles in leukemia and other blood diseases. Usually, the treatment of such diseases include the substitution of the sick bone marrow to a healthy one, and in accordance to such fact, studies involving HSCs are mainly focused on how HSCs behave in different live organisms. Autologous grafts, or the implantation into a genetically similar live organism, may be performed in order to treat blood related diseases, as well as heterologous implantations, or grafting into genetically different live organisms. Presently, these different graft types show paradox behaviours. Heterologous grafts cause immune rejection in the host, requiring the host to be continuously submitted to immunossuppression. This therapy can lead to patient death due to the absence of an immune response to opportunist pathogens, however, this treatment is still commonly used today. In cases where the patient's conditions are good, cells can be extracted from the patient himself. This method is named autologous transplantation and is not susceptible to host rejection (Friedenstein et al, 1966; Owen et al, 1988, Conrad et al, 2004; Davila et al, 2004 and Gregory et al, 2005).

Even though stem cells derived from bone marrow have been well studied, they do not constitute the ideal mesenchymal stem cell source, due to the limited extent of MSC isolation (low extractable quantity of tissue) and donor discomfort. Therefore, new alternative sources have been proposed, including the adipose tissue. Adipose tissue is an excellent tissue to obtain great quantities of mesenchymal stem cells and it presents low discomfort when compared to bone marrow. According to Zuk, 2002, adipose tissue is a viable source to obtain mesenchymal stem cells, and these cells present similar characteristics to bone marrow MSCs.

Since the discovery of so many MSC sources, the International Society for Stem Cell Therapy postulated that a cell will only be considered a MSC if it presents 3 characteristics: 1. Being able to attach to cell culture surface; 2. Specific surface antigen expression; 3. Multipotent differentiation potential (osteoblast, adipocytes, chondroblasts) (Dominici et al, 2006). MSCs must then be capable of differentiating into cartilage, bone and muscular cell lineages, self-renewing and proliferating *in vitro*.

Embryonic stem cells (ESCs) are derived from the inner cell mass of the blastocist and present great moral, religious and ethical barriers due to their isolation technique, which leads to embryo destruction. They constitute a very promising stem cell type considering the tissue engineering field, for their pluripotent differentiation potential and unlimited proliferation capacity. Besides their ethical issues, ESCs also present the possibility of when injected *in vivo,* to produce teratomas. Thus, even though ESC present endless wonderful possibilities to be used in several science fields, more studies are necessary to ensure their safety and efficiency to be used in humans (Takahashi et al, 2006). In 2009, the biotech company Geron received the FDA approval to start the first human clinical trial of embryonic stem cell-based therapy in

order to assess the safety of using differentiated embryonic stem cells to treat spine cord injuries. In 2011, they finally injected the first cells in patients and are waiting for results.
With respect to the potential of these stem cells, researchers have developed methods to trace these cells in both live and post-mortem stages. This is very important, because there are many routes and ways to introduce stem cells in an organism. If we can determine where these cells are going, wheter they can stay inside the 3D scaffold and differentiate or if they can stimulate others cells to migrate and graft, many unanswered questions will be addressed. Therefore, researchers have developed tracing techniques to locate injected cells in the organism. In basic science models, genetically modified transgenic organisms that express fluorescent proteins (FP) have been used to generate cells expressing such markers. Green fluorescent protein, the first FP generated, was derived from fluorescent seaweeds found in the US. After the isolation and characterization of the protein, its gene was introduced in mice and many other animals, so that those animals fluoresce when exposed to UV radiation. Cells taken from these transgenic animal and introduced into other non-transgenic animal (of similar lineages) do not present rejection problems and allow for trafficking of these cells (Ogawa et al, 2004). Many other tracing strategies were also developed including: radioisotopes, DNA and mitochondria dyes, as well as fluorescent microbeads. (Ogawa et al, 2004).

1.2 Tissue engineering

Tissue engineering or Bioengineering, as definided by Langer and Vancanti, constitute a innovation in regenerative medicine and is based on three elements (i) cells; (ii) scaffold and (iii) growth factors. Scaffolds can be bi-dimensional and three-dimensional, bi-dimensional structures allow us to observe only cell behavior with reference to medium composition, cell-cell interaction, cell viability and cell differentiation. However, three-dimensional structures allow us more physiologically realistic factors including dynamic fluids rich in O_2, mechanical forces, and cell adhesion but this interaction is three-dimensional and can be modify cell behaviour. For instance, nowadays it is beyond dispute that scaffolds are sources of instructive signals for cell differentiation, migration, proliferation and orientation, and of paramount role in phenotype maintenance. Therefore, many studies have searched for great biomaterials that can be used as a surrogate for extracellular matrix (ECM) tissue (Ikada et al, 2006; Chiang et al, 2009; Tabata et al, 2009 and Mingliang et al, 2011).
One of the main goals of Tissue engineering is to create a scaffold that can mimic ECM due to better cells, and micro-environment interactions. This interaction permits cells adhesion, migration, proliferation, differentiation and long-term viability (Bacakova et al, 2004). To produce a new organ or tissue we need scaffolds that are biodegradable and biocompatible. These structures need stable and appropriate porosity and architecture to permit formation of a vascular net able to give nutrients and O_2. These scaffolds should be gradually degraded to be occupied by new tissue formed by the interaction among the 3D scaffold, stem cells and growth factors.
To construct our 3D structure, we use chitosan and gelatin. Chitosan is derived from chitin presents in arthropods, including shrimp and crab. It is a polysaccharide very similar to glycosaminoglycans present in cartilage ECM. It is acid soluble, forms like-gel solutions and is water insoluble. Therefore, chitosan is available in nature and easily manipulated beyond its seemed ECM polysaccharides state (Roughley et al, 2006 and Dong et al, 2010).
Gelatin is derived from collagen, mainly proteins presents in cartilage ECM. Cartilage tissue is composed of type II colagen. In spite of this, gelatin is not composed of type II collagen, it present RGD motifs like all types collagen and this motif is able promote cell adhesion and

differentiation and/or promote phenotype maintenance. Gelatin is water and acid soluble and is able to mix to chitosan gels (Tortelli and Cancceda, 2009 review). Both biomaterials have properties similar to cartilage ECM and they are biocompatible and biodegradable and are able to form porous where fluid can pass.

The connection between chitosan and gelatin is termed reticulates. Reticulates have a property to make chemical connections between molecules. These connections are stable and require the maintenance of stable scaffold architecture. In this study, we used two reticulates: Genipin and Glutaraldehyde. These reticulates are used beyond stable architecture, to increase degradation time *in vivo*. The importance of that controllable degradation is that it guarantees new tissue formation (e.g. ECM secretion) by differentiated cells. This way, the scaffold is able to provide a temporary matrix for developing cells, such as a support for cell attachment and tissue neomorphogenesis (Bacakova et al, 2004 and Mironov et al, 2009).

Glutaraldehyde is the reticulate most used in tissue engineering; it helps the 3D matrix creation through freeze and freezing drying. This process creates pores inside the scaffold and these pores are favorable to cell development, adhesion, proliferation and differentiation beyond the exchange of metabolites and food (Hofmann et al, 2009). Despite being most used, glutaraldehyde presents high levels of citotoxicity and limited reactivity with acetylates molecules. Therefore, a new approach is needed to find new reticulate that overcome all prior difficulties.

There is a new reticulate that had been studied, genipin. Genipin is derived from vegetable (Gardennia jasminoides, ELLIS) and it presents good capacity to increase mechanical properties for biomaterial-based protein. It forms pores and delays degradation that favors new tissue formation (Al amar et al, 2009 and Beier et al, 2009). This work verified which reticulates are better for our goal, the design of cartilage tissue.

The ultimate goal of tissue engineering is to design and fabricate close-to-natural functional human organs suitable for regeneration, repair and replacement of damaged, injured or lost human organs. Without tissue engineering, living functional human organs can be produced only during natural embryonic development. Therefore, according of Miranov and colleagues (2009) one of the most logical and obvious ways to look for possible alternatives to solid biodegradable scaffold-based tissue engineering approaches is to understand how tissue and organs are formed during normal embryonic development. Organ printing (one biomedical application of rapid prototyping) is an emerging transforming biomimetic technology that has potential for surpassing traditional solid scaffold-based tissue engineering (Miranov et al, 2009).

1.3 Signalling molecules

As the third pillar to tissue engineering, besides cells and scaffolds, it is important to deal with media constitution. All biochemical molecules present in culture media are able to stimulate cells. These stimuli differentiate all cells in culture, so theses molecules are important in creating new tissue or regenerate damage tissue. Due to stem cell differentiation capacities, they are cultivated in special medium that stimulates them during the differentiation process. Here we describe signaling molecules that stimulate chondrogenic differentiation of stem cells, mainly type II collagen secretion (Raghunath et al, 2005).

There are key molecules to chondrocyte differentiation: TGF-β and dexamethasone (Raghunath et al, 2005; Betre et al, 2006; James et al, 2007; Melrose et al, 2008 and Mueller et al, 2008). According to Lee and colleagues (2004) and Melrose and colleagues (2008), TGF-β induces the synthesis of type II collagen through Sox-9 pathway. Mueller and colleagues (2008) agree that

dexamethasone causes chondrocyte hypertrophy because it induces type X collagen synthesis from cells. If this process occurs, the neotissue will suffer mineralization and will lose its properties (e.g. smoothness). However, most studies that aim to achieve chondrogenic differentiation use such molecules in differentiation medium, as the beneficial aspects outweigh disadvantages and such molecules seem to necessary to the chondrogenic differentiation process (Otto et al, 2004; Medrado et al, 2006; Huang et al, 2006 and Koay et al, 2007).
Still considering the example given, one viable option to obtain cartilage tissue in vitro and adequate to tissue engineering application is the combination of chitosan and gelatin, reticulated either by glutaraldehyde or genipin, seeded with mesenchymal stem cells. Here we show *in vitro* analysys performed to verify stem cell behavior in control (no differentiation stimuli) and differentiation medium. We tested whether differentiated cells in 3D scaffolds maintained differentiated phenotype *in vivo*.

2. Materials and methods

All animals were used and sacrificed in accordance to CETEA – UFMG (Ethical Committee Animals Experiments) # 153/2006. We used 30 rats (Lewis, male and female, 4 to 6 weeks old) from Physiology Department of UFMG and 5 rats (Lewis transgenic GFP - Lew-Tg e-GFP, 4 to 6 weeks old) from Missouri University (USA).

2.1 Cells and characterization by flow cytometry

Mesenchymal stem cells were obtained from rat adipose tissue (Lewis and Lewis eGFP). Rats were killed with anesthetic overdose and adipose tissue was removed from the abdominal region and it was maintained in conical tubes with DMEM supplemented 10% serum bovine fetal (SBF). After a few minutes, this tissue was digested with collagenase type II for 60 minutes, in 37ºC and 5% CO_2. Every 15 minutes this solution was manually shaken. After this procedure, this solution was centrifuged for 10 minutes (1400rpm). The pellet was recovered and cultivated in DMEM with 10%SBF for 3 days. On the third day, the medium was collected, centrifuged to recover the non adherent cells and adhesive cells were cultivated in DMEM + 10% SBF. When this culture became confluent (80 to 90%), cells were trypsinized and expanded to new culture flasks (Zuk et la, 2002).
These cells were phenotyped by flow cytometry and used for differentiation studies at the 4^{th} pass by flow cytometer. This procedure used anti-CDs antibodies to label markers present on the cell surface. The CDs are markers present in mesenchymal stem cells, hematopoietic stem cells and other cell types. We used CD54, CD91 and CD73 as MSC markers and CD45 as HSC (Zuk et al, 2002 and Ucelli et al, 2006). All cells are fixed with formaldehyde (2%) and analyzed by FACScalibur (USA). For control, we use only secondary antibody and selected the gate for cells to be analyzed we used no marker cells. Around 20,000 events (minimum) were used for fluorescence capture in Cell Quest software. All data were analyzed by WinMid 2.8 software. This procedure was performed according Zuk et al, 2002. To conduct flow cytometry we used $1x10^6$ cells and stained with antibodies (anti-CDs) isolated before we used secondary antibody (FITC for Lewis and PE for Lewis eGFP).

2.2 Chondrogenic medium

Chondrogenic medium was based in protocol by Medrado and cols (2006), Huang and cols (2006), Koay and cols (2008) and Breyner and cols (2010), from these we used 10μg/L recombinant TGF-β3 (Bioclone), 10^{-7} M dexamethasone (Sigma) with 1% SBF.

2.3 Cell differentiation

Mesenchymal stem cells were cultivated in T75 flasks with control medium (DMEM + 10%SBF) and chondrogenic medium for 1, 3, 6 and 9 weeks. To verify to differentiation process immunofluorescence was perfomed using antibodies for collagen II, CD54, CD90,CD45 and CD73, osteocalcin (Zuk et al, 2002 and Huang et al, 2006).

2.4 Immuno-fluorescence

Mesenchymal stem cells ($1x10^5$) cultivate in normal medium and chondrogenic medium for 1, 3, 6 and 9 weeks were used for immunofluorescence. To conduct this experiment, we used cells from eGFP rats. Due to green GFP fluorescence, these cells were able to fluoresce under confocal microscopy (green) and we used others markers with red fluorescence. Initially, when we used to identify chondrogenic differentiation, we used markers from msenchymal stem cells (CD90, CD54and CD73), hematopoietic stem cells (CD45), cartilage-specific (type II collagen) and bone-specific (osteopontin). Cells were seeded in glass laminules. By 48 h, all cells were fixed with formaldehyde (2%) and stained with each antibody. Each well was washed with cold PBS 0,15M and the secondary antibodies (polyclonal anti-rat IgG made in rabbit, Molecular Probes) were applie and the cells were observed in confocal microscope (Soliman et al, 2008).

2.5 3D scaffolds

We developed two kinds of 3D scaffolds with similar chitosan (85% deacetylated, Sigma) and gelatin (Sigma) ratios. The difference between scaffolds is the reticulate use: glutaradehyde (0,1%, Sigma) or genipin (0,1%, Challenge Bioproducts). In order to solubilise chitosan we diluted it in acetic acidic (0,5mM) and gelatin was diluted in water. Misture of chitosan to gelatine was performed maintaining the ratio of 3 parts of chitosan solution to 1 part gelatin solution. Immediately, we shook these solutions and distributed 1mL/well in 24 wells plate. These plates were shaken in mechanic shaker overnight, protect from light. On the next day we added 1mL reticulate to each well. After 60min, these plates were frozen at -20°C overnight and then were transferred to -80°C. Plates were freeze-drying for 8 hours. The cilinders (3D scaffolds) were sterililzed (120°C) and used for cell culture (Guo et al, 2006 and Yamane et al, 2005). The scaffolds were analyzed at the Department of Metallurgical and Materials Engineering, Federal University of Minas Gerais. The matrices were covered with gold (Sputter Coater - SPI Supplies) for 90 sec at 13mA. The images were obtained by means of scanning electron microscope (JEOL 6360LV), at 15kV and 750mA, to qualitatively assess the pore interconnectivity and size.

2.6 Cell and 3D scaffold

GFP cells were used because of the need to trace these cells; they were processed the same way as the MSC characterized by IMF. These cells were cultured in a 3D scaffold with chondrogenic medium for 3 weeks and surgically grafted in the rat subcutaneous dorsal region. After 6 weeks, all animals were dead and the samples were analyzed by IMF to verify presence of collagen type II. This assay was conducted because of the need to know whether differentiated cells can dedifferentiate after implantation (Janune et al, 2006).

2.7 Statiscal analyzes

All data are presented as an average ± standard deviation (SD). To test the significance of the observed differences between the study groups, a statistical evaluation was carried

out using a one-way ANOVA. A value of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1 In vitro assay

3.1.1 Cell characterization

Cells were characterized by Immunofluorescence using GFP, MSC membrane surface markers CD90, CD73 and CD54, HSC surface marker, CD45 antibodies to show the absence of cell contamination with another source of stem cells. All cells were derived from GFP-Lewis rats and verified with an anti-GFP primary antibody and PE (phicoerithrine) conjugate secondary antibody. Our results showed that all cells were positively for GFP (red, Fig. 1). For other markers, we used secondary antibodies with FITC (fluorecein isothiacyanate, Fig 1) and we observed that, cells were negative for CD45 and positive for CD90, CD73 e CD54. Nuclei were marked with DAPI (blue, Fig. 1).

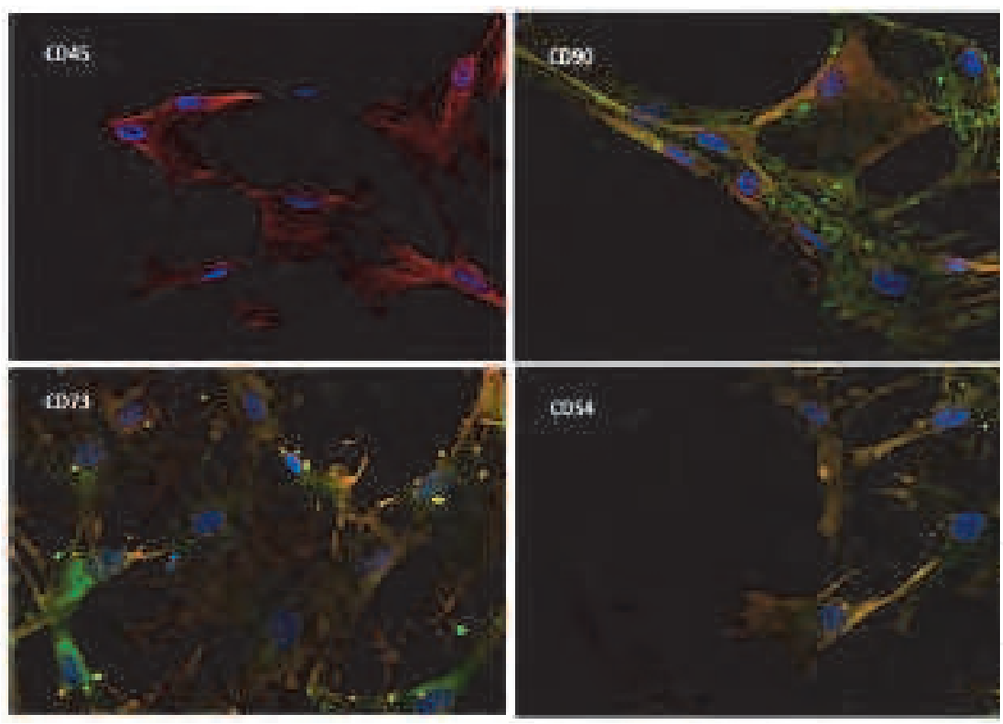

Fig. 1. Characterization of mesenchymal stem cells derived from adipose tissue of GFP-Lewis rats. A- CD45, B-CD90,C CD73 and D-CD54. GFP: red, surface marker: green and nucleus: blue.

3.1.2 Cell differentiation

In order to assess mesenchymal stem cell phenotype changes Cells differentiation was characterized by Immunofluorescence using membrane surface markers, CD90, CD73 and

CD54, MSC markers and CD45 a HSC marker. The goal of this experiment was to observe wether the cellular phenotype changed during the 9 weeks in chondrogenic medium. It is important to note that GFP cells are able to fluoresce without secondary antibody. Therefore anti-GFP were not used , only anti-CDs antibodies and secondary antibodies conjugated to PE to not GFP used. We observed that when cells were cultivated in chondrogenic medium, they were phenotypical alterated. Differentiated cells were positive for CD73 and Collagen II (red, Fig. 2) and they were negative for CD90, CD45, CD54 and osteocalcine (Fig. 2).

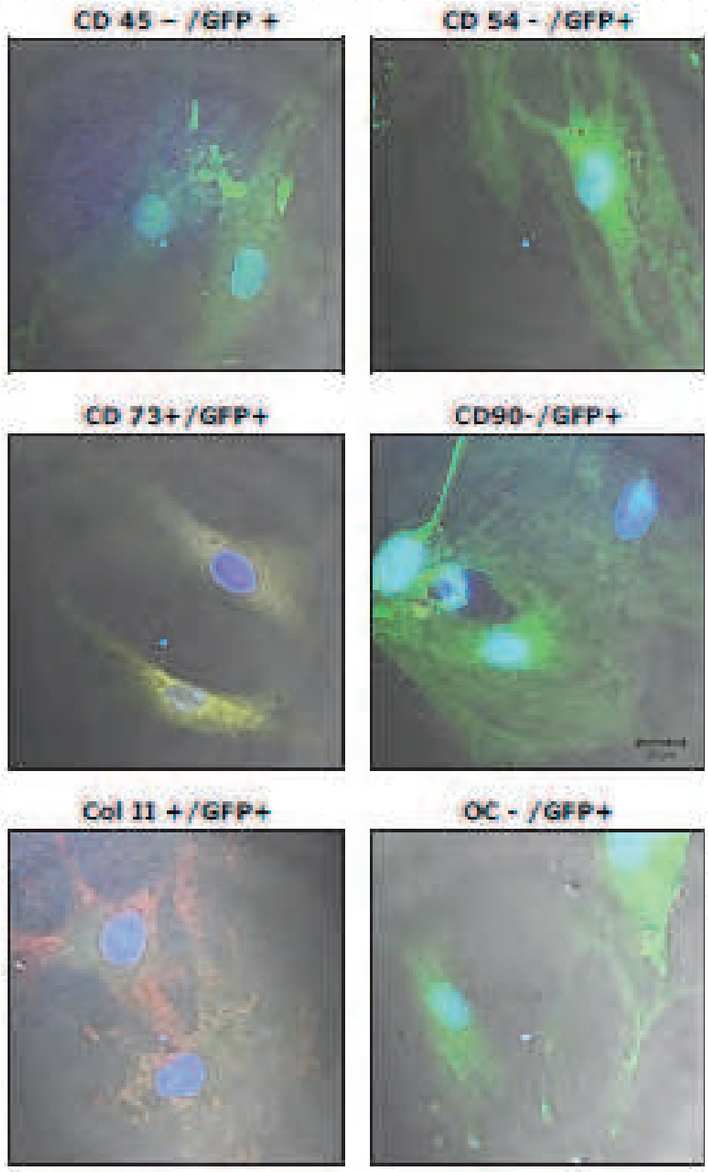

Fig. 2. Cells differentiated with chondrogenic medium for 9 weeks. CD45, CD54, CD73, CD90, Collagen II and osteocalcine. Cells were GFP labeled.

3.2 Biomaterials

3.2.1 Scaffold development and analysis

The characterization of the biomaterials developed began at macroscopic aspects. In in one hand glutaraldehyde – reticulated scaffolds presented yellow color, genipin-reticulated scaffolds were dark blue. Microscopically, glutaraldeyde-reticulated matrices presented round pores with sizes ranging between 100 - 500 μm (Fig. 3A and 3C). On the other hand, genipin-reticulated matrices also presented pores with sizes between 100 - 500 μm, however these pores had the appearance of being more fine and fragile (Fig. 3 B and 3D).

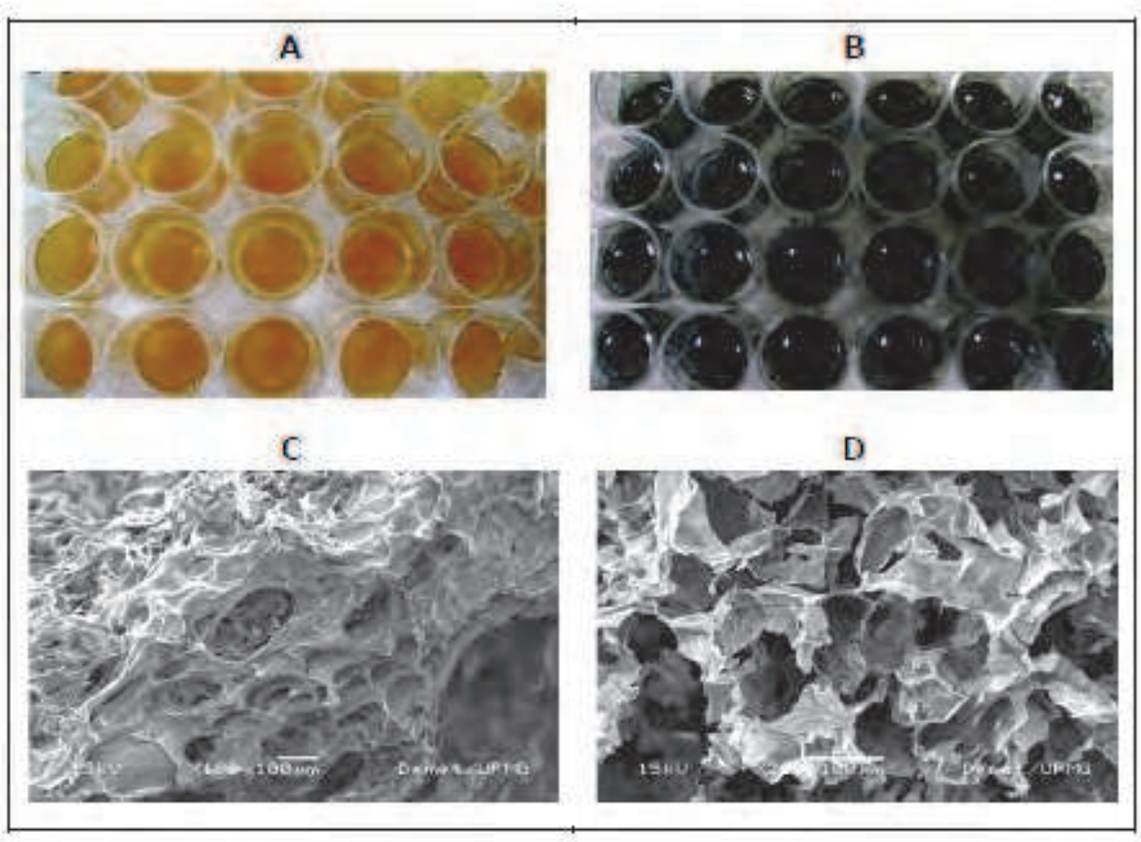

Fig. 3. 3D Scaffolds. (A) and (C) 3D scaffold with glutaraldehyde reticulates. (A) Without magnification and (C) SEM X100. (B) and (D) 3D scaffold with genipin reticulate. (B) Without magnification and (D) SEM X200.

3.2.2 Scaffold and cells

In order to assess the biomaterials' citotoxicity, we seeded cells on the scaffold and verified if the cells were viable after 1 week. To verify this we performed a stablished citotoxicity assay called MTT, which verifies cell viability through the assessment of mitochondria function. It was possible then to verify that cells were kept viable in both scaffolds viable (Fig. 4). The cells colonized both scaffolds as shown in figure 5. Rat MSC extended pseudopodes to link onto wall scaffolds.

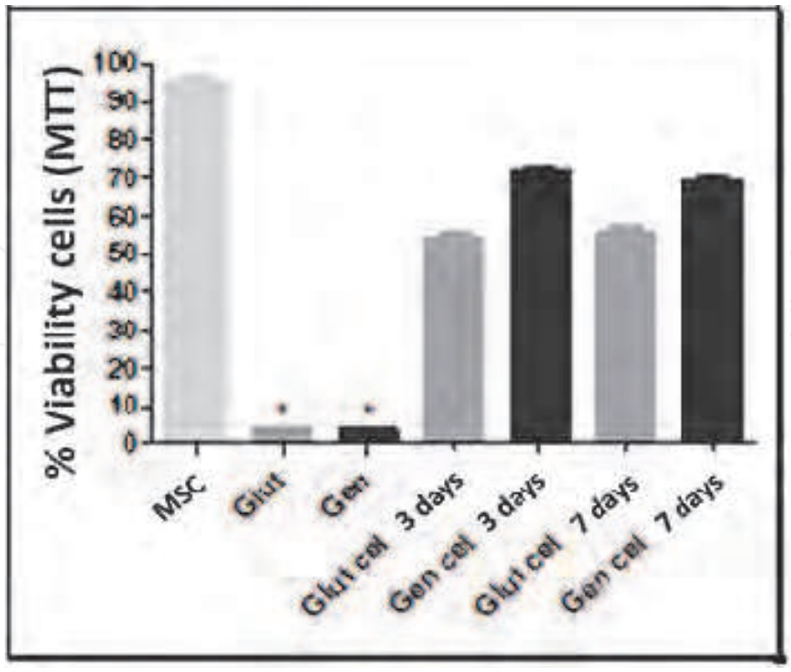

Fig. 4. Cells Viability (MTT). The graph shows cell viability when cultivated on scaffolds for 1 week.

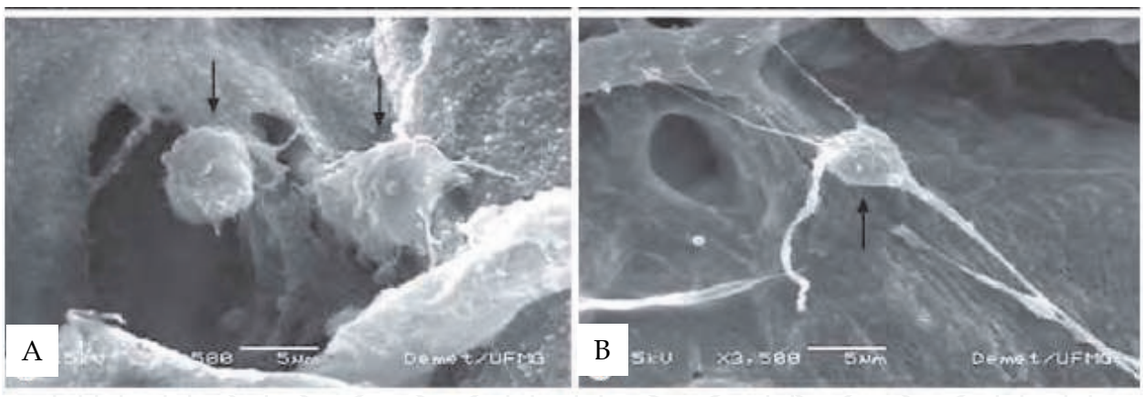

Fig. 5. Scanning electronic microcopy. (A) and (B) Cells attached to scaffolds.

3.3 *In vivo* assay

After 3 weeks of culture in chondrogenic medium in respective scaffolds, we grafted those constructs (association of scaffolds and cells) subcutaneous onto the dorsal region of Lewis rats. Those rats were sacrificed with anesthestic overdose on the 3rd week after implantation. These samples were analyzed by immunofluorescence and we verified that collagen type II was present in both samples. This result showed that once cells differentiated in the scaffold they do not dedifferentiate. The cells were able to maintained the differentiated phenotype inside the scaffolds (Fig. 6).

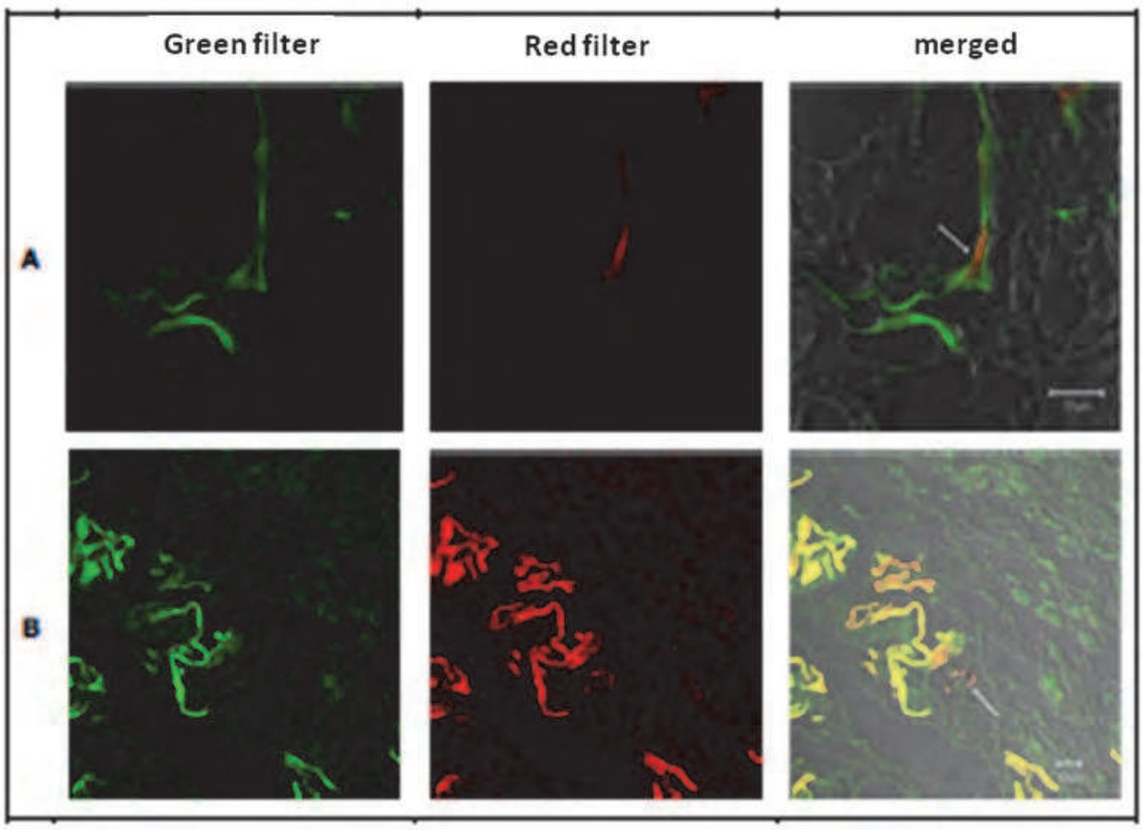

Fig. 6. Collagen II staining *in vivo.* Grafts of scaffolds with differentiated cells. (A) glutaraldehyde scaffold (B) Genipin scaffold.

4. Discussion

An ideal scaffold for been used in cartilage tissue engineering should be biodegradable, tissue compatible and display some degrees of rigidity and mechanical flexibility. It should have a three-dimensional configuration that provides a favorable environment for proliferation of chondrocytes and stem cells, and for cell migration and differentiation (Puppi et al, 2010 and Hutmacher et al, 2000). Furthermore, engineered bio-interfaces covered with biomimetic motifs, including short bioadhesive ligands, are a promising material-base strategy for tissue repair in regenerative medicine.

A 3D culture system that promoted the chondrogenesis of MSCs was established in this study. It was observed that the combination of chitosan and gelatin scaffolds provided a supporting environment for the chondrogenesis of rat MSCs. The MSCs formed cartilage-like tissue formation *in vivo* and *in vitro* via stimulation with common combinations of bioactive substances such as transforming growth factor (TGF-β) and dexamethasone. The chondrogenic differentiation of MSCs is typically detected by the formation of cell spheres in culture expression type II collagen in the extracellular matrix, surface markers alteration and the confirmation of typical gene expressions profiles by PCR analysis as determined by Breyner and colleagues, 2010.

Thus, the chitosan-gelatin scaffold used in our work mimic the natural environment leading to increased ECM synthesis and promoting differentiation of MSCs to chondrocytes.

Other publications have promoted variations in the matrix composition and GAG fine structure among the scaffolds used for cartilage tissue engineering in order to improve articular chondrocyte culture and chondrogenesis of progenitor cells (Mouw et al, 2005 and Melhorn et al, 2007). Our work, however, demonstrates that the 3D structure and chemical composition of the chitosan scaffolds and chondrogenic medium promoted MSC activation, proliferation and differentiation into chondrocytes, as was detected by a decrease in ALP production, an increase in collagen type II production and a lack of osteocalcin, a known osteogenic marker (Breyner et al, 2010; Huang et al, 2008 and Medrado et al, 2006).

The 3D chitosan-gelatin structure is perhaps an indication that the attachment of MSCs to chitosan matrix could improve cell differentiation after matrix deposition as seen in the development of chondrocytes. One of the advantages of 3D systems is the substantial surface area to volume ratio can maximize cell-material contact when compared to monolayer culture systems.

An ideal scaffold for cartilage tissue engineering should be biocompatible, non-cytotoxic and have favorable structural features for cell attachment and proliferation (Lefebvre et al, 1997 and Mingliang et al, 2011). This study showed that cells attached, proliferated and secreted extracellular matrix in the two 3D porous scaffolds used. After 3 days in culture the viability and cell number in the 3D scaffold culture group had increased and was higher than that of the cells in monolayer culture treated with glutaraldehyde or genipin. The percentage of cells in different stages was determined by the MTT metabolization assay. Strong cell attachment and proliferation demonstrated that there was no cytotoxicity in either scaffolds used. The results agreed well with previous studies showing that initial cell adhesion was largely influenced of RGD in gelatin bound to chitosan (Dong et al, 2010). It was reported that immobilization of RGD peptide onto a scaffold enabled the adhesion of stem cells to the scaffold and inhibited the immediate matrix-induced cell aggregation (Re'em et al, 2010). It allowed better access for cells to nutrients, oxygen and chondrogenic inducer. TGF-β1 is the main chondrogenic inducer during MSCs chondrogenesis (Barry et al, 2001). Studies suggested that RGD interaction with α5 and β1 integrin subunits enhanced TGF-β1 secretion, and RGD-dependent integrin activation should be linked to modulation of TGF-β1 activity (Ortega-Velasquez et la, 2003). In this study, the homogeneous spread of MSCs and abundant matrix secretion in scaffold indicated that TGF- β1 had efficiently induced MSCs chondrogenesis. These results may indicate that the 3D scaffold culture is superior to the monolayer culture because it is more effective in promoting ECM secretion or expression.

The immunoflurescence staining also revealed type II collagen accumulation between scaffolds seeded with MSCs after *in vivo* implantation. It is also interesting to note that the 3D culture system not only enhanced chondrogenesis but also increased the cell proliferation of MSCs. This may be a positive effect from chitosan-gelatin combination and TGF-β added to the chondrogenesis medium.

Generally, cell attachment and spreading can occur in a serum-containing environment regardless of surface coating because many factors regulating cell adhesion and spreading, such as fibronectin, vitronectin, and cytokines are found in serum (Underwood et al, 2001). However, during *in vitro* chondrogenic differentiation of MSCs, defined chondrogenic differentiation inducing medium was used without serum. Therefore, cell adhesion was the vital step for MSCs chondrogenic differentiation. However, cell density strongly influences MSCs differentiation and cell-matrix secretion (Hui et al, 2008).

5. Conclusion

Rat mesenchymal stem cells are able to differentiate when they are cultivated in chondrogenic medium. These cells can colonize scaffolds and differentiate inside them. Therefore, when these constructs were subcutaneously grafted in the rat dorsal region we verified that cells maintained a differentiated phonotype after 6 weeks. All together, we concluded that chitosan and gelatin are good candidates for scaffolds used to differentiate stem cells with chondrogenic treatment.

6. References

Williams DF. (2006). To engineering is to create: the link between engineering and regeneration. *Trends in Biotechnol* 24 (1): 4-8.

Ikada Y.(2006). Challenges in tissue engineering. *J.R.Soc Interface* 3:589-601.

Tabata Y.(2006). Biomaterial technology for tissue engineering applications. *J. R. Soc. Interface* 6: S311-S324.

Langer R & Vacanti JP. (1993). Tissue Engineering. *Science* 260: 920-926.

Chiang H & Jiang CC. (2009). Repair of articular cartilage defects: review and perspectives. *J Formos Med Assoc* 108: 87-102.

Zuk PA; Zhu M; Ashjian P; De Ugarte DA; Huang JI; Mizuno H; Alfonso ZC; Fraser JK; Benhaim P; Hedrick MH.(2002). Human adipose tissue is a source of multipotent stem cells. *Molecular Biology of the Cell* 13: 4279-95.

Mountford JC.(2008). Human embryonic stem cells: origins, characteristics and potential for regenerative therapy. *Transfusion Medicine* 18: 1-12.

Friedenstein AJ; Gorskaja JF; Kalugina NN. (1976). Fibroblast precursors in normal and irradiated mouse hematopoietic organs. *Experimental Hematol* 4:267-274.

Owen M & Friedenstein JA. (1988). Stromal stem cells: marrow derived osteogenic precursors. *Ciba Found Symp* 126: 42-60.

Conrad C & Huss R. (2004). Adult stem cell lines in regenerative medicine and reconstructive surgery. *Journal of Surgical Research* 124:201 – 208.

Takahashi K & Yamanaka S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 126: 663-76.

Yu J and Thomson JA. (2008). Pluripotent stem cell lines. *Genes and dev* 22: 1987-97.

Yamanaka S. (2009). Elite and stochastic model for induced pluripotent stem cell generation. *Nature* 460: 49-52.

Davila JC; Cezar G; Thiede M; Strom S; Miki T; Trosko J. (2004). Use and application of stem cells in toxicology. *Toxicological Science* 79: 214-223.

Gregory CA; Prockop DJ; Spees J L. (2005). Non hematopoietic bone marrow stem cells: Mollecular control of expansion and differentiation. *Experimental Cell Research* 306: 330 – 335.

Ogawa R; Mizuno H; Hyakosuko H; Watanabe A; Migita M; Shimada T. (2004). Chondrogenic and osteogenic differentiation of adipose derived stem cells isolated from GFP transgenic mice. *J Nippon Med Sch* 71 (4): 240-1.

Bacakova L; Filova E; Rypacek; Svorcik V; Stary V. (2004). Cell adhesion on artificial materials for tissue engineering. *Physiol Res* 53: 35-45.

Roughley P; Hoemann C; DesRosiers E; Mwale F; Antoniou F; Alini M. (2006). The potencial of chitosan-based gels containing intervertebral disc cells for nucleous pulposus supplementation. *Biomaterials* 27 (3): 388-96.

Tortelli F & Cancedda R. (2009). Three dimensional cultures of osteogenic and chondrogenic cells: a tissue engineering approach to mimic bone and cartilage *in vitro. Eur Cells and Materials* 17:1-14.

Miranov V; Visconti, RP; Kasyanov V; Forgacs G; Drake CJ; Markwald RR. (2009). Organ printing: tissue spheroids as building blocks. *Biomaterials* 30, 2164-74.

Hoffmann B; Seitz D; Mencke A; Kokott A; Ziegler G. (2009). Glutaraldehyde and oxidized dextran as crosslinker reagents for chitosan-based scaffold for cartilage tissue engineering. *J Mater Sci: Mater Med* 1-9.

Al-Ammar A; Drummond JL; Bedran-Russo AKB. (2009). The use of collagen cross-linking agents to enhance dentin bond strength. *J Biomed Mater Res B Appl Biomater*, 91 (1):419-24.

Beier JP; Klumpp D; Rudisile M; Dersh R; Wendorff JH; Bleiziffer O; Arkudas A; Polykandriotis E; Horch RE; Kneser U. (2009). Collagen matrices from sponge to nano: new perspective for tissue engineering of skeletal muscle. *BMC Biotechnology* 9 (34): 1-14.

Raghunath J; Salacinsky HJ; Sales KM; Butler PE and Seifalian AM. (2005). Advanced cartilage tissue engineering: the application of stem cell technology. *Current opinion in biotechnology* 16:503-509.

Betre H; Ong SR; Guilak F; Chilkoti A; Fermor B; Setton LA. (2006). Chondrocytic differentiation of human adipose-derived adult stem cells in elastin-like polypeptide. *Biomaterials* 27: 91-99.

James CG; Ulici V; Tuckermann J; Underhill TM; Beier F. (2007). Expression profiling of dexamethosone-treated primary chondrocytes identifies targets of glucocorticoid signaling in endochondral bone development. *BMC Genomics* 8: 1-27.

Melrose J; Chuang C; Whitelock J. (2008). Tissue engineering of cartilages using biomatrices. *J Chem Technol Biotechnol* 83: 444-63.

Mueller MB & Tuan RS. (2008). Functional characterization of hypertrophy in chondrogenesis of human mesenchymal stem cells. *Arthritis & Rheumatism* 58:1377-88.

Lee JW; Kim YH; Kim SH; Han SH; Hahn SB. (2004). Chondrogenic differentiation of mesenchymal stem cells and its clinical applications. *Yonsei Medical Journal* 45:41-47.

Otto WR and Rao J. (2004). Tomorrow's skeleton staff: mesenchymal stem cell and the repair of bone and cartilage. *Cell Prolif* 37: 97-110.

Medrado GC; Machado CB; Valerio P; Sanches MD; Goes AM. (2006). The effect of a chitosan-gelatin matrix and dexamethasone on the behavior of rabbit mesenchyma stem cells. *Biomed Mater* 13: 155-161.

Hwang NS; Kim MS; Sampattavanich S; Baek JH; Zhang Z; Elisseeff J. (2006). Effects of three dimensional culture and growth factors on the chondrogenic differentiation of murine embryonic stem cells. *Stem cells* 24: 284-291.

Koay EJ; Hoben GMB; Athaunasiou KA. (2007). Tissue engineering with chondrogenically differentiated human embryonic stem cells. *Stem cells* 25: 2183-2190.

Ucelli A; Moretta L and Pistoia V. (2008). Mesenchymal stem cells in health and disease. *Nature* 8:726-37.

Breyner NM; Hell RC, Carvalho LR, Machado CB, Peixoto Filho IN; Valerio P; Pereira MM, Goes AM. (2010). Effect of three dimensional chitosan porous scaffold on the differentiation of mesenchymal stem cells into chondrocytes. *Cell Tissue and Organs*. 191, 119-28.

Soliman EM; Rodrigues MA, Gomes DA; Sheung N; Yu J; Amaya MJ, Nathanson MH, Dranoff JA. (2009). Intracellular calcium signals regulate growth of hepatic stellate cell via specific effects in cell cycle progression. *Cell calcium.* 68: 284-92.

Guo T; Zhao J; Chang J; Ding Z; Hong H; Chen J; Zhang J. (2006). Porous chitosan-gelatin scaffold containing plasmid DNA encoding transforming growth factor-β1 for chondrocytes proliferation. *Biomaterials* 27: 1095-1103.

Yamane S; Iwasaki N; Majima T; Funakoshi T; Masuko T; Harada K; Minami A; Monde K; Nishimura S. (2005). Feasibility of chitosan-based hyaluronic acid hybrid biomaterial for a novel scaffold in cartilage tissue. *Biomaterials* 26: 611-19.

Janune DDJ; Cestari TM; De Oliveira RC; Taga EM; Taga R e Granjeiro JM. (2006). Avaliação da resposta tecidual ao implante de osso bovino misto medular em subcutâneo de ratos. *Innovations implant journal.* 2: 21-28.

Puppi D, Chiellini F, Piras AM, Chiellini E. (2010). Polymeric materials for bone and cartilage repair. *Prog Polym Sci* 35:403-40.

Hutmacher DW. (2000) Scaffolds in tissue engineering bone and cartilage. *Biomaterials* 21:2529-43.

Mouw, J.K., N.D. Case, R.E. Guldberg, A.H. Plaas, M.E. Levenston. (2005). Variations in matrix composition and GAG fine structure among scaffolds for cartilage tissue engineering. *Osteoarthritis Cartilage* 13*:* 828–836.

Mehlhorn, A.T., P. Niemeyer, K. Kaschte, L Muller, G. Finkenzeller, D. Hartl, N.P. Sudkamp, H. Schmal. (2007). Differential effects of BMP-2 and TGF-beta1 on chondrogenic differentiation of adipose derived stem cells. *Cell Prolif* 40*:* 809–823.

Lefebvre V, Huang W, Harley VR, Goodfellow PN, De Crombrugghe B. (1997). SOX9 is a potent activator of the chondrocyte-specific enhancer of the pro alpha1 (II) collagen gene. *Mol Cell Biol* 17:2336-46.

Mingliang You, Gongfeng Peng, Jian, Ping Maa, Zhihui Wang, Weiliang Shu, Siwu Peng, Guo-Qiang Chen. (2011). Chondrogenic differentiation of human bone marrow mesenchymal stem cells on polyhydroxyalkanoate (PHA) scaffolds coated with PHA granule binding protein PhaP fused with RGD peptide. *Biomaterials* 32, 2305-2313.

Dong Y, Li P, Chen CB, Wang ZH, Ma P, Chen GQ. (2010). The improvement of fibroblast growth on hydrophobic biopolyesters by coating with polyhydroxyalkanoate granule binding protein PhaP fused with cell adhesion motif RGD. *Biomaterials* 31:8921-30.

Re'em T, Tsur-Gang O, Cohen S. (2010) The effect of immobilized RGD peptide in macroporous alginate scaffolds on TGF [beta] 1-induced chondrogenesis of human mesenchymal stem cells. *Biomaterials* 31:6746-55.

Ortega-Velázquez R, Díez-Marqués ML, Ruiz-Torres MP, González-Rubio M, Rodríguez-Puyol M, et al. (2003). Arg-Gly-Asp-Ser peptide stimulates transforming growth factor-b1 transcription and secretion through integrin activation. *FASEB J*;17:1529-31.

Underwood PA, Bean PA, Mitchell SM, Whitelock JM. (2001). Specific affinity depletionof cell adhesion molecules and growth factors from serum. *J Immunol Methods* 247:217-24.

Hui TY, Cheung KMC, Cheung WL, Chan D, Chan BP. (2008). In vitro chondrogenic differentiation of human mesenchymal stem cells in collagen microspheres: influence of cell seeding density and collagen concentration. *Biomaterials* 29:3201-12.

Biomaterials Science and Engineering

Edited by Prof. Rosario Pignatello

ISBN 978-953-307-609-6
Hard cover, 456 pages
Publisher InTech
Published online 15, September, 2011
Published in print edition September, 2011

These contribution books collect reviews and original articles from eminent experts working in the interdisciplinary arena of biomaterial development and use. From their direct and recent experience, the readers can achieve a wide vision on the new and ongoing potentials of different synthetic and engineered biomaterials. Contributions were not selected based on a direct market or clinical interest, than on results coming from very fundamental studies which have been mainly gathered for this book. This fact will also allow to gain a more general view of what and how the various biomaterials can do and work for, along with the methodologies necessary to design, develop and characterize them, without the restrictions necessarily imposed by industrial or profit concerns. The book collects 22 chapters related to recent researches on new materials, particularly dealing with their potential and different applications in biomedicine and clinics: from tissue engineering to polymeric scaffolds, from bone mimetic products to prostheses, up to strategies to manage their interaction with living cells.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Natália Martins Breyner, Alessandra Arcoverde Zonari, Juliana Lott Carvalho, Viviane Silva Gomide, Dawidson Gomes and Alfredo Miranda Góes (2011). Cartilage Tissue Engineering Using Mesenchymal Stem Cells and 3D Chitosan Scaffolds – In vitro and in vivo Assays, Biomaterials Science and Engineering, Prof. Rosario Pignatello (Ed.), ISBN: 978-953-307-609-6, InTech, Available from: http://www.intechopen.com/books/biomaterials-science-and-engineering/cartilage-tissue-engineering-using-mesenchymal-stem-cells-and-3d-chitosan-scaffolds-in-vitro-and-in-

INTECH
open science | open minds

InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

IntechOpen