



**HAL**  
open science

# Hypoxia for Mesenchymal Stem Cell Expansion and Differentiation: The Best Way for Enhancing TGF $\beta$ -Induced Chondrogenesis and Preventing Calcifications in Alginate Beads

Christel Henrionnet, Gai Liang, Emilie Roeder, Manuel Dossot, Hui Wang, Jacques Magdalou, Pierre Gillet, Astrid Pinzano

► **To cite this version:**

Christel Henrionnet, Gai Liang, Emilie Roeder, Manuel Dossot, Hui Wang, et al.. Hypoxia for Mesenchymal Stem Cell Expansion and Differentiation: The Best Way for Enhancing TGF $\beta$ -Induced Chondrogenesis and Preventing Calcifications in Alginate Beads. *Tissue Engineering: Parts A, B, and C*, 2017, 23 (17-18), pp.913-922. 10.1089/ten.TEA.2016.0426 . hal-01673606

**HAL Id: hal-01673606**

<https://hal.univ-lorraine.fr/hal-01673606v1>

Submitted on 22 Mar 2022

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

## **Hypoxia for MSC expansion and differentiation: the best way for enhancing TGF $\beta$ -induced chondrogenesis and preventing calcifications in alginate beads**

Christel HENRIONNET (a), PhD*	christel.henrionnet@univ-lorraine.fr
Gai LIANG (a,b), PhD*	lg16-81@163.com
Emilie ROEDER (a), PhD	emilie.roeder@gmail.com
Manuel DOSSOT (c), PhD	manuel.dossot@univ-lorraine.fr
Hui WANG (b), PhD	wanghui19@whu.edu.cn
Jacques MAGDALOU (a), PhD	jacques.magdalou@univ-lorraine.fr
Pierre GILLET (a), MD, PhD	pierre.gillet@univ-lorraine.fr
Astrid PINZANO (a) PhD	astrid.pinzano@univ-lorraine.fr

\* Equal contribution to this work, first co-authors

a UMR 7365 CNRS – Université de Lorraine, 54505 Vandœuvre lès Nancy, France

b Dpt of Pharmacology, Basic Medical School of Wuhan University, Wuhan 430071, China

c LCPME, UMR 7564 CNRS Université de Lorraine, 54602 Villers-lès-Nancy, France

### ***Address for correspondence:***

Pierre GILLET, UMR 7365 CNRS-Université de Lorraine,  
"Ingénierie Moléculaire et Physiopathologie Articulaire" (IMoPA)  
Biopôle de l'Université de Lorraine, Campus biologie-santé,  
Faculté de Médecine, Avenue de la Forêt de Haye,  
BP 20199, 54505 Vandœuvre-lès-Nancy.  
[Pierre.Gillet@univ-lorraine.fr](mailto:Pierre.Gillet@univ-lorraine.fr) tel: +33/(0)372 746 540 (secr) Fax +33/ (0)372 746 545

***Running title:*** Continuous MSC hypoxia enhances chondrogenesis and prevents calcifications

***None of the authors have any conflict/disclosure of interest regarding to this manuscript***

**Abstract (223 words)**

We examined the respective influence of a sequential or a continuous hypoxia during expansion and TGF- $\beta$ 1-driven chondrogenic differentiation of human bone marrow mesenchymal stem cells (MSCs). The differentiation was performed within alginate beads, a classical tool for the implantation of MSCs within the joint. The standard normoxic 2D (expansion) and 3D (differentiation) MSCs cultures served as reference. In order to determine the quality of the chondrogenesis, we analyzed typical markers such as type II and X collagens, *SOX9*, *COMP*, versican, and aggrecan mRNAs using PCR and we assessed the production of type II collagen and HIF-1 $\alpha$  by histological stainings. We simultaneously assessed the expression of osteogenic mRNAs (Alkaline Phosphatase, *RUNX2* and Osteocalcin) and the presence of microcalcifications by alizarin red and Raman spectroscopy. Chondrogenic differentiation is clearly improved by hypoxia in 3D. Best results were obtained when the entire process, *i.e.* 2D expansion and 3D differentiation, was performed under continuous 5% hypoxic condition. In addition, no calcification (hydroxyapatite, proved by RAMAN) was observed after 2D hypoxic expansion even in the case of a normoxic differentiation, in contrast with controls. Finally, a better chondrogenic differentiation of human MSCs is achieved when a reduced oxygen tension is applied during both expansion and differentiation times, avoiding the *in vitro* osteogenic commitment of the cells and subsequently the calcification deposition.

**Key words:** mesenchymal stem cells, hypoxia, alginate, chondrogenesis.

## Abbreviations

*ACAN*: aggrecan gene

ACI: autologous chondrocyte implantation

*ALP*: alkaline phosphatase gene

bFGF: basic Fibroblast Growth Factor

*BGLAP*: Osteocalcin gene

BMP: bone morphogenetic protein

DAB: diaminobenzidine

*COL10A1*: type X collagen gene

*COL2A1*: type II collagen gene

*COMP*: cartilage oligomeric matrix protein gene

DMEM-HG: Dulbecco's modified Eagle medium with high glucose

DMEM-LG: Dulbecco's modified Eagle medium with low glucose

**TGF $\beta$ -1: Transforming growth factor beta 1**

GAG : glycosaminoglycan

HA: hydroxyapatite

HES: Hematoxylin, Eosin, Saffron

HIF: hypoxia-inducible factor

H/H: Hypoxic (5%)/ Hypoxic (5%)

H/N: Hypoxic (5%)/Normoxic (20%)

MSCs: mesenchymal stem cells

N/H: Normoxic (20%)/Hypoxic (5%)

N/N: Normoxic (20%)/ Normoxic (20%)

OA: osteoarthritis

*RP29*: Ribosomal Protein S29 (housekeeping gene)

*SOX9*: SRY (sex-determining region Y)-box 9 gene

TGF- $\beta$ : Transforming growth factor  $\beta$  gene

*VCAN*: Versican gene

## 1. Introduction

Articular cartilage defects such as osteochondritis or focal post-traumatic chondral lesions are a common problem in orthopedic surgery. Due to its limited capacity for regeneration and self-repair as well as the lack of available therapeutic options, chondral defects may have severe consequences (focal/diffuse OA lesions) (1). Some focal injuries can be treated by an autologous chondrocytes implantation (ACI) (2). However, the ACI might result in the synthesis of fibrocartilage/hypertrophic repair tissue and of a subsequent long-term risk of developing OA (3, 4). This current state requires improved therapeutic options for treating cartilage defects such as cell-based and tissue-based methods that can overcome the size limitations of current ACI technologies and be applied to larger defects such as diffuse OA (3).

The mesenchymal stem cells (MSCs) are proved to be a good candidate cell source for treating cartilage defects (5, 6). Multipotent mesenchymal stem cells from adult bone marrow have the potential to renew themselves (auto-replication during expansion) and to differentiate (usually with 3D medium-dedicated conditions) into distinct mesodermal tissues including osteochondral structures(7). Differentiation of MSCs into chondrocytes has been achieved i) by adding growth factors (8), with the aid of co-cultivation with chondrocytes (9), or by transferring, virally or non-virally, dedicated chondrogenic genes (10). TGF- $\beta$  englobes a multi-functional set of growth factors that have been widely used to trigger MSC chondrogenesis in 3D environments, such as pellets, hydrogels, beads, biopolymers and other composite biomaterials (11). More recently BMPs were highlighted as pivotal signals to promote osteochondral commitment in articular engineering (12, 13). However, when pushed towards chondrogenesis, MSCs tend to evolve into the hypertrophic/ osteogenic commitment (14, 15), and many approaches, including low oxygen tension (as hyaline cartilage is non-vascularized) have been investigated to modulate chondrocytic hypertrophy (16, 17).

Articular cartilage is mostly an avascular tissue, surrounded by synovial fluid delivering oxygen and the nutrients diffusing through the extracellular matrix. The lack of blood supply thus generates a hypoxic environment (chondroxia, or physioxia (15)), with oxygen saturation ranging from 1 to 8%, depending on the location and depth within the joint (18, 19). The pioneer work of Nevo *et al.* (20) demonstrated the beneficial influence of hypoxia on embryonic chicken

chondrocyte *in vitro*. Since then, many efforts have been applied in the MSC-based cartilage engineering to mimic this environment during the expansion and/or the 3D differentiation. Generally, hypoxia shows an enhancement of the chondrogenesis (21-30) and strongly inhibits osteogenesis (31, 32).

Recent papers have underlined the importance of priming MSCs by continuous or cyclic hypoxic pre-treatments in regenerative medicine (33, 34): hypoxia promotes the stemness of various types of MSCs, improves tissue regeneration through an enhancement of the MSCs viability, differentiation and paracrine activity. This study was thus designed to clarify the influence of a low oxygen concentration chronology during the hypoxic preconditioning (expansion in 2D) and/or during their chondrogenic differentiation (3D culture in alginate beads). As few studies have studied the impact of an *in vitro* sequential hypoxic preconditioning of MSC-seeded implants (24, 29, 35-41), we have compared *in vitro* the chondrogenic, hypertrophic and osteogenic profiles, this latter being characterized by the occurrence of calcifications within the cellularized implants, under normoxic, mixed and/or continuous hypoxic conditions. We used concomitantly histology and Raman scattering spectroscopy. Raman permits a finer non-destructive chemical characterization of calcium deposits rather than standard Alizarin Red staining (42).

## 2. Materials and Methods

**2.1. Isolation.** Bone marrow human stem cells were isolated from 6 informed patients (mean age  $64,8 \pm 6,3$  y.o., 3 women and 3 men) undergoing total hip arthroplasty, all for hip OA. This study was approved by our local Research Institution. All procedures were done in accordance with our authorization and registration number DC-2008-263 given by the National “Cellule de Bioéthique”.

**2.2. 2D expansion.** The samples were placed in 50 ml tubes containing 1 ml of phosphate buffered saline (PBS) supplemented with 625 UI/ml heparin. The nucleated cells were counted and plated at a density of 50,000 cells/cm<sup>2</sup> in a T75 culture flask at 37°C in a humidified atmosphere containing 5% (v/v) carbon dioxide. The proliferation medium was a Dulbecco’s modified Eagle medium with low glucose (DMEM-LG, Gibco) supplemented with 10% fetal bovine serum (FBS, Sigma), 1% glutamine, 1% penicillin-streptomycin (Gibco) and 1 ng/ml

basic Fibroblast Growth Factor (bFGF, R&D systems). The medium was unchanged for the initial 3 days, and then was changed three times per week until the cells became confluent. Then the MSCs were passaged from P0 to P3 using trypsin-EDTA (0.05%, Gibco). The chondrogenic pre-differentiation medium in P3 (just before 3D differentiation in alginate beads) was DMEM with high glucose (DMEM-HG, Gibco) containing 10% FBS, 1% glutamine, 1% penicillin-streptomycin, sodium pyruvate (110 µg/ml), 40 µg/ml proline,  $10^{-7}$  M dexamethasone, 50 µg/ml L-Ascorbic acid-2-Phosphate and 1 ng/ml bFGF. To evaluate the effect of hypoxia during the expansion phase (preconditioning phase) one monolayer batch of MSCs was incubated in normoxia (20% oxygen, v/v) and another in hypoxia (5% oxygen, v/v) as designed in Suppl 1.

**2.3. 3D differentiation in alginate beads.** Before being mixed with the cells, the alginate (Sigma, France) - hyaluronic acid (Accros, France) powder was autoclaved at 120°C for 20 minutes for sterilization. In order to create alginate gel cultures, the viable cells were suspended at a concentration of  $3 \times 10^6$  cells/ml in 2% wt/vol viscosity alginate medium (16 mg alginate and 4 mg hyaluronic acid in 1 ml 0.9% NaCl solution). The cell/alginate preparation was polymerized into a solution containing 102 mM  $\text{CaCl}_2$  solution for 10 min. Beads were washed three times with 0.9% NaCl solutions and once with basal culture medium. Beads from each monolayer (hypoxia or normoxia expansion) were divided into two parts and cultured in hypoxia and normoxia conditions resulting in four groups: normoxia/normoxia (N/N) normoxia/hypoxia (N/H), hypoxia/normoxia (H/N), and hypoxia/hypoxia (H/H) (Supplemental data 1). The chondrogenic differentiation medium was DMEM-HG supplemented with 1% glutamine, 1% penicillin-streptomycin, 1% sodium pyruvate, 40 µg/ml proline,  $10^{-7}$  M dexamethasone, 50 µg/ml ascorbic acid 2-phosphate and 1 mM  $\text{CaCl}_2$ . In all conditions, the beads were treated from day 3 with ITS 1% (control) and ITS 1% + 10 ng/ml TGF- $\beta$ 1 (chondrogenic condition). The beads were harvested on D28 for analysis. For sake of clarity and lack of accuracy data obtained at D3, D14 are not presented.

**2.4. Histological analysis of the alginate beads.** The alginate beads were fixed in a 4% paraformaldehyde solution containing 10 mM  $\text{CaCl}_2$  and 0.1M sodium cacodylate (pH 7.4) for 4 hours at 4°C. Then they were washed overnight at 4°C in 0.1M sodium cacodylate (pH 7.4) (to irreversibly cross link the alginate matrix) containing 50 mM  $\text{BaCl}_2$  (to irreversibly crosslink the alginate matrix) before dehydrating in ethanol, embedding in paraffin. Sections of 5 µm thickness

were stained with Hematoxylin, Eosin, Saffron (HES) to visualize the cell morphology inside the beads, while Sirius red, Alcian Blue (pH 1.3) and Alizarin Red (pH 4.2) respectively evaluated collagen content, sulfated glycosaminoglycans (GAGs) and calcific depositions.

**2.5. Immunohistochemistry** was performed according to LSAB®+ kit (HRP, Dako) based on avidin-biotin techniques. A type II collagen (6B3, Labvision), and HIF-1 $\alpha$  primary monoclonal antibodies were used at the dilution of 1/100 as we previously published (43).

**2.6. RNA extraction and RT-qPCR.** The alginate beads were dissolved in 55 mM sodium citrate/50 mM EDTA at pH 6.8 for 5 min. The cells were isolated by centrifugation. The RNA extraction was realized using a RNeasy Mini Kit (Qiagen). After extraction, a reverse transcription was performed on 500 ng of RNA by using Omniscript RT Kit (Qiagen). The Real Time PCR was performed using QuantiTect™ SYBR® GreenPCR (Qiagen). The levels of mRNA were compared with the levels obtained from standard curves then normalized by RP29 mRNA (housekeeping gene) (44, 45). The mRNA expressions of each gene studied were compared to ITS and expressed as fold at D28 under various oxygen concentrations. All comparisons were performed versus our control condition, normoxia/normoxia (N/N).

**2.7. Primer design.** The primers sequences for all genes were designed using human mRNA information published on the NCBI website. The genes examined in this study were *RP29* (housekeeping gene), type II collagen (*COL2A1*), type X collagen (*COL10A1*), aggrecan (*ACAN*), versican (*VCAN*), SRY (sex-determining region Y)-box 9 (*SOX9*), cartilage oligomeric matrix protein (*COMP*), alkaline phosphatase (*ALP*) and osteocalcin (*BGLAP*). The sequences and product sizes are listed in the Table 1 and the PCR primers were purchased from MWG-Biotech.

**2.8. Raman Study.** To assess/characterize the presence of calcifications (hydroxyapatite and other possible mineral phases), Raman mapping experiments were performed at D28, as we previously published (46). The laser irradiance was kept below 5 kW.cm<sup>-2</sup> to avoid heating of the sample. Data presented are homogeneous for each sample in the batches studied.

**2.9. Statistical analysis.** The results were performed for each group in triplicate for each patient. The data are expressed as a normalized gene expression (TGF- $\beta$ 1 versus ITS) at D28 with various oxygen concentrations and presented as mean  $\pm$  standard deviation (SD). The significance was



determined by a non-parametric Kruskal-Wallis comparison with a Dunn's post-hoc test compared with our reference: continuous normoxia (N/N). Statistics analysis were performed with JMP®, Version 11. SAS Institute Inc., Cary, NC, 1989-2007. A p-value less than 0.05 was considered as significant.

### 3. Results

#### 3.1. Gene expression under TGF- $\beta$ 1-driven differentiation of MSCs.

*3.1.1. Effect of normoxia.* As predicted, TGF- $\beta$ 1 induced a classical 3D MSCs-driven chondrogenic differentiation (Figure 1): we noticed a high overexpression of *COL2A1* (12069 fold versus ITS condition), *COL10A1* (3802 fold), *ACAN* (174 fold), *SOX9* (7 fold) and *COMP* (64 fold). On the contrary, in our experimental conditions TGF- $\beta$ 1 supplementation induces a minor overexpression of osteogenic markers like *BGLAP* (osteocalcin, 2.6 fold), *RUNX2* (1.5 fold) and even a slight decrease of *ALP* (alkaline phosphatase, 0.8 fold). *VCAN* (versican), a fibrogenic marker was only modestly overexpressed (1.6 fold).

*3.1.2. Effect of sequential hypoxia.* A classical terminal hypoxia (N/H) during 3D differentiation boosted *ACAN* and *COL2A1* expressions while it repressed *COL10A1* suggesting a more pronounced chondrogenic profile. An initial hypoxia during 2D expansion, followed by a reoxygenation during normoxic 3D differentiation (H/N) had no influence on *COL2A1*, *ACAN*, *COL10A1* and *VCAN* expressions, while it boosted *SOX9* and decreased *ALP* and *BGLAP*.

*3.1.3. Effect of a continuous hypoxia.* This particular condition promotes the best chondrogenic gene patterns by robustly and significantly improving *COL2A1*, *ACAN*, *SOX9* and *COMP* expressions, and by simultaneously repressing fibrotic (*VCAN*) and osteogenic (*ALP*, *RUNX2* and *BGLAP*). In contrast, no influence was noticed on the hypertrophic marker *COL10A1*.

#### 3.2. Histology and type II collagen immunostaining

HES staining clearly shows that all cells present in the biomaterial are alive (purple color). These results are also confirmed by a mitochondrial activity test (test MTT) performed throughout the study showed no toxicity (Results not shown). Moreover, under normoxic conditions, the TGF- $\beta$ 1 beads displayed less cellularity than others TGF- $\beta$ 1 groups (Figure 2). During sequential

hypoxia, either terminal (N/H) or preceding re-oxygenation (H/N), proliferating chondrogenic islets were present.

A continuous hypoxia (H/H) markedly promoted chondrocytic-like proliferation. The Alcian Blue stain was done to appreciate proteoglycan content of the TGF- $\beta$ 1-driven constructs. In continuous normoxia (N/N), the GAG (Glycosaminoglycan) content was very faint, because Alcian Blue stained only alginate. An initial hypoxia promoted a weak peri-cellular staining. On the contrary, a distinct blue staining was observed in final hypoxic condition (N/H), even more marked under continuous hypoxia (H/H), with a more pronounced extracellular matrix synthesis.

The results are similar to the Sirius Red staining, giving information about fibrillar collagen content in alginate beads. A 3D differentiation under hypoxia promoted a good collagenous synthesis (N/H), enhanced by a hypoxic preconditioning during MSC expansion (H/H). This increase was particularly observed for the type II collagen immunostaining in the H/H group.

### **3.3. HIF-1 $\alpha$ immunostaining.**

Two typical patterns were observed, only depending on oxygen level during 3D differentiation, without influence of the 2D hypoxic preconditioning. In the 3D normoxic groups (N/N and H/N), the MSCs exhibited a significant cytoplasmic accretion of the HIF-1 $\alpha$  protein. In the hypoxic 3D culture conditions, we have observed a decrease of this cytoplasmic accumulation, due to the transport of HIF-1 $\alpha$  into to the nucleus indicating there was a difference in HIF-1 $\alpha$  expression only depending on the 3D hypoxia, without any influence of the oxygen tension (N/N and H/N) during the expansion time (2D).

### **3.4. Occurrences of calcific depositions.**

It is worth noting that, after a normoxic expansion, the TGF- $\beta$ 1-driven differentiation led to numerous calcifications on the Alizarin Red staining, suggesting a switch towards an osteogenic commitment in both 3D normoxic (N/N) and sequential hypoxic (N/H) conditions. In fact, Figures 2 and 3Ba clearly demonstrates the presence of Alizarin Red spots surrounding the cells, indicating the presence of calcifications in the matrix. Raman spectroscopy exploration of these calcifications confirmed a typical spectrum of the hydroxyapatite (HA). The Figure 3A indicates the typical Raman spectra obtained for the HA compound and also for the paraffin embedding.

The peak at  $962\text{ cm}^{-1}$  is a spectroscopic sign of the presence of HA in a sample and is clearly noticeable in spectrum b. The paraffin embedding may introduce some minor peaks in this spectrum (indicated by stars in Figure 3A) as some residues of paraffin slightly overlaid the HA structures.

However, the two spectra are sufficiently different and the  $962\text{ cm}^{-1}$  peak is in a spectral region completely silent for the paraffin medium. No confusion was possible, and this distinctive feature can be integrated to generate Raman maps. The Figure 3B compares the visible observation (Alizarin red stained samples, or pictures obtained by visible light in reflection mode) and the Raman mapping of the HA compound. The Raman identification was clearly useful as with the visible light, it is very difficult to identify calcified structures due to the paraffin medium.

The vibration analysis is unambiguous and revealed that HA structures were formed with the N/N and N/H modalities for the TGF- $\beta$ 1 samples. Besides, a hypoxic monolayer expansion prevented any HA calcifications in both 3D normoxic (H/N) and 3D hypoxic conditions (H/H). More than 8 areas were scanned randomly in the samples to statistically confirm the presence or the lack of HA compounds within the samples. The Raman mapping also confirmed that in our case, the Alizarin red stained deposits for N/N and N/H modalities matched to HA compounds, as seen on the Figure 3Bc.

#### 4. Discussion

In our study, the chondrogenesis of bone marrow MSCs (bMSCs) has been induced *in vitro* by incubation of cells in a defined medium containing TGF- $\beta$ 1, ascorbic acid and dexamethasone in combination with an alginate beads 3D culture. We confirmed that the chondrogenic induction of human bMSCs in alginate beads was enhanced with a reduced oxygen tension (5%) compared to a normoxic environment (38). No influence of sequential/continuous hypoxia was observed on MTT assay and DNA quantification using Hoechst (data not shown). Interestingly, a continuous hypoxia strongly upregulated mRNA overexpressions of the archetypal chondrogenic genes (*SOX9*, *ACAN*, *COMP* and *COL2A1*) and, on the other hand repressed the hypertrophic/osteogenic genes (*RUNX2* and *ALP*) as previously studied (30, 47, 48). *COL10A1* was only decreased during the terminal hypoxia as formerly reported in pellets (38, 49, 50) and it was not boosted by hypoxic priming, contrasting with some previous results (29, 38),

demonstrating a differential effect of hypoxia depending on the scaffold used. Nevertheless, it is generally accepted that hypoxia can stimulate TGF- $\beta$ -driven MSCs chondrogenesis. However, the optimal oxygen tension level remains discussed (2% or 5%) (51).

Few studies assessed the specific beneficial influence of hypoxia during the expansion time. Our results with bone marrow MSCs are consistent with those of adipose-derived human MSCs under hypoxic conditions promoting the chondrogenicity (35, 52, 53), down regulating the hypertrophic phenotype (54) and increasing the GAG content (24, 52). The inhibition of *RUNX2* was also observed for adipose-derived adult stromal cells during hypoxic expansion conditions (26). Moreover, the hypoxic differentiation of MSCs in pellets, scaffolds and hydrogels was revealed to suppress some markers of the hypertrophy such as *COL10A1* and *ALP* as observed in alginate beads (25, 29, 55). After 7 days in hypoxic conditions, the human bone marrow MSCs are able to undergo a chondrogenic differentiation in alginate beads without any growth factor addition and to display a similar specific gene profile (56). However, the benefits of a hypoxic preconditioning seemed both time- and oxygen-dependent varying on the respective times of normoxic/hypoxic expansion and differentiation (53, 57).

Two oxygen-sensitive transcription factors, the hypoxia-inducible factor 1 $\alpha$  and 2 $\alpha$  (HIF-1 $\alpha$  and HIF-2 $\alpha$ ), play a crucial role in the chondrogenicity of hypoxia. HIF-1 $\alpha$  was shown to improve MSC chondrogenesis via interactions with the SOX9 promoter (58). In addition, these hypoxic related chondrogenic and anti-catabolic effects disappeared with the addition of an HIF-1 $\alpha$  inhibitor (52, 59). In our continuous and terminal hypoxic conditions, HIF-1 $\alpha$  was translocated into the nucleus, as observed by the lack of cytoplasmic immunostaining, and then targeted the hypoxia-inducible elements helping the cells to switch towards the glycolytic metabolism. This indicated that HIF-1 $\alpha$  is not implicated in preventing calcifications, but only in promoting chondrogenesis.

The most original result in our study remained the lack of calcification/ossification inherent to the hypoxic preconditioning during the MSCs expansion, as previously reported in hydrogels and pellets (55). Interestingly, the calcifications were absent even if the 3D chondrogenic differentiation was performed under normoxia following a hypoxic expansion, this is contrasting with the results of Sheehy et al. (55). A beneficial influence of early hypoxia on calcium deposition during ATDC5 2D culture has already been reported (50) by using Alizarin red

staining. To the best of our knowledge, the qualitative RAMAN characterization, authenticating the hydroxyapatite crystal formation, has never been formerly used during *in-vitro* MSC chondrogenic differentiation studies.

The real interest for the hypoxia-mediated expansion and chondrogenic differentiation might be to improve the clinical results of *in vivo* cartilage repair and/or regeneration. Under normoxic expansion/differentiation, the MSCs, independently of their origin (bone, adipose or synovial tissue) do not undergo hypertrophic path when they are implanted intra-articularly in a cartilage environment in preclinical or in clinical conditions (3). Surprisingly, although hypoxia stimulated adipose MSCs chondrogenic commitment, it failed to enhance chondrogenesis after *in vivo* implantation in the rabbit knee (35). Besides, after a normoxic expansion/differentiation, a subcutaneous implantation of chondrogenic pellets issued from both bone marrow and adipose MSCs in SCID mice led to enhanced matrix calcifications along with a vascular invasion and even micro-ossicle ossification (47, 60). Synovium-derived pellets, exhibiting a lower ALP activity compared to bone marrow and adipose MSCs, showed only small-calcified areas after a subcutaneous transplantation (61).

Conversely, differentially hypoxic preconditioned adipose MSCs incorporated in hydrogels were injected subcutaneously in nude mice. After 5 weeks, the GAG and the type II collagen were present in the cartilage as aggregates and no calcification was mentioned (35). Similarly, after a transient hypoxic culture in a dedicated environment (2 weeks 5% O<sub>2</sub> followed by 1 week at 1% O<sub>2</sub>), human embryonic stem cells were cultured in pellets under normoxia. These constructs, expressing chondrogenic markers, were implanted subcutaneously in nude mice for 28 days: the pellets remained phenotypically stable with hyaline ECM, without dedifferentiation or tumorigenesis (37). These results are consistent with those of Leitjen et al. (41), demonstrating that hypoxia-preconditioned alginate implants (human bMSCs) remain cartilaginous after implantation, whereas normoxia preconditioned implants underwent calcification, vascular invasion and subsequent enchondral ossification.

***In conclusion***, we have shown that combining a 2D expansion and a 3D differentiation (alginate beads) under 5% hypoxic condition increases TGF-β1-driven human bone marrow MSC chondrogenesis by up regulating the chondrogenic genes, (e.g. aggrecan and type II collagen) and by down regulating the osteogenic genes. A hypoxic priming during expansion seems crucial to

prevent the development of *in vitro* calcification/ossification. A continuous hypoxic conditioning should be promoted in the clinic to standardize the GLP dedicated to cartilage engineering, like proposed for Wharton's Jelly MSCs (62).

***Acknowledgements:***

This work was supported by grants from:

- Agence Nationale de la Recherche: programme Jeunes Chercheuses et Jeunes Chercheurs. Project: « Cellules Souches Mésenchymateuses et Ingénierie du cartilage » (CESOMIC, JC05\_51629);
- Agence Nationale de la Recherche - programme ANR Blanc 2009: Cartilage Engineering and MRI : Assessment of Biointegration and Biofunctionality in the Rat (CEMABIR, ANR-09-BLANC-0150-01);
- Grants from the Région Lorraine Communauté Urbaine du Grand Nancy, and Conseil Général de Meurthe et Moselle.

***Authors' contributions:***

- Christel HENRIONNET: Collection and assembly of data, data analysis and interpretation, manuscript writing
- Gai LIANG: Collection and assembly of data, data analysis and interpretation, manuscript writing
- Emilie ROEDER: Data analysis, manuscript writing
- Manuel DOSSOT: Data analysis and interpretation, manuscript writing
- Hui WANG: Final approval of manuscript
- Jacques MAGDALOU: Final approval of manuscript
- Pierre GILLET: Data analysis and interpretation, final approval of manuscript
- Astrid PINZANO: Conception and design, financial support, data analysis and interpretation, manuscript writing

## References

1. Pelttari, K., Steck, E., and Richter, W. The use of mesenchymal stem cells for chondrogenesis. *Injury* **39 Suppl 1**, S58, 2008.
2. Mollon, B., Kandel, R., Chahal, J., and Theodoropoulos, J. The Clinical Status of Cartilage Tissue Regeneration in Humans. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society* 2013.
3. Orth, P., Rey-Rico, A., Venkatesan, J.K., Madry, H., and Cucchiari, M. Current perspectives in stem cell research for knee cartilage repair. *Stem Cells Cloning* **7**, 1, 2014.
4. Harris, J.D., Siston, R.A., Brophy, R.H., Lattermann, C., Carey, J.L., and Flanigan, D.C. Failures, re-operations, and complications after autologous chondrocyte implantation--a systematic review. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society* **19**, 779, 2011.
5. Roelofs, A.J., Rocke, J.P., and De Bari, C. Cell-based approaches to joint surface repair: a research perspective. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society* **21**, 892, 2013.
6. Cucchiari, M., de Girolamo, L., Filardo, G., Oliveira, J.M., Orth, P., Pape, D., and Rebol, P. Basic science of osteoarthritis. *J Exp Orthop* **3**, 22, 2016.
7. Pittenger, M.F., Mackay, A.M., Beck, S.C., Jaiswal, R.K., Douglas, R., Mosca, J.D., Moorman, M.A., Simonetti, D.W., Craig, S., and Marshak, D.R. Multilineage potential of adult human mesenchymal stem cells. *Science* **284**, 143, 1999.
8. Weiss, S., Hennig, T., Bock, R., Steck, E., and Richter, W. Impact of growth factors and PTHrP on early and late chondrogenic differentiation of human mesenchymal stem cells. *Journal of cellular physiology* **223**, 84, 2010.
9. Shi, S., Xie, J., Zhong, J., Lin, S., Zhang, T., Sun, K., Fu, N., Shao, X., and Lin, Y. Effects of low oxygen tension on gene profile of soluble growth factors in co-cultured adipose-derived stromal cells and chondrocytes. *Cell proliferation* **49**, 341, 2016.
10. Frisch, J., Rey-Rico, A., Venkatesan, J.K., Schmitt, G., Madry, H., and Cucchiari, M. TGF-beta gene transfer and overexpression via rAAV vectors stimulates chondrogenic events in human bone marrow aspirates. *J Cell Mol Med* **20**, 430, 2016.
11. Freyria, A.M., and Mallein-Gerin, F. Chondrocytes or adult stem cells for cartilage repair: the indisputable role of growth factors. *Injury* **43**, 259, 2012.
12. Mehlhorn, A.T., Niemeyer, P., Kaschte, K., Muller, L., Finkenzeller, G., Hartl, D., Sudkamp, N.P., and Schmal, H. Differential effects of BMP-2 and TGF-beta1 on chondrogenic differentiation of adipose derived stem cells. *Cell proliferation* **40**, 809, 2007.
13. Handorf, A.M., and Li, W.J. Induction of mesenchymal stem cell chondrogenesis through sequential administration of growth factors within specific temporal windows. *Journal of cellular physiology* 2013.
14. Johnstone, B., Alini, M., Cucchiari, M., Dodge, G.R., Eglin, D., Guilak, F., Madry, H., Mata, A., Mauck, R.L., Semino, C.E., and Stoddart, M.J. Tissue engineering for articular cartilage repair--the state of the art. *European cells & materials* **25**, 248, 2013.



15. Anderson, D.E., Markway, B.D., Bond, D., McCarthy, H.E., and Johnstone, B. Responses to altered oxygen tension are distinct between human stem cells of high and low chondrogenic capacity. *Stem Cell Res Ther* **7**, 154, 2016.
16. Frisch, J., and Cucchiari, M. Gene- and Stem Cell-Based Approaches to Regulate Hypertrophic Differentiation in Articular Cartilage Disorders. *Stem Cells Dev* 2016.
17. Chen, S., Fu, P., Cong, R., Wu, H., and Pei, M. Strategies to minimize hypertrophy in cartilage engineering and regeneration. *Genes Dis* **2**, 76, 2015.
18. Studer, D., Millan, C., Ozturk, E., Maniura-Weber, K., and Zenobi-Wong, M. Molecular and biophysical mechanisms regulating hypertrophic differentiation in chondrocytes and mesenchymal stem cells. *European cells & materials* **24**, 118, 2012.
19. Pfander, D., and Gelse, K. Hypoxia and osteoarthritis: how chondrocytes survive hypoxic environments. *Current opinion in rheumatology* **19**, 457, 2007.
20. Nevo, Z., Beit-Or, A., and Eilam, Y. Slowing down aging of cultured embryonal chick chondrocytes by maintenance under lowered oxygen tension. *Mechanisms of ageing and development* **45**, 157, 1988.
21. Meyer, E.G., Buckley, C.T., Thorpe, S.D., and Kelly, D.J. Low oxygen tension is a more potent promoter of chondrogenic differentiation than dynamic compression. *Journal of biomechanics* **43**, 2516, 2010.
22. Merceron, C., Vinatier, C., Portron, S., Masson, M., Amiaud, J., Guigand, L., Cherel, Y., Weiss, P., and Guicheux, J. Differential effects of hypoxia on osteochondrogenic potential of human adipose-derived stem cells. *American journal of physiology Cell physiology* **298**, C355, 2010.
23. Markway, B.D., Tan, G.K., Brooke, G., Hudson, J.E., Cooper-White, J.J., and Doran, M.R. Enhanced chondrogenic differentiation of human bone marrow-derived mesenchymal stem cells in low oxygen environment micropellet cultures. *Cell transplantation* **19**, 29, 2010.
24. Zscharnack, M., Poesel, C., Galle, J., and Bader, A. Low oxygen expansion improves subsequent chondrogenesis of ovine bone-marrow-derived mesenchymal stem cells in collagen type I hydrogel. *Cells, tissues, organs* **190**, 81, 2009.
25. Koay, E.J., and Athanasiou, K.A. Hypoxic chondrogenic differentiation of human embryonic stem cells enhances cartilage protein synthesis and biomechanical functionality. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society* **16**, 1450, 2008.
26. Xu, Y., Malladi, P., Chiou, M., Bekerman, E., Giaccia, A.J., and Longaker, M.T. In vitro expansion of adipose-derived adult stromal cells in hypoxia enhances early chondrogenesis. *Tissue engineering* **13**, 2981, 2007.
27. Khan, W.S., Adesida, A.B., and Hardingham, T.E. Hypoxic conditions increase hypoxia-inducible transcription factor 2alpha and enhance chondrogenesis in stem cells from the infrapatellar fat pad of osteoarthritis patients. *Arthritis research & therapy* **9**, R55, 2007.
28. Robins, J.C., Akeno, N., Mukherjee, A., Dalal, R.R., Aronow, B.J., Koopman, P., and Clemens, T.L. Hypoxia induces chondrocyte-specific gene expression in mesenchymal cells in association with transcriptional activation of Sox9. *Bone* **37**, 313, 2005.

29. Adesida, A.B., Mulet-Sierra, A., and Jomha, N.M. Hypoxia mediated isolation and expansion enhances the chondrogenic capacity of bone marrow mesenchymal stromal cells. *Stem Cell Res Ther* **3**, 9, 2012.
30. Lee, H.H., Chang, C.C., Shieh, M.J., Wang, J.P., Chen, Y.T., Young, T.H., and Hung, S.C. Hypoxia enhances chondrogenesis and prevents terminal differentiation through PI3K/Akt/FoxO dependent anti-apoptotic effect. *Sci Rep* **3**, 2683, 2013.
31. Malladi, P., Xu, Y., Chiou, M., Giaccia, A.J., and Longaker, M.T. Effect of reduced oxygen tension on chondrogenesis and osteogenesis in adipose-derived mesenchymal cells. *American journal of physiology Cell physiology* **290**, C1139, 2006.
32. Potier, E., Ferreira, E., Andriamanalijaona, R., Pujol, J.P., Oudina, K., Logeart-Avramoglou, D., and Petite, H. Hypoxia affects mesenchymal stromal cell osteogenic differentiation and angiogenic factor expression. *Bone* **40**, 1078, 2007.
33. Muscari, C., Giordano, E., Bonafe, F., Govoni, M., Pasini, A., and Guarnieri, C. Priming adult stem cells by hypoxic pretreatments for applications in regenerative medicine. *Journal of biomedical science* **20**, 63, 2013.
34. Pei, M. Environmental preconditioning rejuvenates adult stem cells' proliferation and chondrogenic potential. *Biomaterials* **117**, 10, 2016.
35. Portron, S., Merceron, C., Gauthier, O., Lesoeur, J., Sourice, S., Masson, M., Fellah, B.H., Geffroy, O., Lallemand, E., Weiss, P., Guicheux, J., and Vinatier, C. Effects of in vitro low oxygen tension preconditioning of adipose stromal cells on their in vivo chondrogenic potential: application in cartilage tissue repair. *PloS one* **8**, e62368, 2013.
36. Lennon, D.P., Edmison, J.M., and Caplan, A.I. Cultivation of rat marrow-derived mesenchymal stem cells in reduced oxygen tension: effects on in vitro and in vivo osteochondrogenesis. *Journal of cellular physiology* **187**, 345, 2001.
37. Yodmuang, S., Marolt, D., Marcos-Campos, I., Gadjanski, I., and Vunjak-Novakovic, G. Synergistic effects of hypoxia and morphogenetic factors on early chondrogenic commitment of human embryonic stem cells in embryoid body culture. *Stem Cell Rev* **11**, 228, 2015.
38. Bornes, T.D., Jomha, N.M., Mulet-Sierra, A., and Adesida, A.B. Hypoxic culture of bone marrow-derived mesenchymal stromal stem cells differentially enhances in vitro chondrogenesis within cell-seeded collagen and hyaluronic acid porous scaffolds. *Stem Cell Res Ther* **6**, 84, 2015.
39. Muller, J., Benz, K., Ahlers, M., Gaissmaier, C., and Mollenhauer, J. Hypoxic conditions during expansion culture prime human mesenchymal stromal precursor cells for chondrogenic differentiation in three-dimensional cultures. *Cell transplantation* **20**, 1589, 2011.
40. Hudson, K.D., and Bonassar, L.J. Hypoxic Expansion of Human Mesenchymal Stem Cells Enhances 3D Maturation of Tissue Engineered Intervertebral Discs. *Tissue Eng Part A* 2016.
41. Leijten, J., Georgi, N., Moreira Teixeira, L., van Blitterswijk, C.A., Post, J.N., and Karperien, M. Metabolic programming of mesenchymal stromal cells by oxygen tension directs chondrogenic cell fate. *Proc Natl Acad Sci U S A* **111**, 13954, 2014.

42. Flausse, A., Henrionnet, C., Dossot, M., Dumas, D., Hupont, S., Pinzano, A., Mainard, D., Galois, L., Magdalou, J., Lopez, E., Gillet, P., andRousseau, M. Osteogenic differentiation of human bone marrow mesenchymal stem cells in hydrogel containing nacre powder. *J Biomed Mater Res A* **101**, 3211, 2013.
43. Reppel, L., Schiavi, J., Charif, N., Leger, L., Yu, H., Pinzano, A., Henrionnet, C., Stoltz, J.F., Bensoussan, D., andHuselstein, C. Chondrogenic induction of mesenchymal stromal/stem cells from Wharton's jelly embedded in alginate hydrogel and without added growth factor: an alternative stem cell source for cartilage tissue engineering. *Stem Cell Res Ther* **6**, 260, 2015.
44. Cailotto, F., Reboul, P., Sebillaud, S., Netter, P., Jouzeau, J.Y., andBianchi, A. Calcium input potentiates the transforming growth factor (TGF)-beta1-dependent signaling to promote the export of inorganic pyrophosphate by articular chondrocyte. *J Biol Chem* **286**, 19215, 2011.
45. Henrionnet, C., Roeder, E., Gillet, R., Galois, L., Bensoussan, D., Mainard, D., Netter, P., Gillet, P., andPinzano, A. Expression of chondrogenic genes by undifferentiated vs. differentiated human mesenchymal stem cells using array technology. *Biomed Mater Eng* **20**, 175, 2010.
46. Brion, A., Zhang, G., Dossot, M., Moby, V., Dumas, D., Hupont, S., Piet, M.H., Bianchi, A., Mainard, D., Galois, L., Gillet, P., andRousseau, M. Nacre extract restores the mineralization capacity of subchondral osteoarthritis osteoblasts. *J Struct Biol* **192**, 500, 2015.
47. Pelttari, K., Winter, A., Steck, E., Goetzke, K., Hennig, T., Ochs, B.G., Aigner, T., andRichter, W. Premature induction of hypertrophy during in vitro chondrogenesis of human mesenchymal stem cells correlates with calcification and vascular invasion after ectopic transplantation in SCID mice. *Arthritis and rheumatism* **54**, 3254, 2006.
48. Mueller, M.B., andTuan, R.S. Functional characterization of hypertrophy in chondrogenesis of human mesenchymal stem cells. *Arthritis and rheumatism* **58**, 1377, 2008.
49. Munir, S., Foldager, C.B., Lind, M., Zachar, V., Soballe, K., andKoch, T.G. Hypoxia enhances chondrogenic differentiation of human adipose tissue-derived stromal cells in scaffold-free and scaffold systems. *Cell Tissue Res* **355**, 89, 2014.
50. Portron, S., Hivernaud, V., Merceron, C., Lesoeur, J., Masson, M., Gauthier, O., Vinatier, C., Beck, L., andGuicheux, J. Inverse regulation of early and late chondrogenic differentiation by oxygen tension provides cues for stem cell-based cartilage tissue engineering. *Cell Physiol Biochem* **35**, 841, 2015.
51. Demoor, M., Ollitrault, D., Gomez-Leduc, T., Bouyoucef, M., Hervieu, M., Fabre, H., Lafont, J., Denoix, J.M., Audigie, F., Mallein-Gerin, F., Legendre, F., andGalera, P. Cartilage tissue engineering: molecular control of chondrocyte differentiation for proper cartilage matrix reconstruction. *Biochim Biophys Acta* **1840**, 2414, 2014.
52. Duval, E., Bauge, C., Andriamanalijaona, R., Benateau, H., Leclercq, S., Dutoit, S., Poulain, L., Galera, P., andBoumediene, K. Molecular mechanism of hypoxia-induced chondrogenesis and its application in in vivo cartilage tissue engineering. *Biomaterials* **33**, 6042, 2012.
53. Ghone, N.V., andGrayson, W.L. Recapitulation of mesenchymal condensation enhances in vitro chondrogenesis of human mesenchymal stem cells. *Journal of cellular physiology* **227**, 3701, 2012.

54. Ronziere, M.C., Perrier, E., Mallein-Gerin, F., and Freyria, A.M. Chondrogenic potential of bone marrow- and adipose tissue-derived adult human mesenchymal stem cells. *Biomed Mater Eng* **20**, 145, 2010.
55. Sheehy, E.J., Buckley, C.T., and Kelly, D.J. Oxygen tension regulates the osteogenic, chondrogenic and endochondral phenotype of bone marrow derived mesenchymal stem cells. *Biochem Biophys Res Commun* **417**, 305, 2012.
56. Duval, E., Leclercq, S., Elissalde, J.M., Demoor, M., Galera, P., and Boumediene, K. Hypoxia-inducible factor 1alpha inhibits the fibroblast-like markers type I and type III collagen during hypoxia-induced chondrocyte redifferentiation: hypoxia not only induces type II collagen and aggrecan, but it also inhibits type I and type III collagen in the hypoxia-inducible factor 1alpha-dependent redifferentiation of chondrocytes. *Arthritis and rheumatism* **60**, 3038, 2009.
57. Meretoja, V.V., Dahlin, R.L., Wright, S., Kasper, F.K., and Mikos, A.G. The effect of hypoxia on the chondrogenic differentiation of co-cultured articular chondrocytes and mesenchymal stem cells in scaffolds. *Biomaterials* **34**, 4266, 2013.
58. Kanichai, M., Ferguson, D., Prendergast, P.J., and Campbell, V.A. Hypoxia promotes chondrogenesis in rat mesenchymal stem cells: a role for AKT and hypoxia-inducible factor (HIF)-1alpha. *Journal of cellular physiology* **216**, 708, 2008.
59. Strobel, S., Loparic, M., Wendt, D., Schenk, A.D., Candrian, C., Lindberg, R.L., Moldovan, F., Barbero, A., and Martin, I. Anabolic and catabolic responses of human articular chondrocytes to varying oxygen percentages. *Arthritis research & therapy* **12**, R34, 2010.
60. Boeuf, S., and Richter, W. Chondrogenesis of mesenchymal stem cells: role of tissue source and inducing factors. *Stem Cell Res Ther* **1**, 31, 2010.
61. Dickhut, A., Pelttari, K., Janicki, P., Wagner, W., Eckstein, V., Egermann, M., and Richter, W. Calcification or dedifferentiation: requirement to lock mesenchymal stem cells in a desired differentiation stage. *Journal of cellular physiology* **219**, 219, 2009.
62. Reppel, L., Margossian, T., Yaghi, L., Moreau, P., Mercier, N., Leger, L., Hupont, S., Stoltz, J.F., Bensoussan, D., and Huselstein, C. Hypoxic culture conditions for Mesenchymal Stromal/Stem Cells from Wharton's jelly: a critical parameter to consider in a therapeutic context. *Curr Stem Cell Res Ther* **9**, 306, 2014.