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ENGINEERING OSTEOCHONDRAL TISSUE BY HUMAN ADIPOSE STEM CELLS USING BIOMECHANICAL AND BIOCHEMICAL STIMULI

Cristina Correia[1,2,3] , Sarindr Bhumiratana[3], Rui A. Sousa[1,2], Gordana Vunjak-Novakovic[3], Rui L. Reis[1,2]

[1]3B's Research Group – Biomaterials, Biodegradables and Biomimetics, University of Minho, Portugal
[2] ICVS/3B's - PT Government Associate Laboratory, Braga/Guimarães, Portugal
[3]Department of Biomedical Engineering, Columbia University, New York, USA
E-mail: ccorreia@dep.uminho.pt

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ABSTRACT

There is a worldwide demanding clinical need for osteochondral grafts to repair joint ailments. To fulfill this need, we propose a tissue engineering approach where human adipose tissue derived stem cells (hASCs) are biomechanically or biochemically stimulated to develop a complete osteochondral graft, employing a complete autologous therapy. Hydrostatic pressure culturing increased cartilage matrix protein deposition, in comparison to standard static culture, while shear stress applied by pulsatile fluid flow enhanced bone tissue development, relatively to usual continuous fluid flow. A temporal nourishment with angiogenic and osteogenic growth factors allowed the differentiation of both lineages in the same graft, proving the possibility to vascularize bone grafts using the same cell source. The promising in vitro cartilage and vascularized bone tissue development suggest that hASC may be successfully used as single cell source for the complete regeneration of osteochondral defects.

INTRODUCTION

Joint ailments are a major health drawback affecting population worldwide. Population of all ages are affected, and usually, these ailments are lifetime enduring. At a worldwide scale, nearly 15 million people suffer from knee joint failure due to articular cartilage rupture and its inherente incapacity for self repair with normal body healing processes. Consequently, the direct costs in healthcare, and the indirect costs due to incapacity to work and early retirement, are virtually unsustainable. Most articular cartilage defects are caused by trauma, which can occur by a single impact or repeated micro trauma (Erggelet 2008). On the other hand, degenerative or rheumatic diseases are the main causes of joints cartilage degradation. Currently, no therapeutic approach have experienced wide clinical acceptance. The most common surgical treatment approaches include arthroscopic lavage and debridement (Gibson, White et al. 1992), microfracture (Steadman, Rodkey et al. 2001), mosaicplasty (Hangody, Kish et al. 1998) and autologous chondrocyte transplantation (ACT) (Brittberg, Lindahl et al. 1994). In a best-case scenario these approaches render a temporary effect, being their long time therapeutic effect still questionable.

A tissue engineering approach, where the appropriate employment of cells, biomaterials and stimulation may generate a fully replaceable functional tissue.

Human adipose tissue derived stem cells (hASCs) present excellent features and appear to be a promising alternative cell source for cell based therapies (Gimble, Guilak et al. 2010), as well as for cartilage and bone tissue regeneration. ASCs may be easily isolated from adipose tissue, proliferate quickly, and their osteogenic (Schaffler and Buchler 2007), chondrogenic and angiogenic (Scherberich, Muller et al. 2010) differentiation potential have been proven.

Both articular cartilage and underlying subchondral bone are affected by several, very distinct, biomechanical forces that are responsible to maintain its structure and function. These include tensile or shear forces, fluid perfusion, compression or hydrostatic pressurization. Hydrostatic pressure is known to be the predominant mechanical signal governing normal articular cartilage (Hu and Athanasiou 2006), while bone cells experience mechanical shear force due to the flow of interstitial fluid that is generated by physical movement. The application of mechanical stimuli to

foster tissue development during culturing, as a complement to the use of growth factors solely, is a scientific and experimental challenge.
This known, we hypothesise if ASC may be used to fully develop osteochondral grafts, and if biomechanical stimulation could be employed to enhance and ameliorate tissue development in vitro, in order to reduce time to therapy, and increase graft implantation success.

MATERIALS AND METHODS

hASCs were isolated according to previously described methods (McIntosh, Zvonic et al. 2006) from liposuction aspirates of subcutaneous adipose tissue, donated with written consent by patients undergoing elective liposurgery. hASC were expanded to the fourth passage in high-glucose Dulbecco's Modified Eagle's Medium (DMEM) (Gibco 11965) supplemented with 10% fetal bovine serum (FBS) (Gibco 26140), penicillin–streptomycin (1%) (Gibco 15140) and 1 ng/mL basic fibroblast growth factor (bFGF) (Peprotech 100-18B). p0 cells were examined for surface marker expression using flow cytometry. The presence of specific antigens such as CD29, CD105, CD45, CD34, CD44, CD73 and CD90 were analyzed, as previously published (McIntosh, Zvonic et al. 2006; Mitchell, McIntosh et al. 2006). hASCs were tested for their differentiation capacity into the osteogenic, chondrogenic and adipogenic lineages.

Chondrogenesis

Gellan gum hydrogel (1.5%) (Oliveira, Martins et al. 2010) was used to encapsulate hASC for cartilage tissue development. Briefly, powdered gellan gum (Gelzan™ Sigma/G1910) was dissolved in distilled water into a 1.5% solution, and heated up to 90ºC. Temperature was subsequently decreased down to 37-40ºC, for cell encapsulation at a final concentration of $10x10^6$ cells/mL. Cylindrical discs were made with a mould and phosphate buffer saline (PBS) (Sigma/P4417) was used as a cross-linking agent to stabilize the hydrogel structure. Human adipose stem cells encapsulated in gellan gum hydrogel were cultured up to 28 days in two distinct conditions:

1) High Hydrostatic Pressure - Culture medium pressurized at 50 Bar, at a frequency of 0.5 Hz, 4 hours/day, 5 days/week;
2) Static culturing - Constructs were cultured at free swelling conditions during the total culturing period.

Chondrogenic media was used in both dynamic and static culture, for 4 weeks. A custom-designed hydrostatic pressure bioreactor was used to apply the dynamic regimes. A standard 6-well plate was used for static control.

Osteogenesis

HFIP-derived silk fibroin scaffolds were prepared as previously described (Kim, Park et al. 2005). Expanded hASCs at passage 4 were suspended at $30x10^6$ cells/mL. A 40 µL aliquot of cell suspension was pipetted to the top of blot-dried scaffods, pipetted up and down to ensure even distribution of cells. After 15 minutes in the incubator, scaffolds were rotated 180º, and 10 µL of cell-free medium was added to prevent these to dry. This process was repeated four times, to allow uniform cell distribution, after which, osteogenic media (low glucose DMEM, 10% FBS, 1% penicillin–streptomycin, 10 mM sodium-b-glycerophosphate, 10 mM HEPES, 100 nM dexamethasone and 50 µg/mL ascorbic acid-2-phosphate) was added and cultured in static conditions (well-plate) for 3 days to allow cell attachment. Constructs were then transferred to the perfusion bioreactor, set-up as described in previous work (Frohlich, Grayson et al. 2010; Bhumiratana, Grayson et al. 2011; Grayson, Marolt et al. 2011).

Four groups were defined by varying fluid flow regimens applied to constructs (Fig.1):

C_0P_5 - No period of Continuous Fluid Flow (CFF) and 5 weeks (w) of Pulsatile Fluid Flow (PFF);
C_1P_4 - CFF the first week of culture, followed by 4 weeks of PFF;
C_2P_3 - CFF the first 2 weeks, followed by 3 w PFF;
C_5P_0 - CFF the total 5 weeks of culture, no PFF.

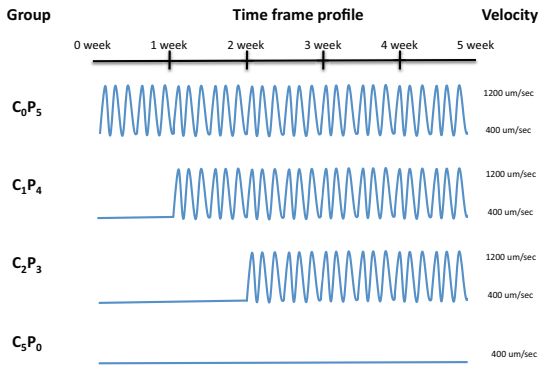

Figure 1. Perfusion profile applied along 5 weeks of culture

Pulsatile fluid flow regime was composed of flow velocities ranged from 400 µm/s to 1200 µm/s, at 0.5 Hz frequency for 2h every 10h (total of 4h/day). CFF was applied for the remaining 20h a day, at low flow velocity – 400 µm/s, as well as for group C_5P_0.

Osteogenesis + Vasculogenesis

Expanded hASCs were suspended at $20x10^6$ cells/mL and seeded to HFIP-derived silk scaffolds as before-mentioned.
These hASCs, attached to silk fibroin scaffolds were intended for bone formation. At day 1, fibrinogen (Sigma F8630) was prepared at a concentration of 5 mg/mL and thrombin (Sigma T6200-1KU) was used at 10 Units/mL. Expanded hASCs intended to form vasculature network were encapsulated in fibrin at a density of $20x10^6$ cells/mL. Thrombin was added to cross-link the gel, giving a final fibrin concentration of 4 mg/mL. Before cross-linking occurred, 20 µl of cell/gel suspension was pipetted into blot-dried scaffolds to allow uniform cell seeding throughout the scaffolds. This step was performed on groups 1 to 5 at day 1, and on groups 6 and 7 only after 3 weeks of osteogenic culture.
Seven experimental groups were defined in this study. Two of these were 'controls':

Group 1: constructs were cultured in osteogenic medium (**OM**), which consisted of low glucose DMEM, 10% FBS, 1% Pen–Strep supplemented with osteogenic factors (all purchased from Sigma-Aldrich): 100 nM dexamethasone, 10 mM sodium-β-glycerophosphate, 10 mM HEPES and 50 µg/mL ascorbic acid-2-phosphate, for 5 weeks, to evaluate osteogenic differentiation and bone tissue development;

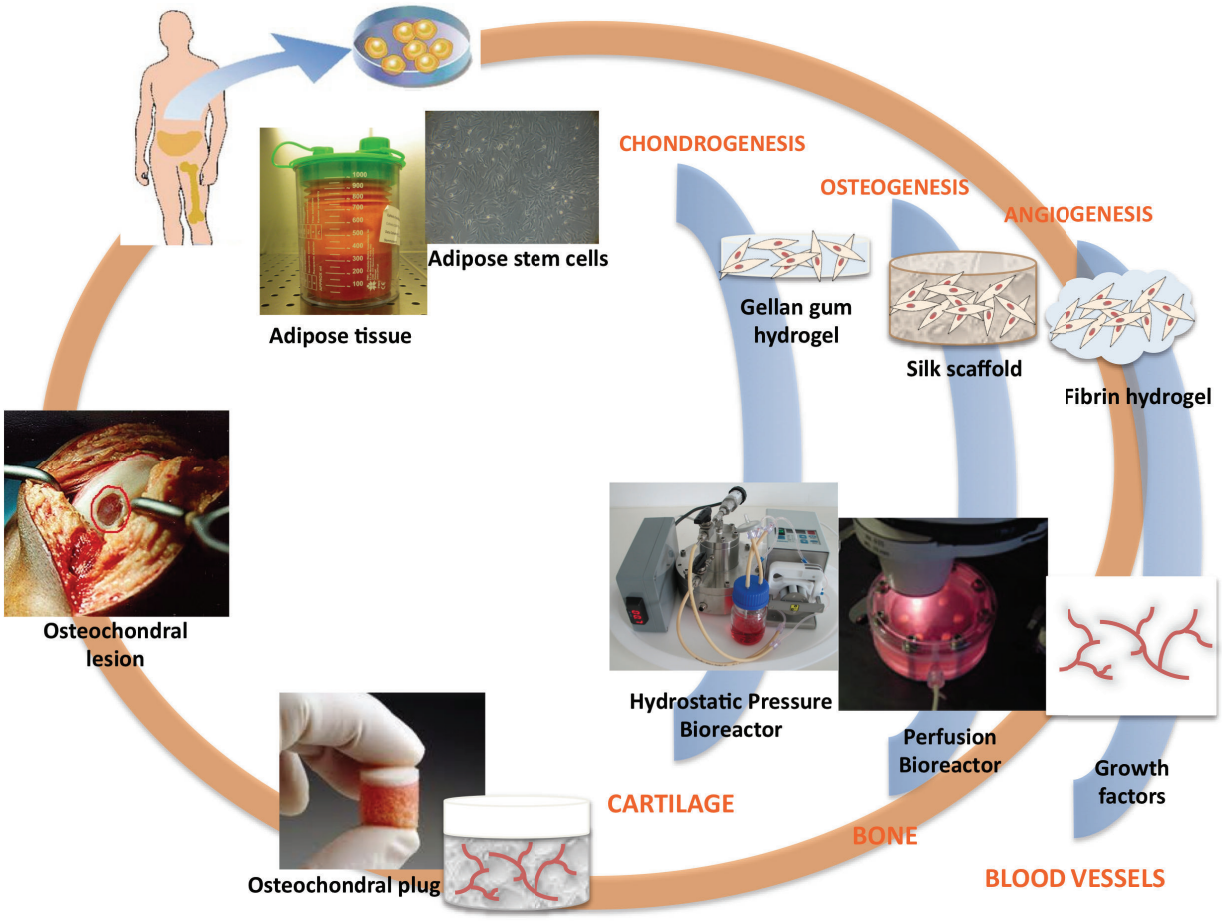

Figure 2. Engeneering osteochondral tissue wuth human adipose stem cells: the overall approach

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Group 2: endothelial growth media was used (**EGM**) (Lonza CC-3162), to evaluate endothelial differentiation and vascular tissue development.
Five further groups were chosen for growing vascularized bone constructs.
Group 3: both osteogenic and vasculogenic supplements were provided simultaneously throughout the total period of culture - constructs were cultured for 5 weeks in a cocktail medium composed by EGM and OM at 1:1 ratio (**cocktail** group).
Further, two sequential approaches were established: a) to induce vasculogenesis prior to osteogenesis (groups 4 and 5), and b) reverse the process, and induce osteogenesis prior to vasculogenesis (groups 6 and 7).
Group 4: constructs were cultured in EGM for 2 weeks and in cocktail medium for remaining 3 weeks (**EGM|cocktail**).
Group 5: cultured exactly as in Group 4 except that fresh hASCs were added into scaffold pore spaces at this 2-week time point. This is indicated as **EGM|cocktail+ASC**.
Groups 6 and 7: hASCs were only seeded into the scaffolds and cultured in osteogenic media for 3 weeks. Subsequently, hASCs in fibrin were added to the constructs and cultured either in EGM media (Group 6 - **OM|EGM**), either in cocktail medium (Group 7 - **OM|cocktail**).

RESULTS AND DISCUSSION

Chondrogenesis

A novel custom easy-to-use device was developed to generate hydrostatic pressure forces, which uniformly pressurize the constructs in culture. This device encloses particular key characteristics, such as:

a) Possibility to perform long term culturing, up to several weeks;
b) Culture of multiple 3D constructs within a high range of dimensions;
c) Pressurization is tunable from ~15-100 Bar and frequency between 0-1 Hz;
d) It is operated inside standard biohazard hoods, and CO_2 incubators at 37ºC, in complete sterile conditions;
e) It is reusable, composed majorly by safe stainless steel components.

This device was used to dynamically culture human adipose stem cells encapsulated in gellan gum hydrogel up to 4 weeks. Collagen Type II, the major articular hyaline cartilage extracellular matrix protein, and collagen type I, marker of the development of a fibrocartilage tissue, were immunolocalized in cross-sections of constructs harvested after 4 weeks of culture (Fig.3). Constructs of human adipose stem cells encapsulated in gellan gum hydrogel cultured under high hydrostatic pressure – 50 Bar, demonstrated increased deposition of collagen type II, and lower deposition of collagen type I, when compared to same constructs cultured under static, free-swelling conditions. Although gene expression for collagen type II was not significantly different among both groups, gene expression of aggrecan, one of the major negatively charged proteoglycans found in articular cartilage tissue, was significantly higher in the dynamically cultured group (data not shown). Glycosaminoglycan deposition was also quantified in cultured constructs. Concordantly, in comparison to static culturing, high hydrostatic pressure culture stimulated to a higher extent the hASC chondrogenic differentiation, and deposition of cartilage extracellular matrix, measured by glicosaminoglycan content in the constructs.
We suggest that hASCs are capable to sense changes of environment pressure and therefore differentiate better into the chondrogenic lineage under these dynamic conditions.

Osteogenesis

Bone extracellular matrix proteins, namely osteopontin (OPN) and bone sialoprotein (BSP) were immunolocalized in cross-sections of constructs harvested after 5 weeks of culture (Fig. 3). Among the three groups subjected to PFF, we observed increased bone protein deposition (both OPN and BSP) in C_2P_3 group, where cell-seeded scaffolds were cultured under constant fluid flow (CFF) for 2 weeks, before the application of pulsatile fluid flow (PFF). Constructs cultured under any regimen of PFF also demonstrated increased deposition, and better distribution of mineral throughout the scaffold when compared to constructs cultured under CFF, as observed by µCT reconstruction. Among the three pulsatile regimens, C_2P_3 was that which stimulated best the hASC into mineralization, as shown by compact mineral deposition detected by µCT (Fig.3).

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Osteopontin gene expression profile corroborate with bone protein deposition. Highest OPN gene expression was quantified at C_2P_3 group. The lowest expression of OPN was quantified for C_5P_0 group. These values were demonstrated as significantly different ($p<0.05$) (data not shown). OPN gene expression (GE) was progressively increased with the increase of osteogenic pre-differentiation period, before application of a pulsatile regimen: $C_0P_5 < C_1P_4 < C_2P_3$. BSP gene expression was not different in between groups studied. Gene expression of prostaglandin E2 synthase (PGE_2S) was also quantified, since prostaglandin E 2 (PGE_2) release is stimulated by shear stress in a dose-dependent matter (Reich and Frangos 1991). This paracrine factor was also expressed to the highest levels by hASCs cultured in C_2P_3 pulsatile regimen, and significantly different ($p<0.05$) from C_5P_0 group, that was not subjected to pulsatile fluid flow during culturing period. As observed for OPN gene expression, PGE_2S gene expression was also progressively increased with the increase of osteogenic pre-differentiation period, before application of a pulsatile regimen: $C_0P_5 < C_1P_4 < C_2P_3$.
Mineralization was assed through calcium quantification, and bone volume normalized by tissue volume (BV/TV). The highest amount of calcium was obtained with the C_2P_3 culturing regimen, again significantly different ($p<0.05$) from C_5P_0 group where the lowest amount was quantified (data not shown). BV/TV ratio corroborate with calcium data and µCT reconstruction images, where C_2P_3 present the highest ratio, and C_5P_0 the lowest.

Perfusion bioreactors have been widely employed in tissue engineering with the main purpose to improve gas transfer, nutrient supply and waste removal into/out tri-dimensional constructs, in which these biological/ biochemical processes tend to diminish from periphery to bulk of the engineered tissue (Martin, Wendt et al. 2004; Coletti, Macchietto et al. 2006). Several studies have explored the use of these dynamic culturing methods to improve osteogenic differentiation and bone tissue formation, by primary cells, bone marrow mesenchymal stem cells (Gomes, Holtorf et al. 2006; Grayson, Bhumiratana et al. 2008; Martins, Saraf et al. 2010; Bhumiratana, Grayson et al. 2011), and even human adipose stem cells (Frohlich, Grayson et al. 2010).

We hypothesized that osteogenic cells do not only response to shear force but also have the propensity in detecting and reacting to the change in shear force. In this study, we simplified tissue dynamic flow pattern by employing pulsatile fluid flow (PFF) regime to study the effect of change in shear force on human adipose stem cells (hASCs) differentiation and tissue engineered bone formation in contrast to conventional continuous fluid flow (CFF) regime. An estimation of shear stress in silk scaffolds was performed by reducing the interconnected pore network to a bundle of parallel, cylindrical channels of an average of 550 µm diameter, in order to estimate shear rates. The number of channels was estimated taking into account 90% scaffold porosity. Therefore, shear stress within tissue constructs fluctuated in average from 13.44 mPa (0.134 dyn/cm^2) and 4.48 mPa (0.045 dyn/cm^2) at flow velocities of 1200 µm/s and 400 µm/s, respectively. According to to theoretical model proposed by Weinbaum et al (1994), the magnitude of the fluid induced shear stresses in vivo is predicted to vary between 8 - 30 dyn/cm^2. In our study, we did not reach these high levels of shear stress, but we attempted to mimic the in vivo pulsatile profile, where maximum shear stress is 3x higher than minimum (0.045 - 0.134 dyn/cm^2), fluctuating at a frequency of 0.5 Hz, approximate to the frequency spectra of the forces acting on the human hip during walking (1 - 3 Hz) (Bacabac, Smit et al. 2004). Effectively, in our study we observed that constructs receiving pulsatile fluid flow for 5 weeks demonstrated superior bone development in terms of bone protein deposition and gene expression as well as increased mineralization comparing to constructs cultured under continuous fluid flow. Therefore, we may valid our hypothesis that pulsatile fluid flow culture improves human adipose stem cells (hASCs) differentiation and tissue engineered bone formation in contrast to conventional continuous fluid flow.
Furthermore, we determined the temporal development of the sensing mechanism for the exerted pulsatile forces in hASCs osteogenic differentiation. We may find in the literature that communicating osteocytes are the bone cells responsible for sensing the very small in vivo strains in the calcified matrix components of bone, through their osteocytic processes and not through the cell body (Weinbaum, Cowin et al. 1994; Anderson, Kaliyamoorthy et al. 2005). Human adipose stem cells lack these processes, fact that may lead to failure on attempting to mechanically stimulate these. Therefore, we hypothesized if hASC should be pre-differentiated into the osteogenic lineage in order to be capable to sense the pulsatile fluid flow, and consequent pulsatile shear stress, applied by dynamic culture of hASCs seeded silk scaffolds. For that, cells were cultured in

osteogenic media, and continuous fluid flow (to improve nutrient nourishment and waste removal) for 1 and 2 weeks, before applying pulsatile fluid flow for the reamianing culturing period (4 and 3 weeks respectively). In fact, the best bone tissue development was achieved when hASC were pre-differentiated for 2 weeks under CFF, and only then subjected to the PFF regime. As a result, increase in bone protein deposition (osteopontin and bone sialoprotein), correspondent gene expression, as well as calcification and bone volume were detected. Mechanical loading induces prostaglandin E_2 (PGE_2), an important early stage bone formation marker (Murakami, Naraba et al. 2000; Nauman, Satcher et al. 2001). Thus, we looked into gene expression of PGE_2 synthase to assess if this marker would be differently expressed in response to change in shear force. Interestingly, we observed a progression of expression, with progression of osteogenic differentiation: $C_0P_5 < C_1P_4 < C_2P_3$. We may valid our hypothesis that there is a temporal development of the sensing mechanism on hASC in order to respond to the exerted pulsatile forces towards osteogenic differentiation.

Osteogenesis + Vasculogenesis

Vascular development

Endothelial cells line the interior of all blood vessels, composing the endothelium. Therefore, endothelial cell markers were used to identify and characterize specific endothelial cell types and therefore evaluate vascular development on cultured tissues.

CD31 adhesion molecule is a non-exclusive marker,

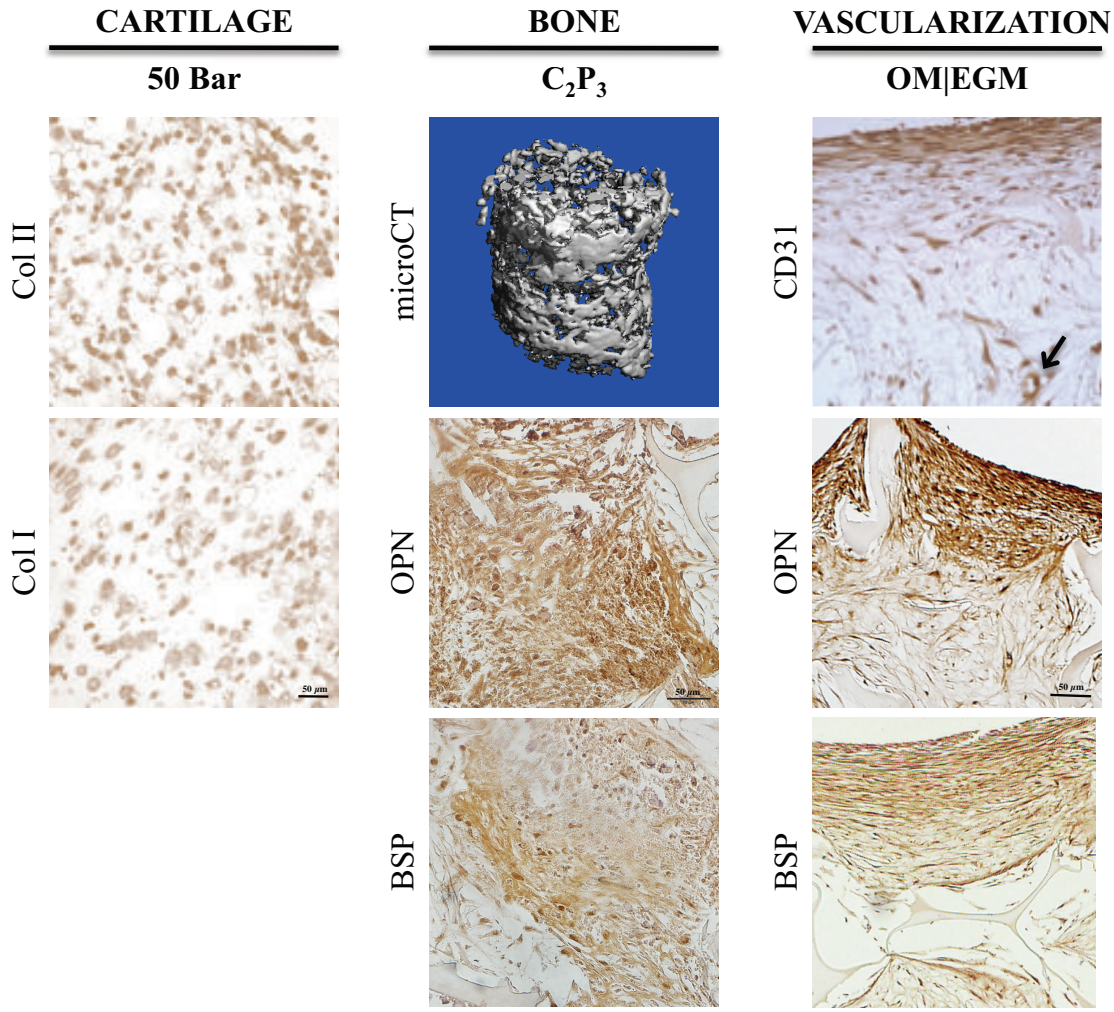

Figure 3. Immunolocalization of Cartilage (collagen type II, collagen type I), Bone (OPN – osteopontin, BSP – Bone Sialoprotein) and Endothelial (CD31) specific proteins. **Top-middle:** microCT reconstruction images of mineralized bone tissue.

present in all endothelials. It is expressed in large amounts on endothelial cells at intercellular junctions.
Both gene expression and protein deposition of this endothelial cell marker was assessed. When analyzing CD31 gene expression we verify that similar gene expression was detected in all groups 1, 2, 3 and 4 - **OM, EGM, cocktail** and **EGM|cocktail** respectively, and that these values were not superior than that obtained at day 1, before culture. Group 5 - **EGM|cocktail+ASC** was that where CD31 gene expression was the highest. Nonetheless, at groups 6 – **OM|EGM** and 7 – **OM|cocktail**, in which osteogenic induction preceded vasculogenic, CD31 gene expression was increased above values obtained for groups 1 – 4, and close to expression levels detected at group 5. What we find in common in these three groups is that ASC were added to the construct at later stages of culture, therefore we may estimate that this step is crucial for endothelial cell differentiation.
CD31 protein deposition correlates with gene expression profile. Groups 1, 2, 3 and 4 are those that demonstrate less staining for this protein, while groups 5 and 6 demonstrate the highest. Additionally, we observe that culture conditions at group 6 – **OM|EGM** promoted the development of more elongated structures, as well as small circumferential arrangements, which may correspond to vascular lumen. Relatively to group 7 – **OM|cocktail**, although CD31 gene expression was higher than groups 1 - 4, protein staining was not detected in this group. We suppose that 2 weeks of cocktail nourishment was not sufficient for ASC differentiation into endothelial cells at a higher degree of maturation. Accordingly, EGM nourishment at this stage was more efficient in endothelial differentiation than cocktail media: group 6 – **OM|EGM.**
vWF protein deposition corroborate with data for CD31 protein staining. Groups 1, 2, 3 and 4 - **OM, EGM, cocktail** and **EGM|cocktail** respectively, demonstrate less staining for this protein, while groups 5 and 6 demonstrate the highest. Again, elongated structures were stained for vWF at group 6 – **OM|EGM**. Concordantly to data obtained for CD31 protein staining, vWF was not detected at group 7 – **OM|cocktail**. We suppose that 2 weeks of cocktail nourishment was not sufficient for ASC differentiation into endothelial cells at a higher degree of maturation. Accordingly, EGM nourishment at this stage was more efficient in endothelial differentiation than cocktail media: group 6 – **OM|EGM.**

As an overall conclusion of vascular development, it seems that a sequential nourishment of growth factors is beneficial. Increased vascular outcomes are obtained either when: 1) vasculogenesis is induced before osteogenesis, with the addition of a new batch of ASC at this 2 week time point; 2) osteogenesis is induced before vasculogenesis, with supply of ASC for endothelial differentiation with EGM media, at this 3 week time point.

Bone tissue development
Bone tissue markers were assessed in order to evaluate bone development in the proposed vascularized bone tissue culture. Bone Sialoprotein II (BSP) is one of the major noncollagenous proteins (phosphorylated glycoprotein) in the extracellular matrix of bone. Osteopontin is a highly phosphorylated sialoprotein, a prominent component of the mineralized extracellular matrices of bones. These proteins were detected in cultured grafts through immuno-localization on histological slides. **EGM** group was the less stained, as this group was not nourished with osteogenic growth factors. The group that mostly stained for both these proteins was **cocktail** group. It seems that simultaneous presence of osteogenic and vasculogenic growth factors throughout the 5 weeks of culture improved bone protein deposition, even more than osteogenic control, group 1 - **OM**. Groups 4 and 5 - **EGM|cocktail** and **EGM|cocktail+ASC** respectively, demonstrated similar bone protein deposition. Groups 6 – **OM|EGM** and 7 – **OM|cocktail**, although cultured for 3 weeks in osteogenic media before vasculogenic induction, were not the groups that demonstrated best bone protein outcomes.
Mineralization of engineered tissue was evaluated by calcium quantification and by BV/TV ratio measured by μCT. After 5 weeks of culture, constructs cultured in **OM** for total culture period presented the highest amount of calcium, as well as correspondent BV/TV ratio. Among groups where sequential induction of both lineages were evaluated, Group 7 - **OM|cocktail** deposited the highest amount of calcium. Graft 3D reconstruction demonstrate that mineral deposition occurred homogeneously throughout the engineered graft, although to a less extent than that observed for **OM** group. Analyzing calcium quantification, group 5 demonstrated 1.7x higher calcium content than group 4, suggesting that the addition of ASC at the osteogenic induction stage improved calcification of the overall system. The outcomes were superior than those obtained

by ASC cultured 5 weeks in cocktail media (**cocktail** group).

As an overall conclusion of bone tissue development, we observed that culture conditions provided at group 6 – **OM|EGM** were those that promoted the best co-deposition of calcium and bone proteins. The outcomes of this group were even better than those achieved by group 1 and 7, where osteogenic factors were present in culture for extended time – both these groups presented high mineralization, but not increased protein deposition.

Vascularized bone outcomes

It is definitely challenging to achieve the ideal microenvironment, cell-cell interactions and local release of cytokines / growth factors for the in vitro development of vascularized bone. In this study we evaluated distinct culture conditions, by combining the sequential nourishment of cells and growth factors into a 3D constructs, and, although we have not achieved a fully developed vascularized tissue, we clarified some doubts and opened new paths to explore. We determine that group 6 – **OM|EGM** promoted the best outcome, once the culturing procedure adopted for this group provided the best results by co-promoting endothelial cell differentiation – demonstrated by CD31 and vWF gene expression and protein detection in elongated and lumen-like structures; and simultaneously bone cell differentiation – demonstrated by OPN and BSP bone protein deposition together with tissue mineralization detected by calcium quantification and µCT evaluation.

CONCLUSIONS

The adoption of a holistic approach concerning the single use of hASCs for the full engineering of osteochondral tissues proved successful. In this regard, the combined application of dynamic culturing conditions, namely of hydrostatic pressure and fluid flow, with biochemical cues, such as chondrogenic, osteogenic and angiogenic growth factors can be integrated in the manufacturing of osteochondral grafts.

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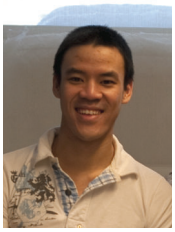
Portuguese Foundation for Science and Technology for C. Correia's PhD grant SFRH/BD/42316/2007 and NIH for R01 DE016525 and P41 EB002520 funding.

AUTHORS' BIOGRAPHIES

CRISTINA CORREIA was born in Miami, USA. She graduated in Applied Biology by University of Minho (2003-2007), after which she started her PhD research at 3B's Research Group, with Professor Rui L. Reis and Doctor Rui A. Sousa. Her major scientific interest is to understand differentiation of human adipose tissue derived stem cells in precise biomechanical systems to develop osteochondral tissue. Under this scope, she worked for one year at the Laboratory for Stem Cells and Tissue Engineering, Columbia University, NY, USA, in collaboration projects with Professor Gordana Vunjak-Novakovic. E-mail: ccorreia@dep.uminho.pt



SARINDR BHUMIRATANA is originally from Thailand, and has been studing in the United States. He graduated in Biomedical Engineering by Northwestern University, and obtained his masters degree in Biomedical Engineering by University of Michigan – Ann Arbor, He has participated in wide range of research in biological science: protein engineering and shrimp genome project, and in biomedical engineering: transport across eye tissue, and cell processing and culturing. For his PhD project, his current research interest is on engineering functional osteochondral tissue from human mesenchymal stem cells (hMSCs) using specialized bioreactors at the Laboratory for Stem Cells and Tissue Enhineering, Columbia University, NY, USA, with Professor Gordana Vunjak-Novakovic. E-mail: bs2385@columbia.edu

RUI A. SOUSA graduated in Metallurgical and Materials Engineering at the University of Porto, 1997. In the same year he initiated his PhD at Brunel University (UK) in cooperation with the University of Minho (Portugal).

Semana da Escola de Engenharia
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During the biennium 1999/2000, he concluded the disciplines of the Master Degree on Polymer Engineering. His PhD targeted the development of polymer based composites aimed at being used in orthopaedic applications. In 2002, Rui A. Sousa was awarded his PhD in Mechanical Engineering by the University of Brunel. Rui Amandi de Sousa is post-doc research fellow at the 3B´s Research Group – Biomaterials, Biodegradables, and Biomimetics. His current research interests focus on non-conventional manufacturing methods for application within the biomedical and tissue engineering fields. Since 2005, Rui Sousa has been involved in the creation of a spin-off company in the area of biomaterials, tissue engineering and regenerative medicine. In 2005, initiated his Master of Business Administration (MBA) at Escola de Gestão do Porto, University of Porto (Portugal), where he has specialized in business strategy, innovation and entrepreneurship. E-mail: rasousa@dep.uminho.pt

GORDANA Vunjak-NOVAKOVIC obtained her Ph.D. degree in chemical engineering at the University of Belgrade, in her homeland of Serbia. She stayed there as a faculty, raising in the ranks from an Assistant Professor to a regular Professor. Upon moving to the USA, Gordana spent twelve years at the Harvard-MIT Division for Health Sciences and Technology. As a Fulbright Fellow, she became fascinated by the concept of tissue engineering, and emerging technologies for improving and saving human life. She then joined Columbia University in 2005, where her laboratory hosts the Bioreactor Core of the NIH Center for Tissue Engineering. In 2002, she was elected a Fellow of the American Institute for Medical and Biological Engineering. In 2007, she gave the Director's lecture at the NIH, as the first woman engineer to receive this distinction. In 2008, she was inducted into the Women in Technology International Hall of Fame "for developing biological substitutes to restore, maintain or improve tissue function". In 2009, she was elected to the New York Academy of Sciences. E-mail: gv2131@columbia.edu

RUI L. REIS was born in Porto, Portugal, where he still lives. He is a Full Professor of Tissue Engineering, Regenerative Medicine and Stem Cells at the Department of Polymer Engineering, School of Engineering of University of Minho (UM), being the Director of the *3B's Research Group – Biomaterials, Biodegradables and Biomimetics* at the U. Minho in Portugal *and* the Director of PT Government *Associate Laboratory ICVS/3B´s*, jointly run with the UM Medical School. He is also, since 2000, the main responsible for LABMAT the general materials characterization Lab. of U. Minho and the President/Chairman and Chief Scientific Officer (CSO) of the company Stemmatters that he has founded as a spin-off of the 3B´s Research Group. Rui L. Reis is the CEO of the European Institute of Excellence on Tissue Engineering and Regenerative Medicine (TERM) Rui L. Reis education background includes: (i) a graduation in Metal. Eng., U. Porto, Portugal, 1990, (ii) a Master degree by research on Mater. Sci. and Eng. – Biomaterials - obtained in a joint program of the six major technical Universities in Portugal, awarded by U. Porto, Portugal, 1994 (iii) a PhD on Polymer Eng. – Biomaterials & Tissue Engineering, U. Minho, Portugal, degree that was prepared in co-operation with Brunel University, London, UK, 1999, (iv) a Doctor of Science (D.Sc.) degree on Biomedical Engineering - Biomaterials & Tissue Engineering, by U. Minho, Portugal, 2007. E-mail: rgreis@dep.uminho.pt.