CIFRE 2012/1161

# EVALUATION OF THE CONTRIBUTION OF A MARINE HEMOGLOBIN IN THE CULTURE OF MESENCHYMAL STEM CELLS FOR BONE AND MENISCUS TISSUE ENGINEERING.


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**INSERM U1078 : Génétique et génomique fonctionnelles et biotechnologie**

**HEMARINA S.A – Morlaix, France**

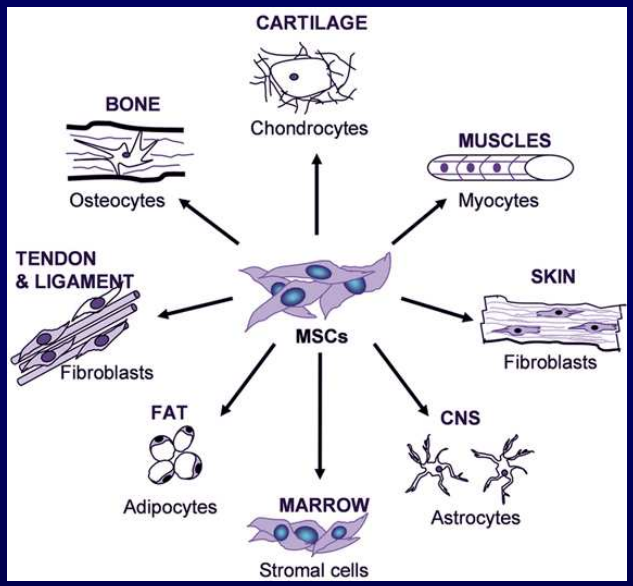
# Mesenchymal stem cells (MSC)

Adult stem cells:

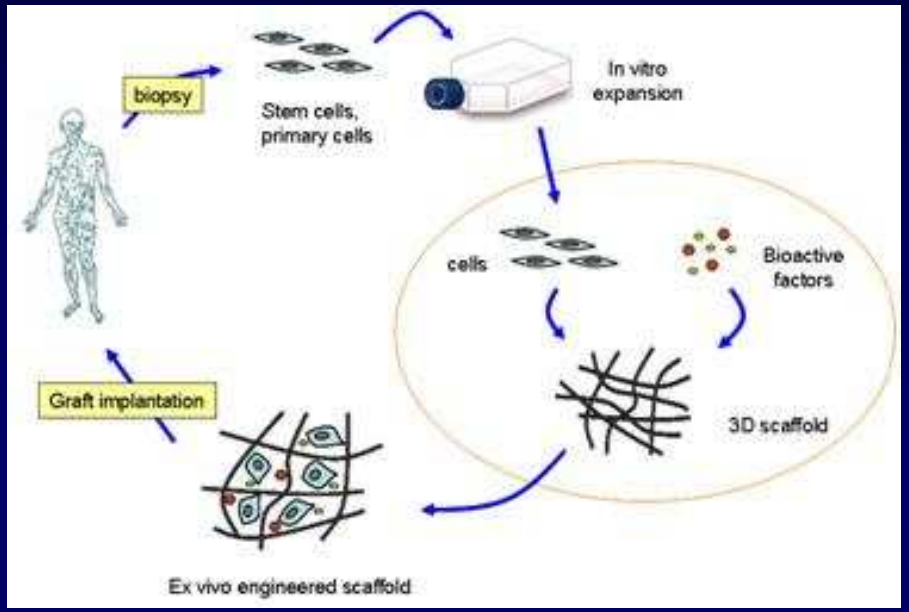
Undifferentiated cells

Self-renewal capacity

Multipotency



# MSCs and tissue engineering




- **Advantages**:

**MSCs**

- *In vitro* isolation and expansion
- Plasticity
- Immunomodulating capacity
- Homing capacity
- No ethical controverse (O'Byrne, 2013)

# Origines of MSCs

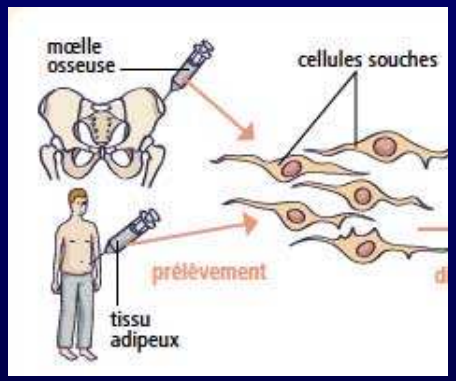
•**Discovered in the 1970' by Friedensten and his collaborators**

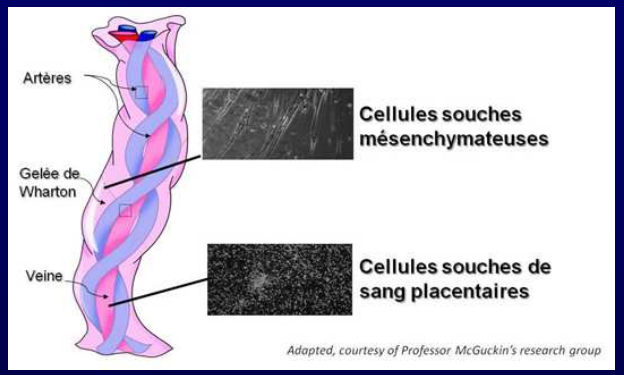
**<u>Classical sources of MSCs:</u>**

1- Bone arrow

2- Fate

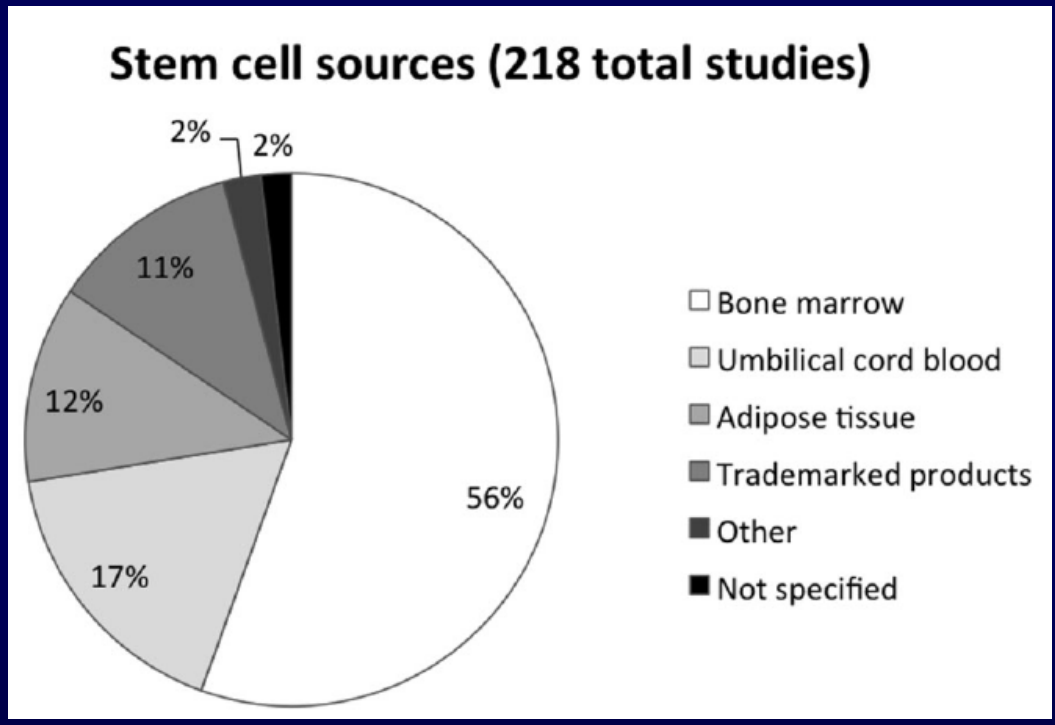
3- Cord blood (- 12 h, few cells)



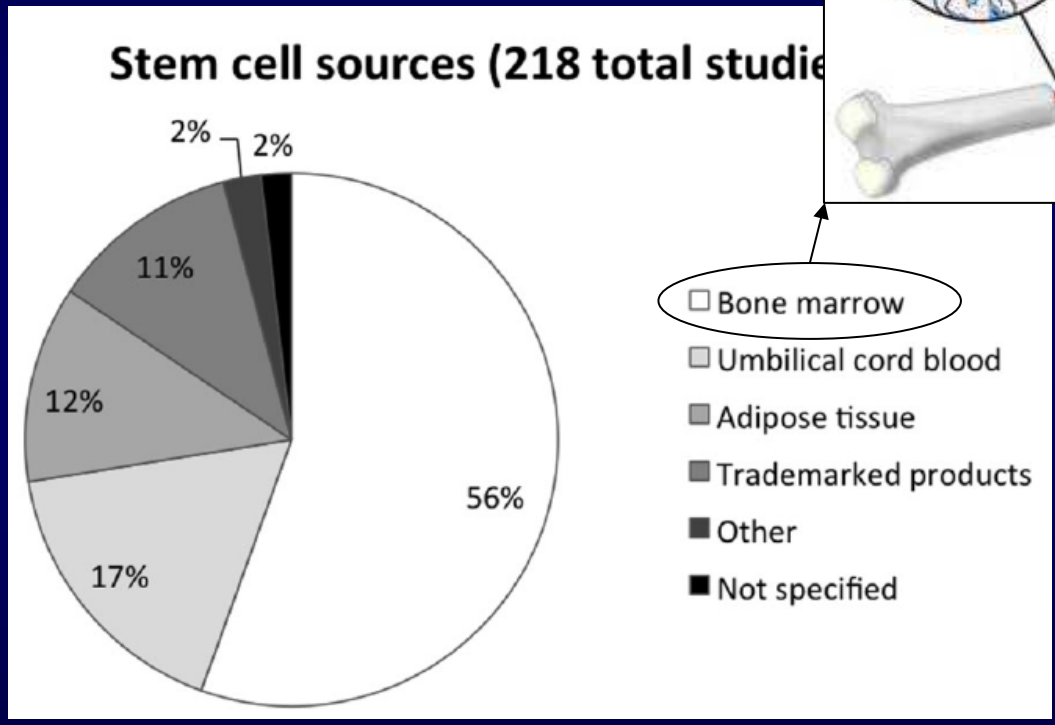

•Also in: amniotic fluid, menstrual blood, human scalp, dental pulp, synovial fluid, periosteum, skeletal muscle, conjunctiva of the eye.

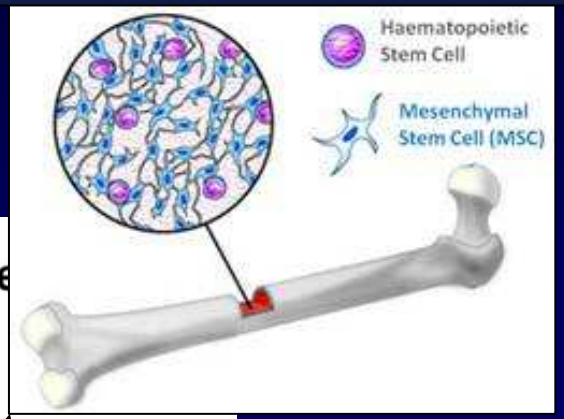
# Experimental studies with MSCs




(Sharma *et al*, 2014.Transfusion.)

# Experimental studies with MSCs




(Sharma *et al*, 2014.Transfusion.)

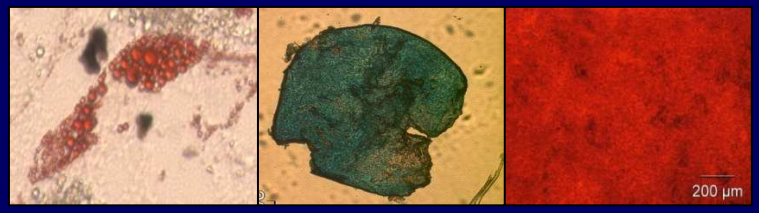
# *In vitro* characterization

**ISCT** (**I**nternational **S**ociety for **C**ellular **T**herapy)

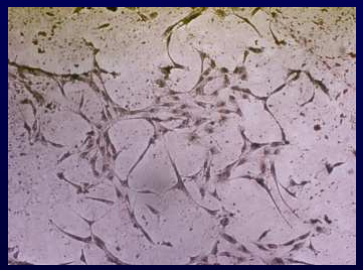
(Dominici, 2006. *cytotherapy)*

**Adipocytes** **Chondrocytes** **Osteoblastes**

(*in vivo*: O, rarely A, pas C)

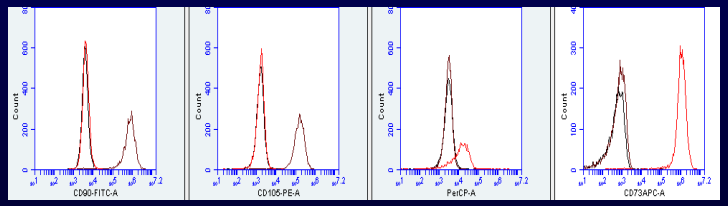



**Plastic adherence**



**Specific membrane markers**

CD105+, CD73+, CD90+ et CD14-, CD20-, CD34-, CD45-



# MSC and Tissue Engineering

Introduction
Objectives
Meniscal cells
2D culture
3D culture
Conclusion
Prospects
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## •Current studies: Tissue Repair

- Régénération vasculaire
- Réparation osseuse et maladie des os (ostéogenèse imparfaite) (Grayson, 2015)
- Régénération du cartilage (Pastides, 2013)
- Régénération cornéenne (*in vitro* et *in vivo*) (Harking, 2014)
- Ulcère chronique de jambe (d'origine veineuse) (Dash, 2009)
- Maladie neuronale (différenciation dopaminergique) (Nadri, 2008)
- Régénération cardiaque (Mietten, 2012; Yang, 2010, Hare, 2009)
- Réparation de la peau (Guhathakurta, 2009; Lataillade, 2007)

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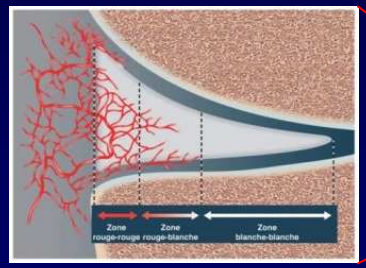
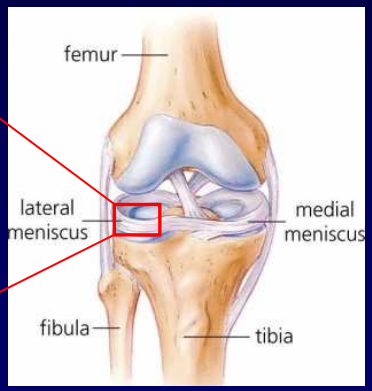
# Meniscus Tissue Engineering




(from P.Beaufils, MO 2011)



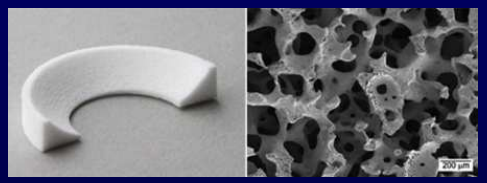

dailysportstack.com

- Partially avascular tissue
- No spontaneous healing
- Cell type not totally characterized
- Important for joint stability and articular congruence

Examples of meniscal substitutes:

Fresh grafts

Frezz grafts



Synthetic substitute (Actifit®, Orteq)

Biological substitute MENISC-T (TBF)

# Meniscus Tissue Engineering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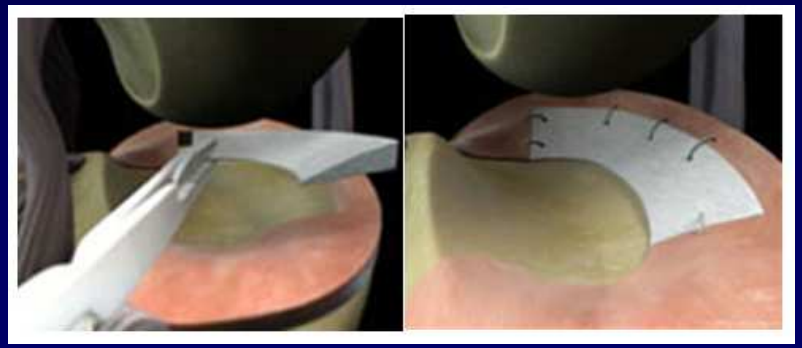
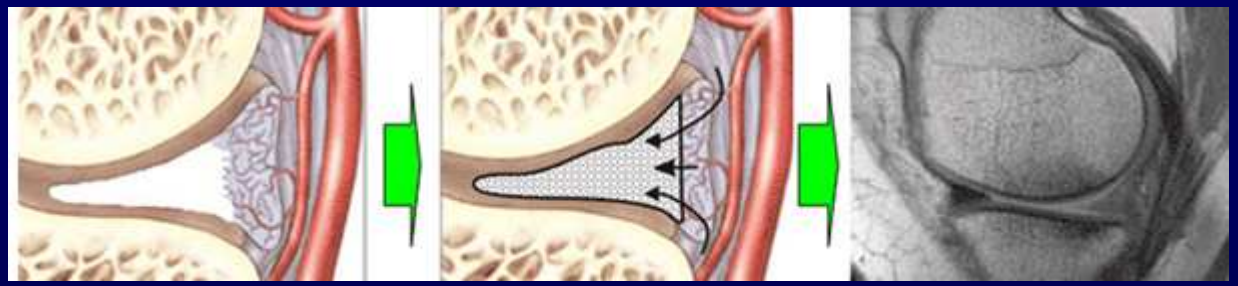




# Bone Tissue Engineering

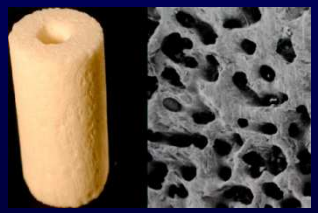
## Case of dental implantology :

http://www.dr-julien-sin.chirurgiens-dentistes.fr

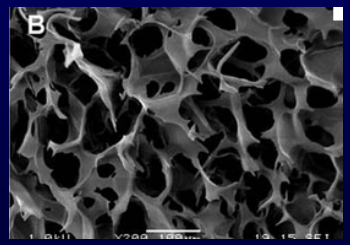
The sypply of oxygen and nutrients is critical for graft survival and long-term integration. (Grayson *et al*, 2015.)

- Bone maxillofacial grafting
- To build a stable base for a denture
- Osteo-integration

## Examples of bone subtitutes:



Calcium carbonate (Corail)




Albumine-based scaffold (Li *et al*, 2014.)

Decellularized human bone (BIOBank®)

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# Bone Tissue Engineering

- 2 grandes étapes :

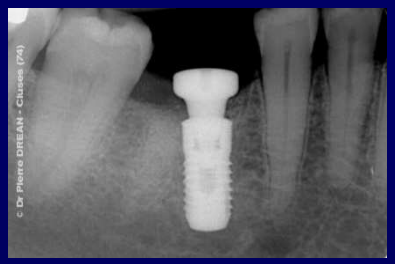

JC Cursolle

Gamma radiosterilization

Biomaterial

MSCs

Growth factors: BMP7...



Osteo-conduction

Osteo-genicity

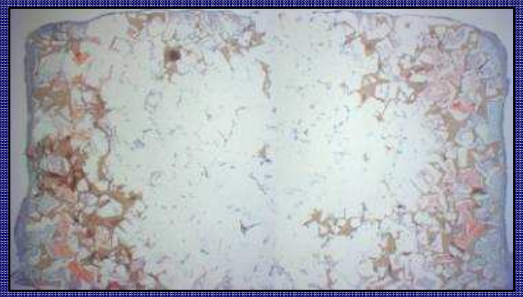
Osteo-induction

Osteo-integration / osteogenesis

# Tissue Engineering

Association of MSCs and biocompatible substituts to dynamize scaffolds

→ cellularization remains incomplete in the innermost (Buma *et al*, 2004; Wendt *et al*, 2003)



Causes?

Lack of oxygen diffusion through the scaffold

Exhaustion of nutrients

Accumulation of cellular wastes

Solutions:

To work with an oxygen carrier to provide oxygen to the cells according to their needs

To improve nutrients renewal

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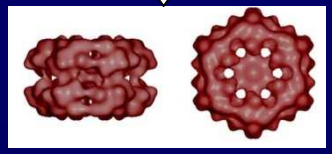
# Marine hemoglobin

North Atlantic coast beaches

*Arenicola marina*

*Nereis virens*

(Rousselot *et al*, 2006; Zal *et al*, 1997)



Extracellular hemoglobin

French biotechnology startup HEMARINA (Morlaix, France)

**Characteristics:**

- Extracellular
- Autonomous
- Universal Hb
- *A.marina* Hb = 3.6 MDa *vs* Human Hb = 68 KDa
- Can bind 156 oxygen molecules (only 4 for humans)
- Antioxidant (SOD-like activity)

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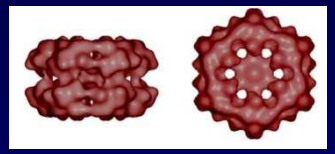
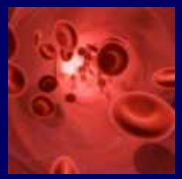
# Marine hemoglobin

Hemoglobin Oxygen Carrier
HEMARINA. SA



Organ preservation
**(HEMO2Life®)**

Management and control of hemorrhagic shock
**(HEMOXYCarrier®)**



Oxygenating dressing
**(HEMO2Ling®)**

(diabetic foot ulcer, etc.)

Cell culture and recombinant protein production (**HEMOXCell®**)

Does HEMOXCell® have any interest for MSC culture?

LeGall *et al*, 2014; Mallet *et al*, 2014; Zal *et al*, 2014; Thillier *et al*, 2011; Rousselot *et al*, 2006.

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# Objectives

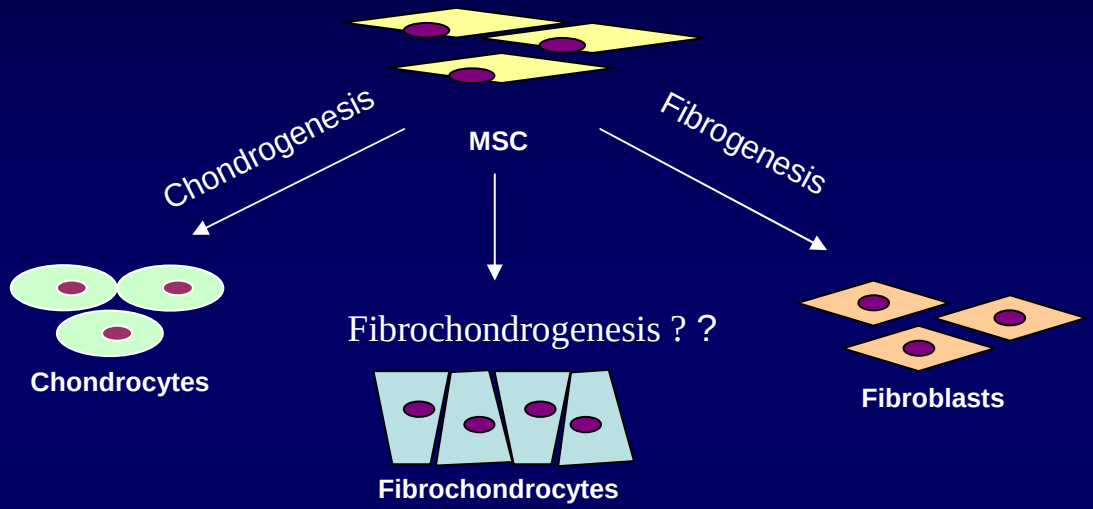
1- Characterization of meniscal cell type (fibrochondrocytes)

2- Impact of HEMOXCell® in MSC 2D culture

3- Impact of HEMOXCell® in scaffold cellularization

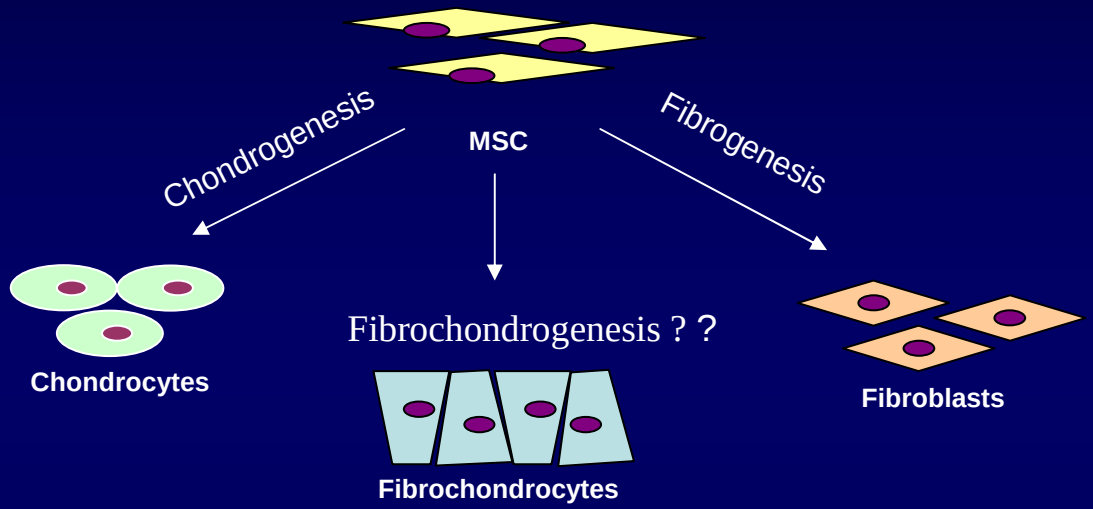
# Axis 1- Characterization of meniscal cell type (fibrochondrocytes)

## 1- Induction of fibrochondrogenesis

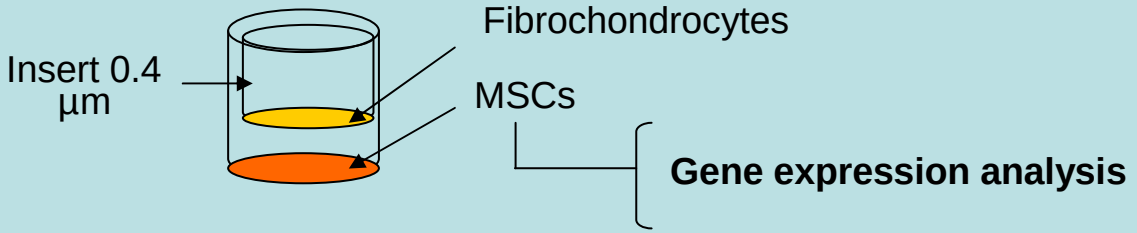


# Axis 1- Characterization of meniscal cell type (fibrochondrocytes)

## 1- Induction of fibrochondrogenesis




- Induction of differentiation with MSC/ fibrochondrocyte co-culture system

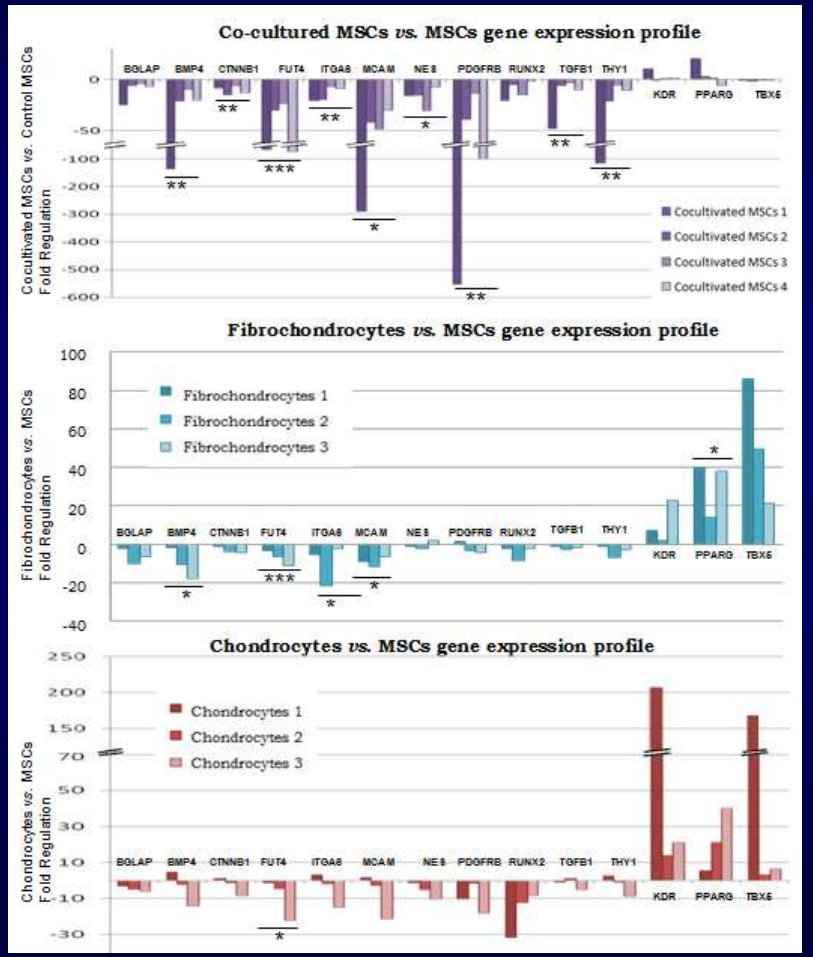


# Axis 1- Characterization of meniscal cell type (fibrochondrocytes)

## 2 - Gene expression analysis by RT-qPCR in a panel of 84 MSC-related genes

| | **Overexpressed** genes (*vs* MSCs) | **Underexpressed** genes (*vs* MSCs) |
|---|---|---|
| Co-cultured MSCs | 1 | **40** |
| Fibrochondrocytes | 5 | 7 |
| Chondrocytes | 7 | 6 |

**Data indicate similarities in gene expression profiles between cell types. They also suggest that co-culturing induces differentiation of MSCs towards the cell type of interest**




*p<0.05, ** p<0.005, *** p<0.0005 (n=3)

# Axis 1- Characterization of meniscal cell type (fibrochondrocytes)

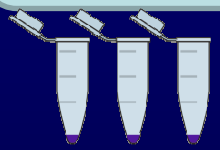
## 3-Searching for protein biomarkers

- Comparative studies between chondrocytes, fibrochondrocytes, and MSCs

**Laboratoire de Spectrométrie de Masse des Interactions et des Systèmes (LSMIS)**
UMR7140 UdS - CNRS « Chimie de la Matière Complexe » , Strasbourg.

Cell culture
(MSC, Fibro et Chondro)

Membrane protein extraction



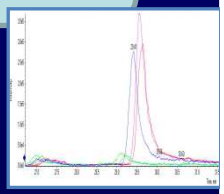
Triplicat (3 patients/ cell type)
MEM Per Plus Kit, PerBio

Nano LC-MSMS analysis
(Label Free)

Database search

Protein identification

Quantification

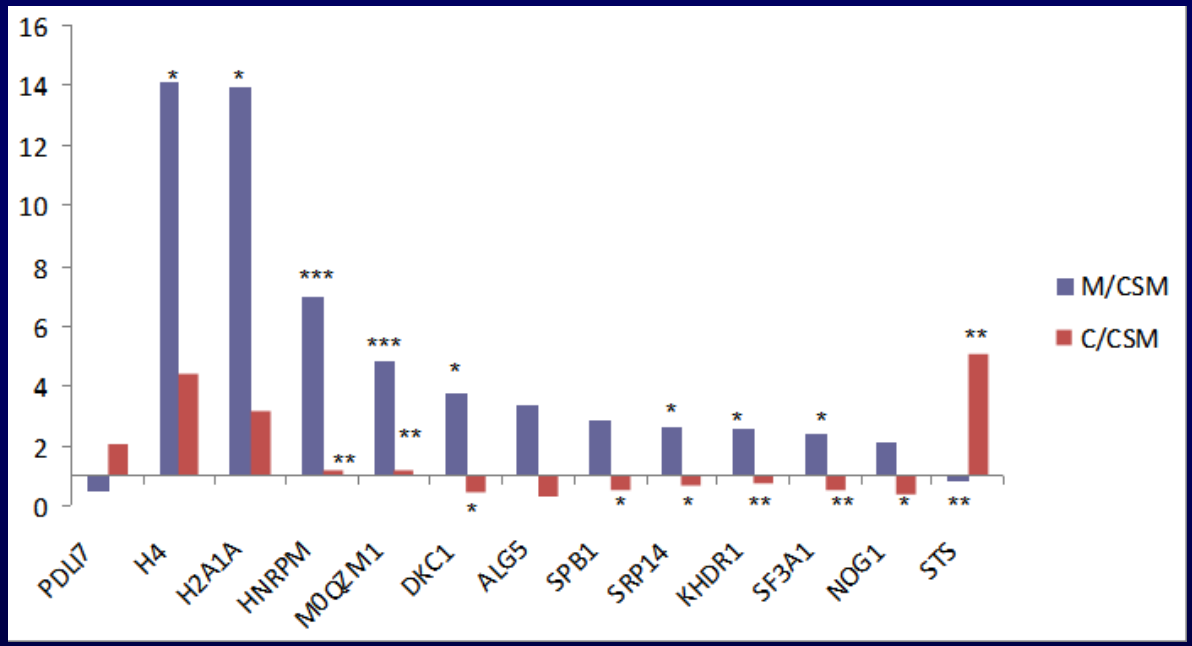


Calculation of protein ratios

# Axis 1- Characterization of meniscal cell type (fibrochondrocytes)

## 3-Searching for protein biomarkers

Comparative studies between chondrocytes, fibrochondrocytes, and MSCs proteomes



# Axis 1- Characterization of meniscal cell type (fibrochondrocytes)

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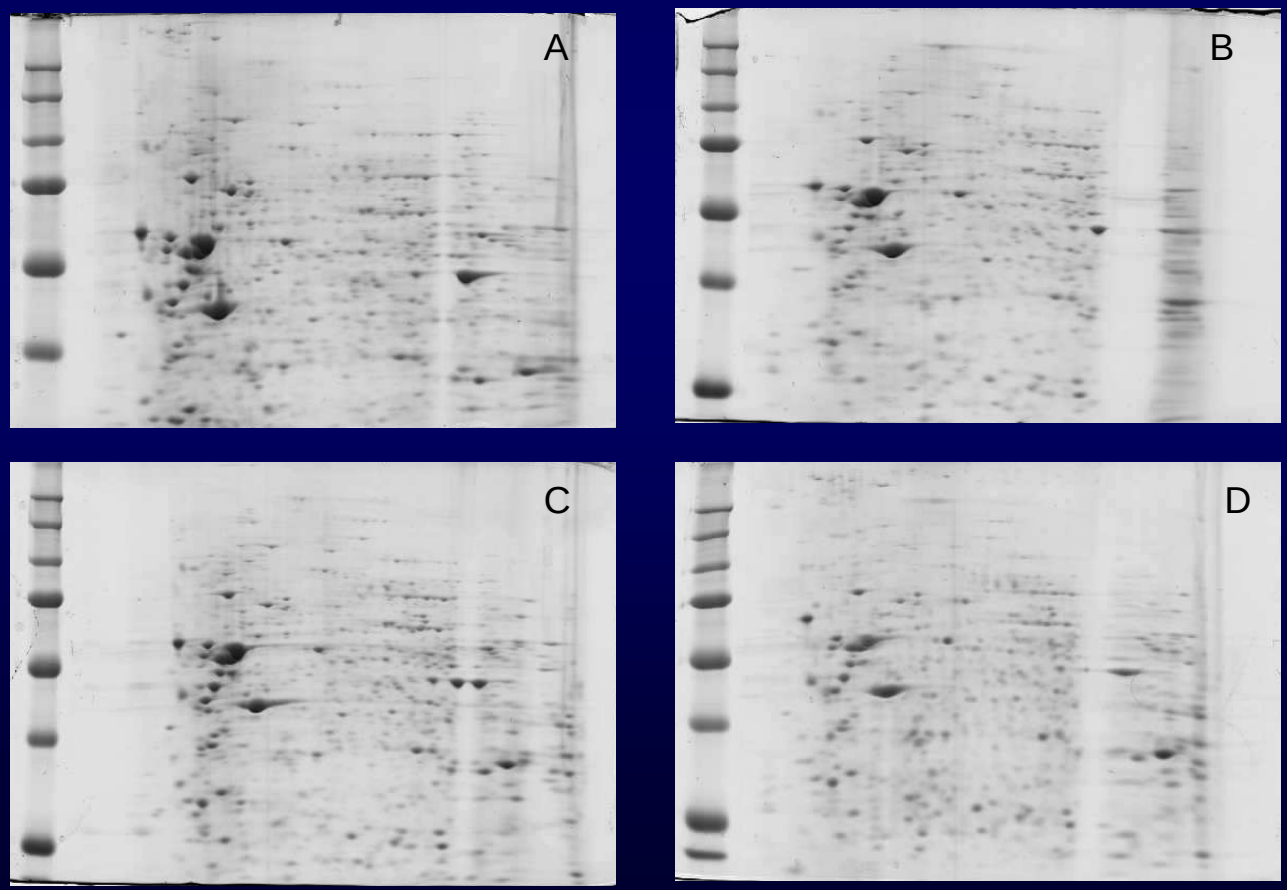
Conclusion

4- Development of two-dimensional gel electrophoresis (2D)

Examples of 2D electrophoretic gels

(100µg total protein extract/ MSC sample):

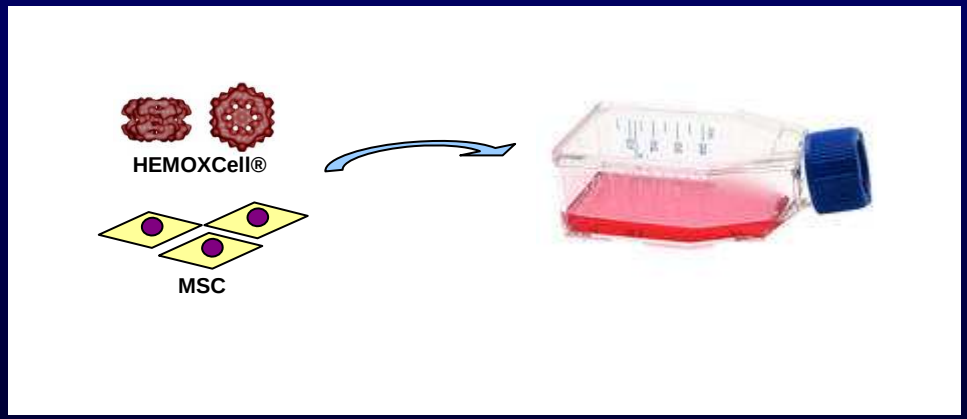


# Axis 2- Impact of HEMOXCell® in MSC 2D culture

**Optimisation of Mesenchymal Stem Cell functions and proliferation: Investigation of the benefits of a new oxygen carrier, HEMOXCell®, in platelet lysate-supplemented media.**

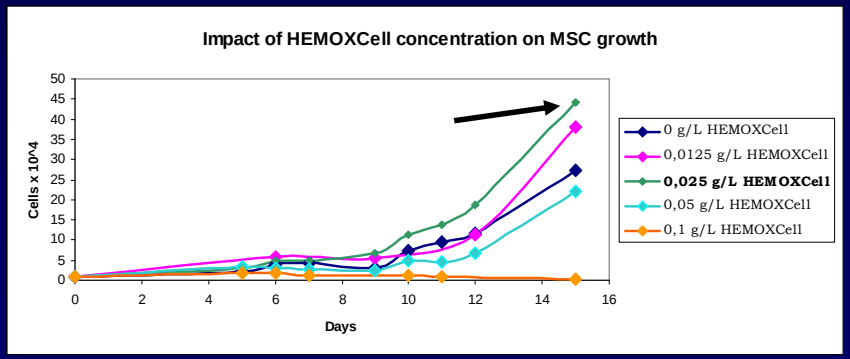
Fiona Le Pape 1,2, Lucie Cosnuau-Kemmat 2, Elisabeth Leize 1,3, Frédéric Dubrana 4, Claude Férec 1,4,5, Morgane Rousselot 2, Franck Zal 2, and Pascal Delépine 1,5*

En soumission au journal *Stem Cell research*

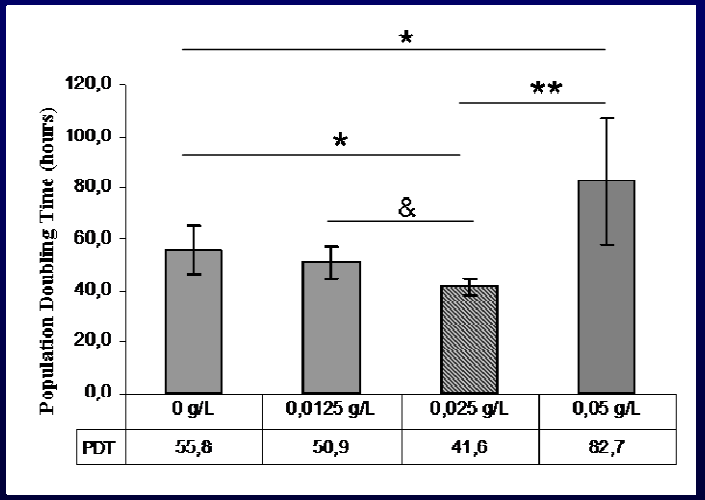


# Axis 2- Impact of HEMOXCell® in MSC 2D culture

## 1 – *In vitro* growth analysis




Low concentrations of HEMOXCell® improve MSC 2-D culture




*p<0.05

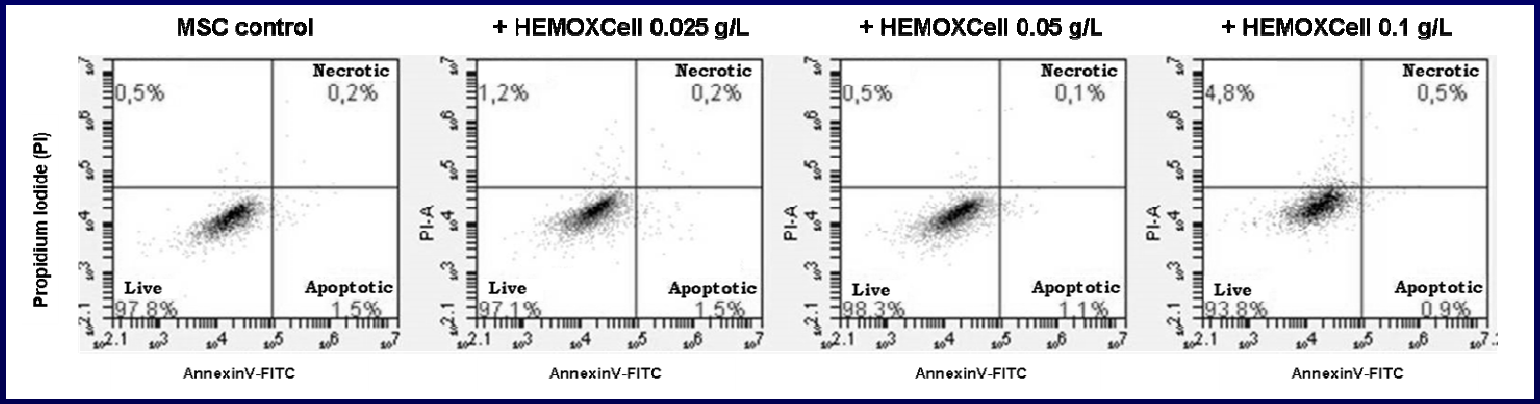
# Axis 2- Impact of HEMOXCell® in MSC 2D culture

## 2 – Cell viability analysis

Normal: 97% ± 2%

Early apoptotic state: 1.3 % ± 0.2%

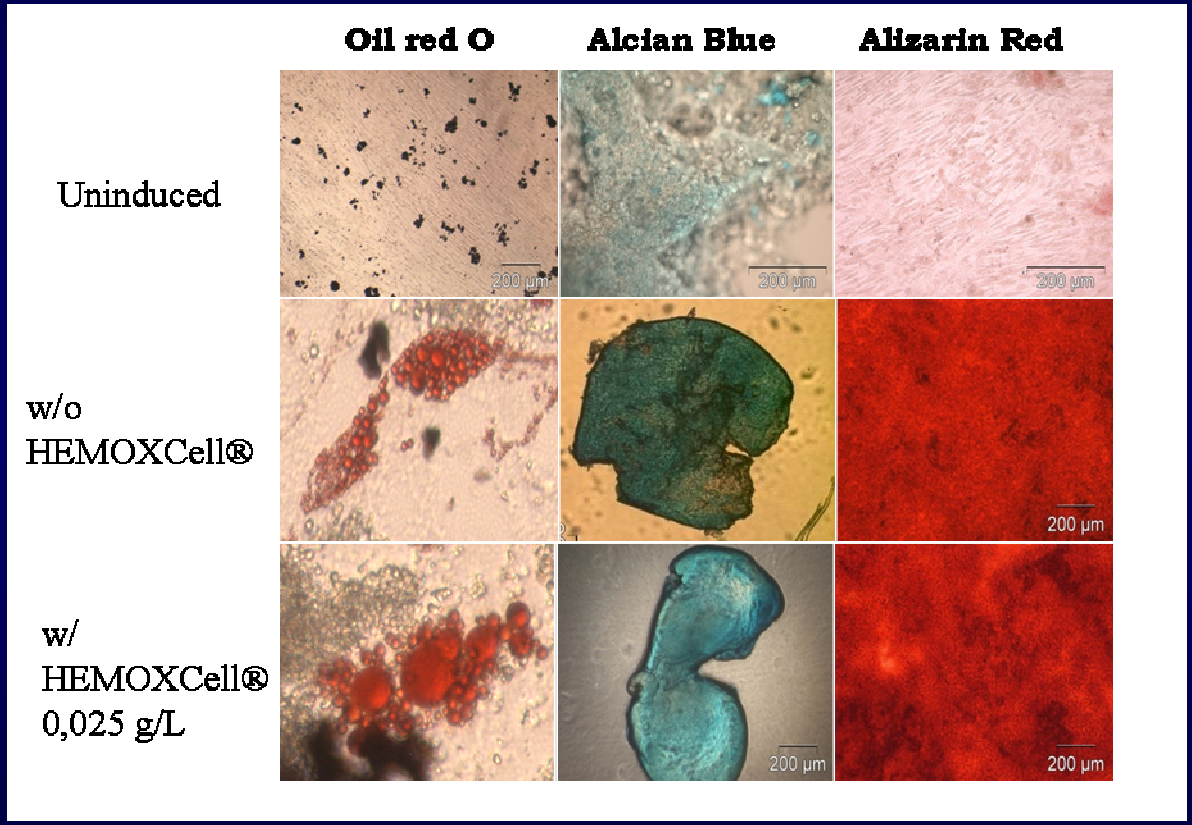
Late apoptotic state: 0.3% ± 0.1%




Annexin V-FITC
Apoptosis Detection Kit
(SIGMA)

# Axis 2- Impact of HEMOXCell® in MSC 2D culture

## 3 - *In vitro* induction assay into adipocytes, chondrocytes and osteoblasts



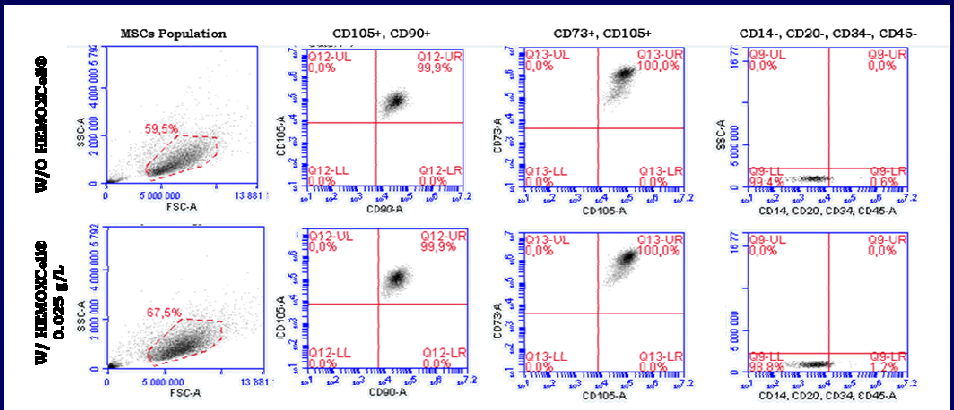

MSCs conserved their plasticity + HEMOXCell® 0.025 g/L

# Axis 2- Impact of HEMOXCell® in MSC 2D culture

## 4- Flow cytometry analysis of MSCs phenotype

Co-expression: CD105+/CD90+ and CD105+/CD73+

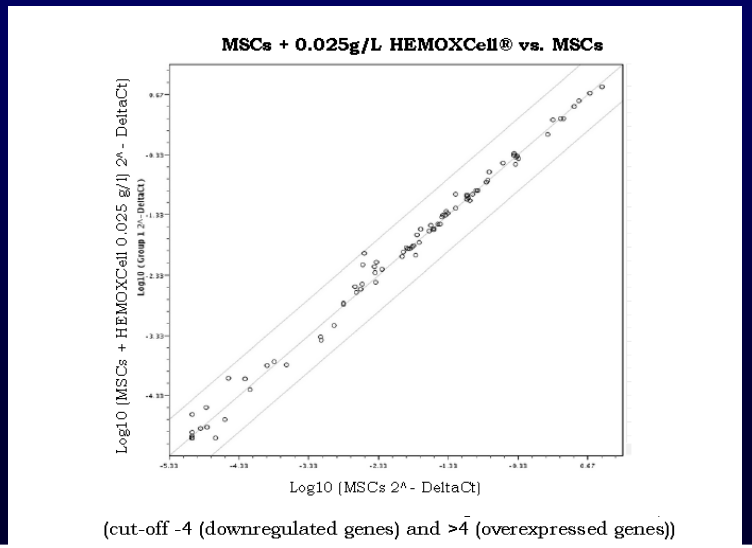
Negative markers: CD14- CD20- CD34- CD45-




**MSCs conserved their phenotype with HEMOXCell® 0.025 g/L**

## 5- Analysis of 84 MSC related gene expression

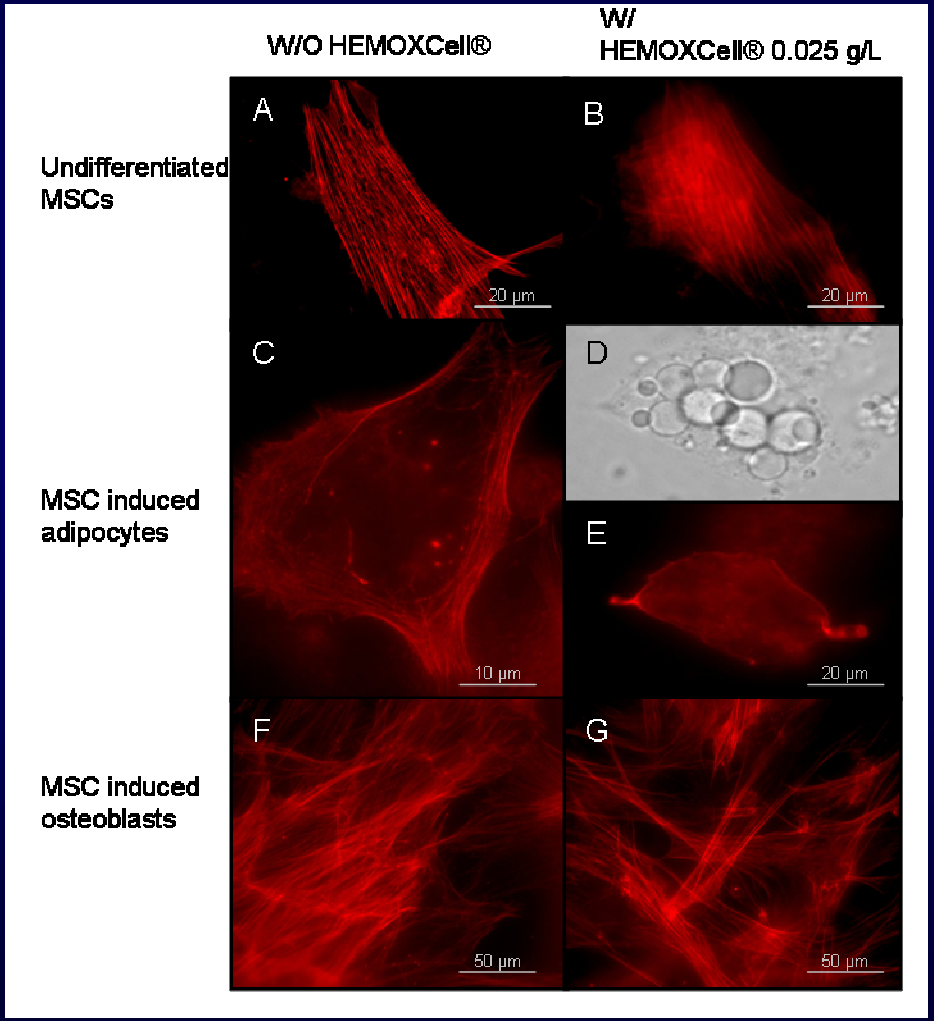
(RT$^2$ Profiler PCR Array (Qiagen)




(cut-off -4 (downregulated genes) and >4 (overexpressed genes))

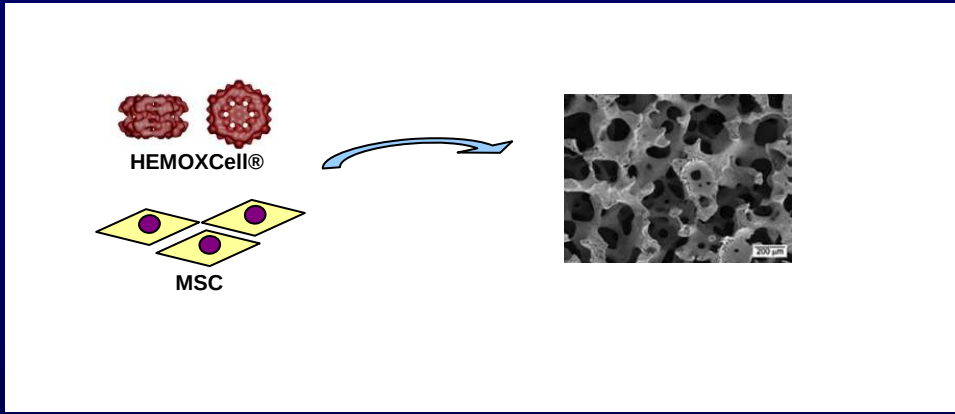
# Axis 2- Impact of HEMOXCell® in MSC 2D culture

## 6 – F-actin cytoskeleton analysis




Alexa Fluor® 594 Phalloidin

# Axis 3- Impact of HEMOXCell® in MSC 3D culture



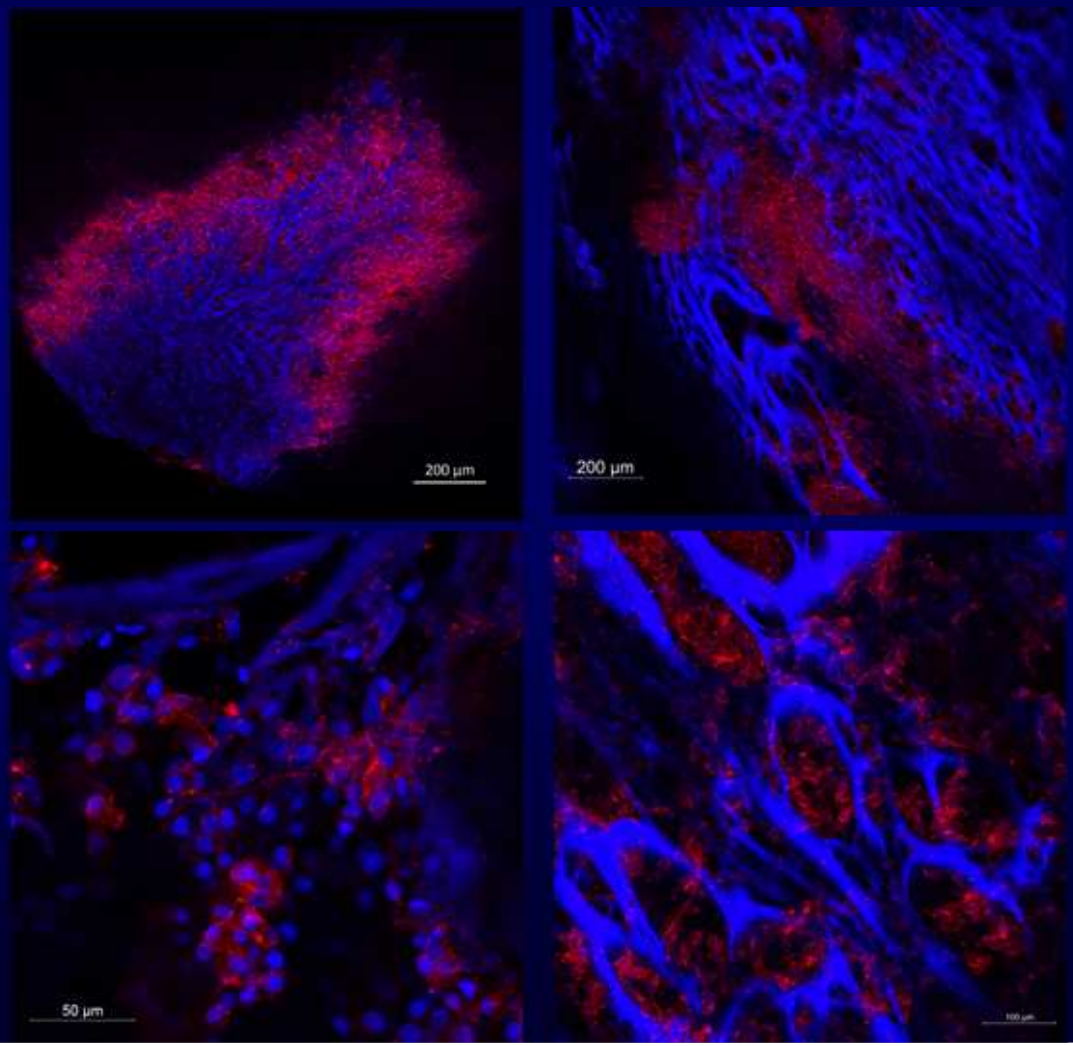
# Axis 3- Impact of HEMOXCell® in MSC 3D culture

## 1- Development of analysis techniques for the meniscal substitute (MENISC-T, TBF)

Culture medium: DMEM

Confocal microscopy photographs of MSC cultured 4 weeks in roller stirring (2 RPM)

Control + HEMOXCEll®

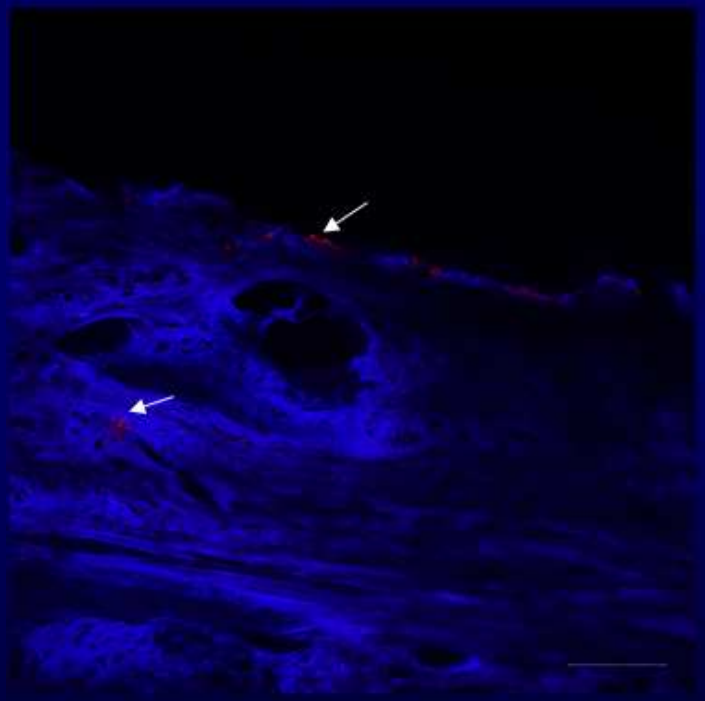


# Axis 3- Impact of HEMOXCell® in MSC 3D culture

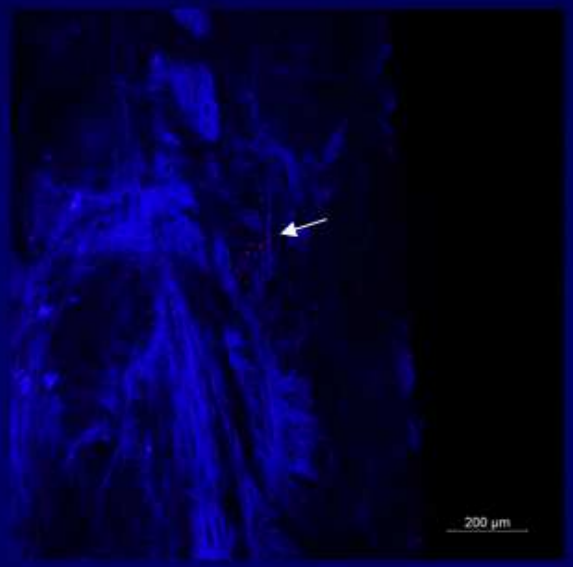
## 1- Development of analysis techniques for the meniscal substitute (MENISC-T, TBF)

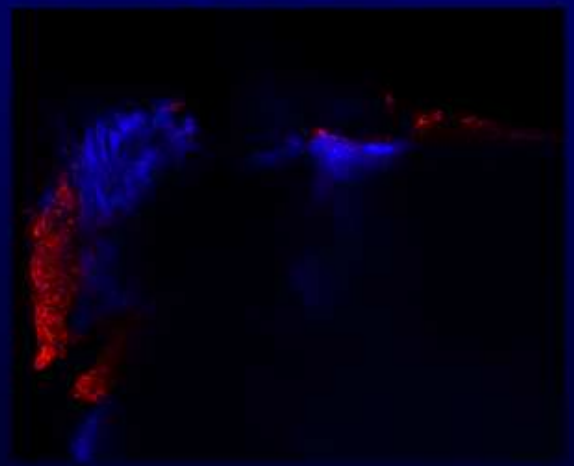
Culture medium: DMEM

Control



+ HEMOXCell® 0.025 g/L

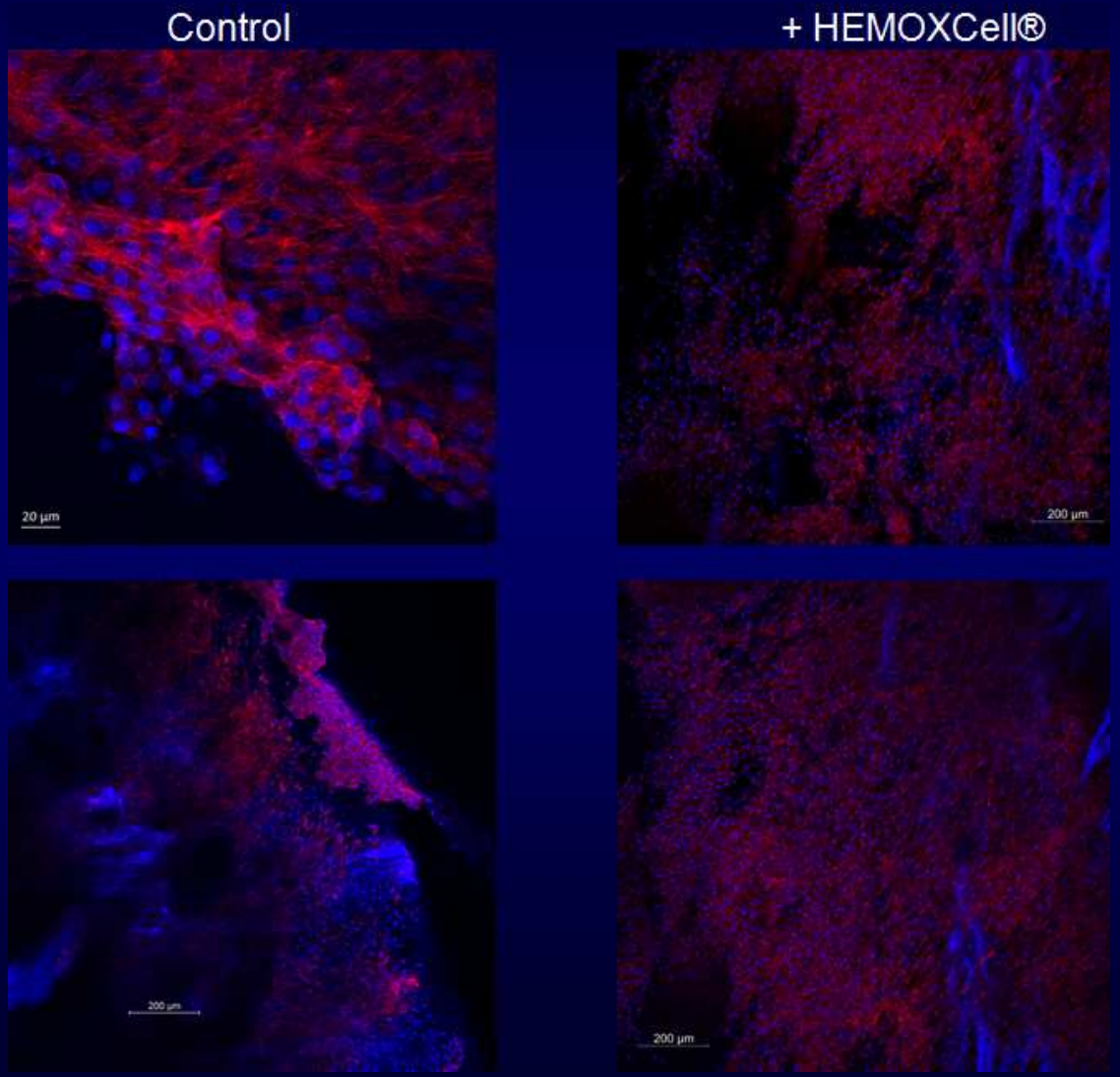


# Axis 3- Impact of HEMOXCell® in MSC 3D culture

## 1- Development of analysis techniques for the meniscal substitute (MENISC-T, TBF)

Culture medium: DMEM, Top of the scaffolds

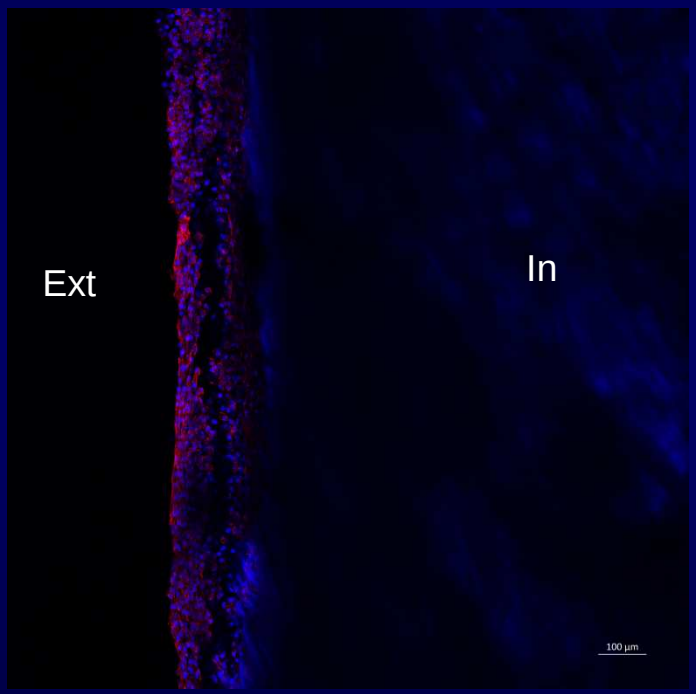


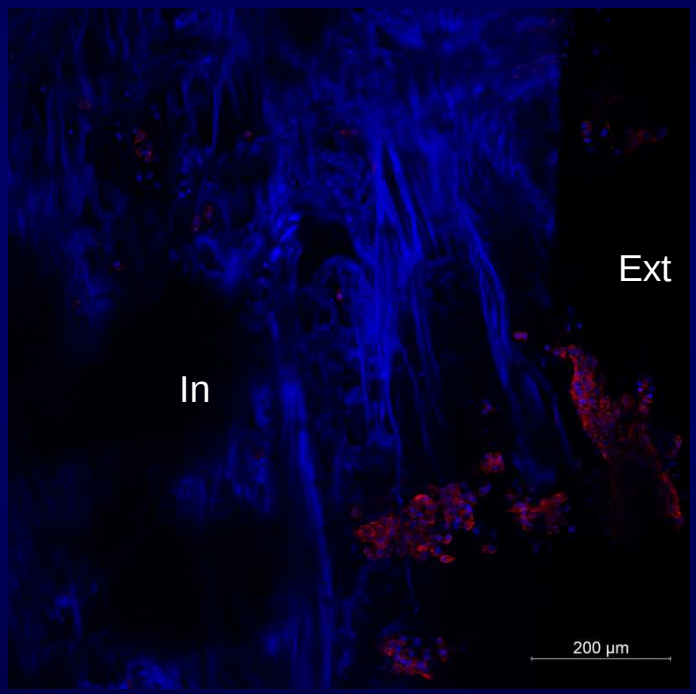
# Axis 3- Impact of HEMOXCell® in MSC 3D culture

## 1- Development of analysis techniques for the meniscal substitute (MENISC-T, TBF)

Culture medium: Chondrogenic differentiation

Control w/o HEMOXCell®



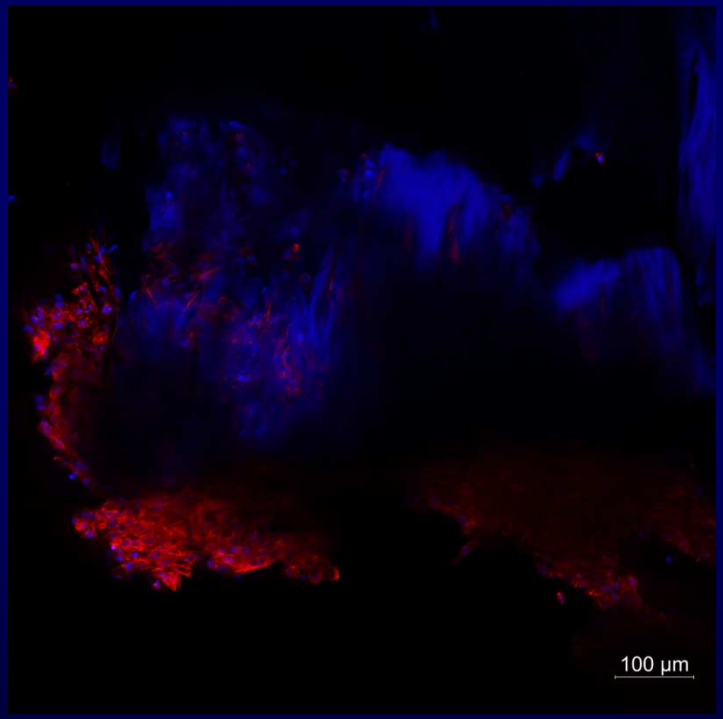



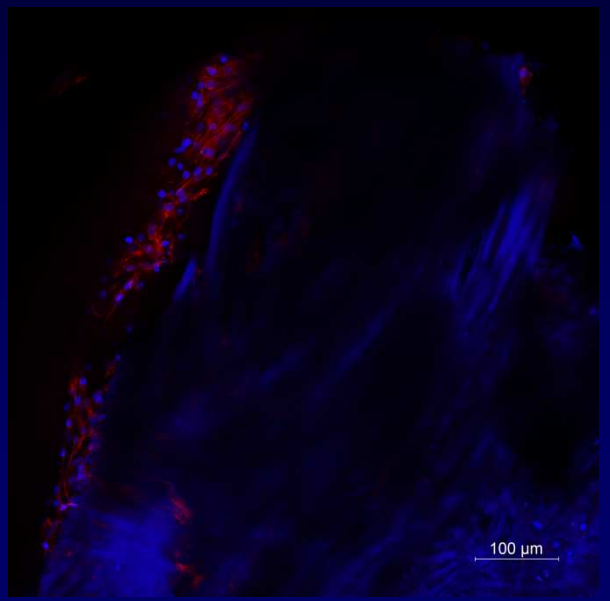
# Axis 3- Impact of HEMOXCell® in MSC 3D culture

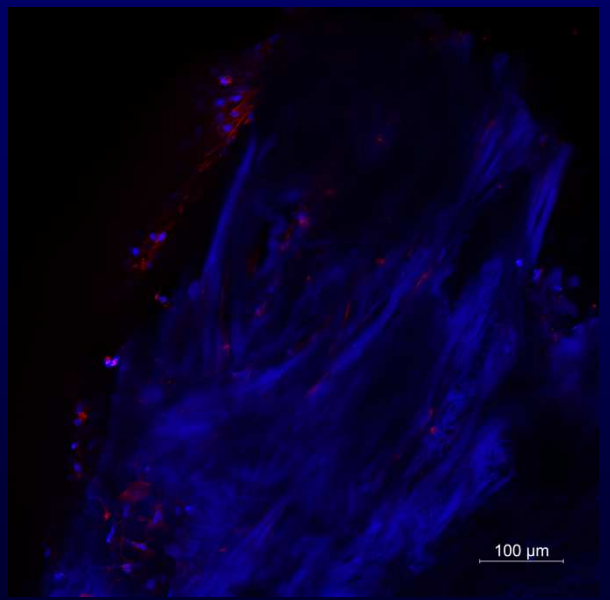
## 1- Development of analysis techniques for the meniscal substitute (MENISC-T, TBF)

Culture medium: Chondrogenic differentiation

+ HEMOXCell®



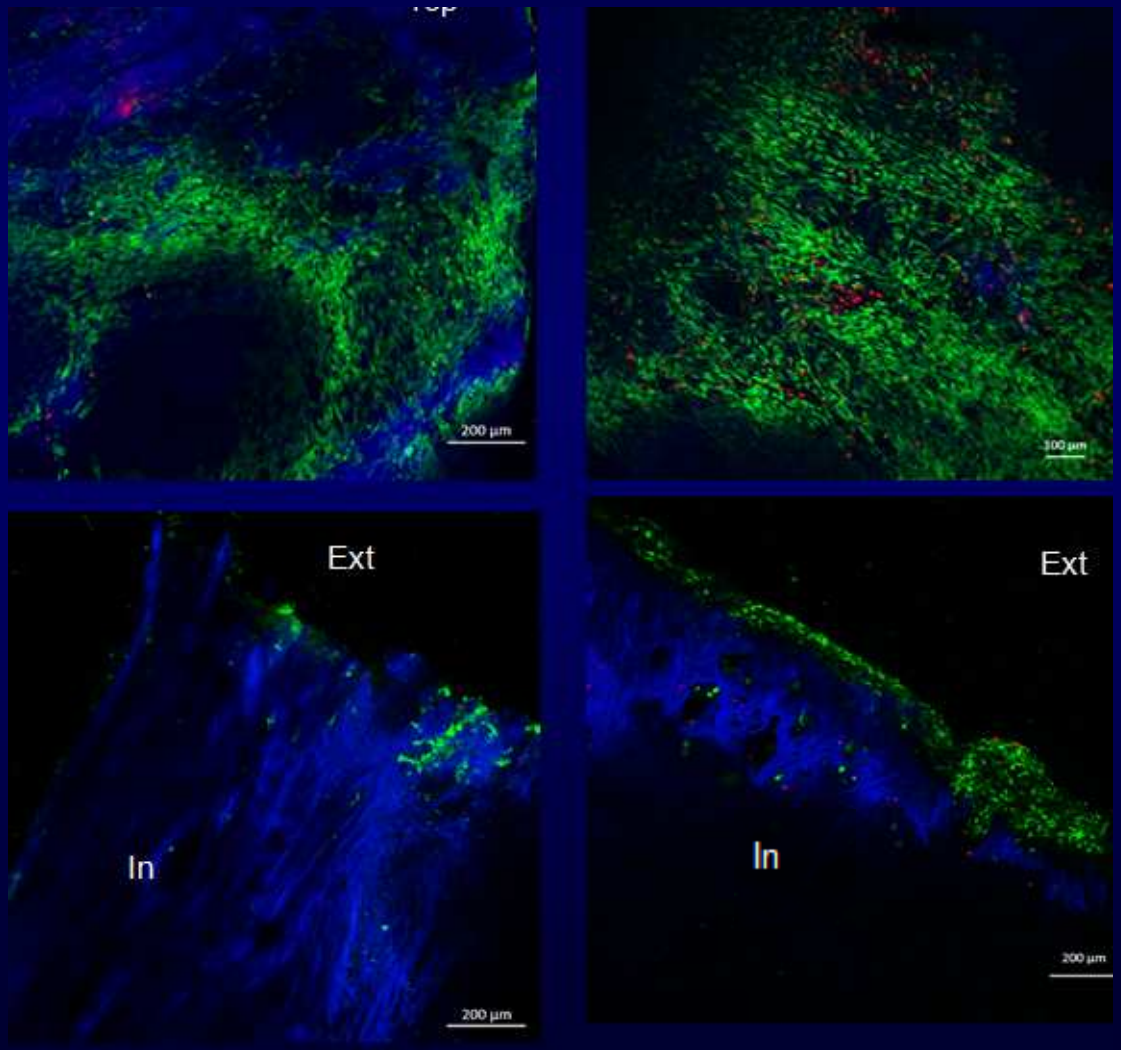
# Axis 3- Impact of HEMOXCell® in MSC 3D culture

## 1- Development of analysis techniques for the meniscal substitute (MENISC-T, TBF)

Controls W/O HEMOXCell®

**DMEM** **Milieu chondrogénique**

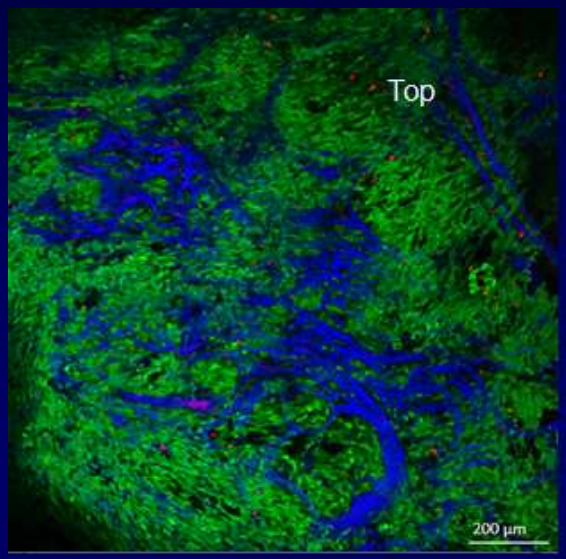
Confocal microscopy photographs of MSC cultured 4 weeks in roller stirring (2 RPM)

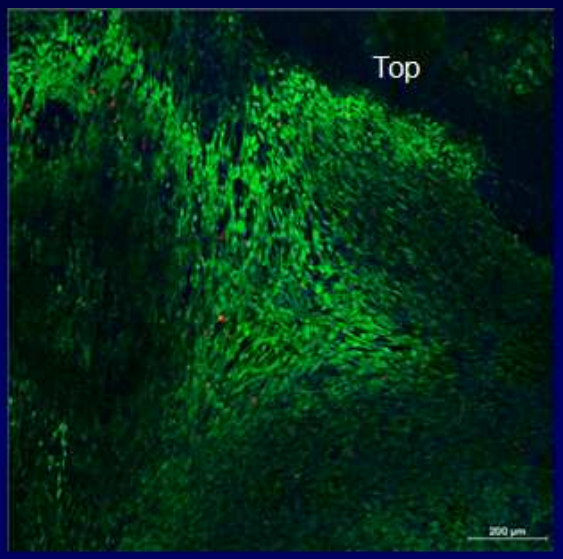


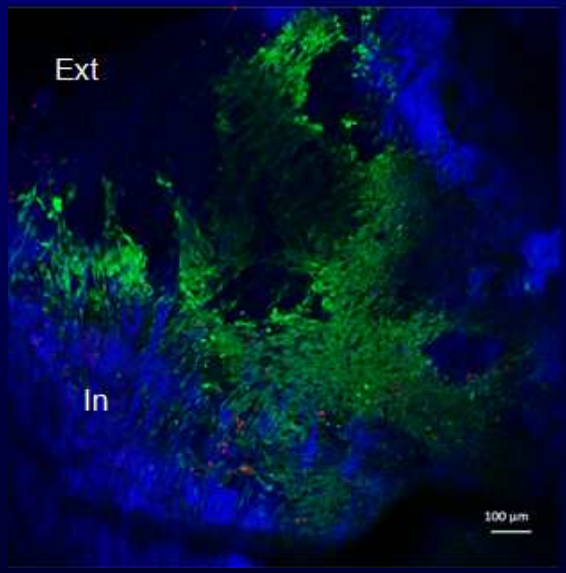
# Axis 3- Impact of HEMOXCell® in MSC 3D culture

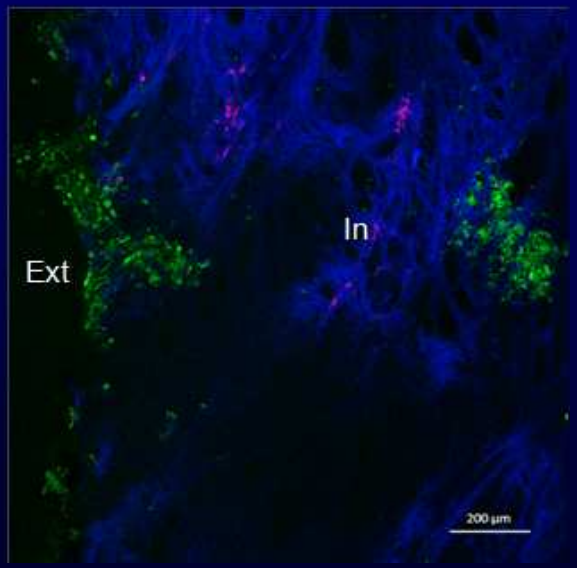
## 1- Development of analysis techniques for the meniscal substitute (MENISC-T, TBF)

+ HEMOXCell® 0.025 g/L



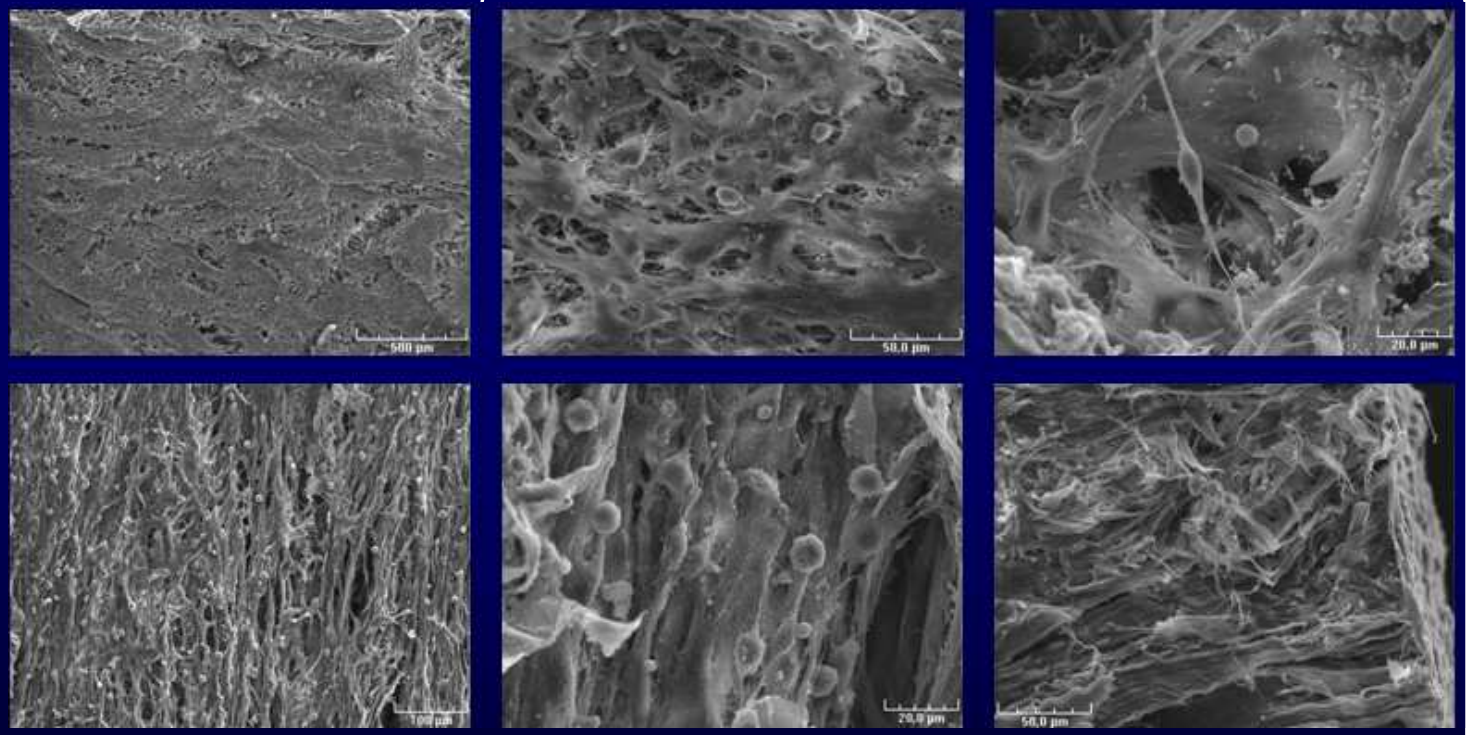



# Axis 3- Impact of HEMOXCell® in MSC 3D culture

## 1- Development of analysis techniques for the meniscal substitute (MENISC-T, TBF)

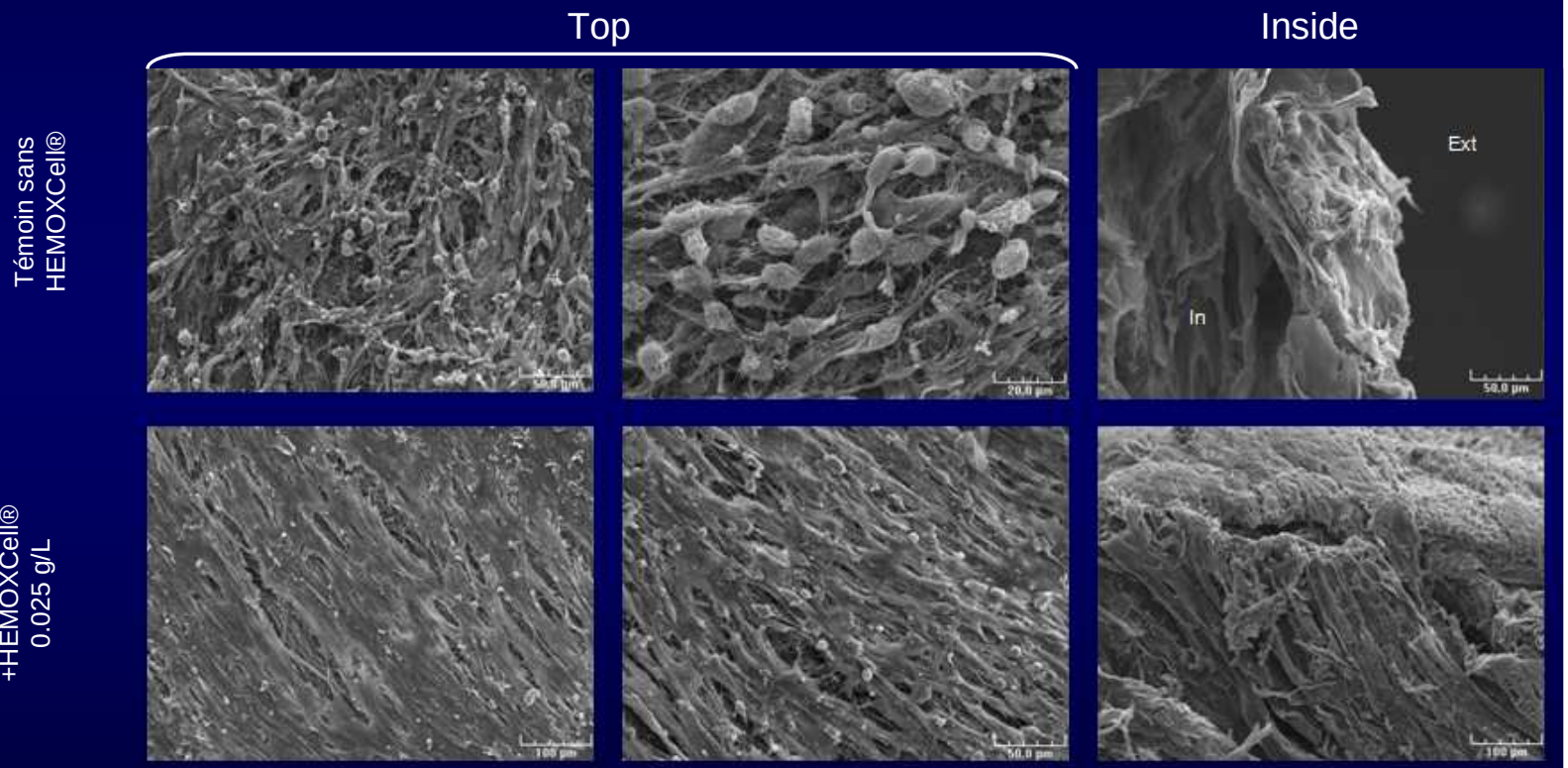
- **Scanning electron microscopy (SEM) photographs (3 weeks)**
  - **Culture medium: DMEM**



# Axis 3- Impact of HEMOXCell® in MSC 3D culture

## 1- Development of analysis techniques for the meniscal substitute (MENISC-T, TBF)

- **Scanning electron microscopy (SEM) photographs (3 weeks)**
  - **Culture medium: Chondrogenic medium**



# Axis 3- Impact of HEMOXCell® in MSC 3D culture

## 1- Development of analysis techniques for the meniscal substitute (MENISC-T, TBF)

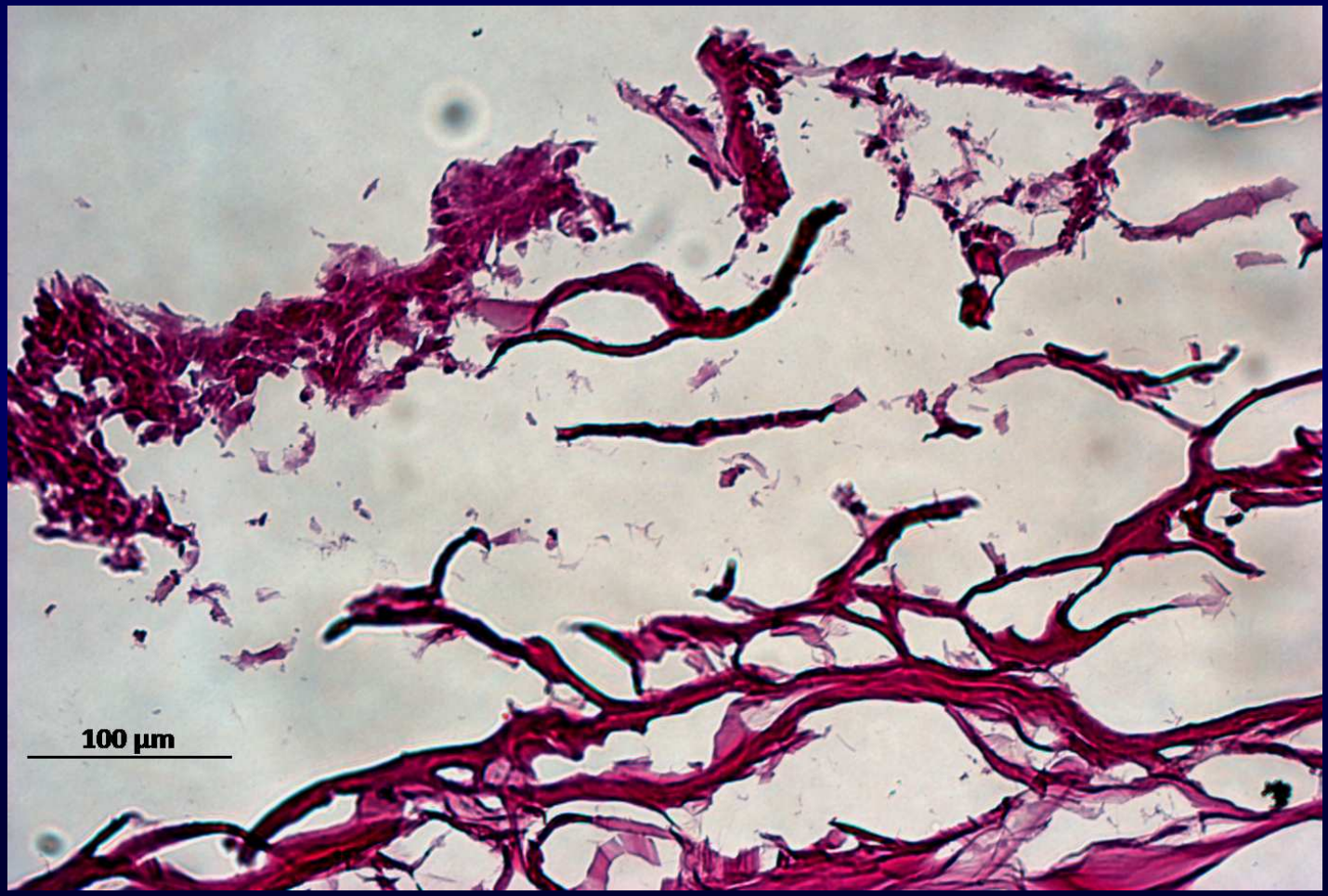
- **Cryostat histological section: H&E staining**
  - **DMEM medium**

# Axis 3- Impact of HEMOXCell® in MSC 3D culture

## 1- Development of analysis techniques for the meniscal substitute (MENISC-T, TBF)

**- DMEM medium**

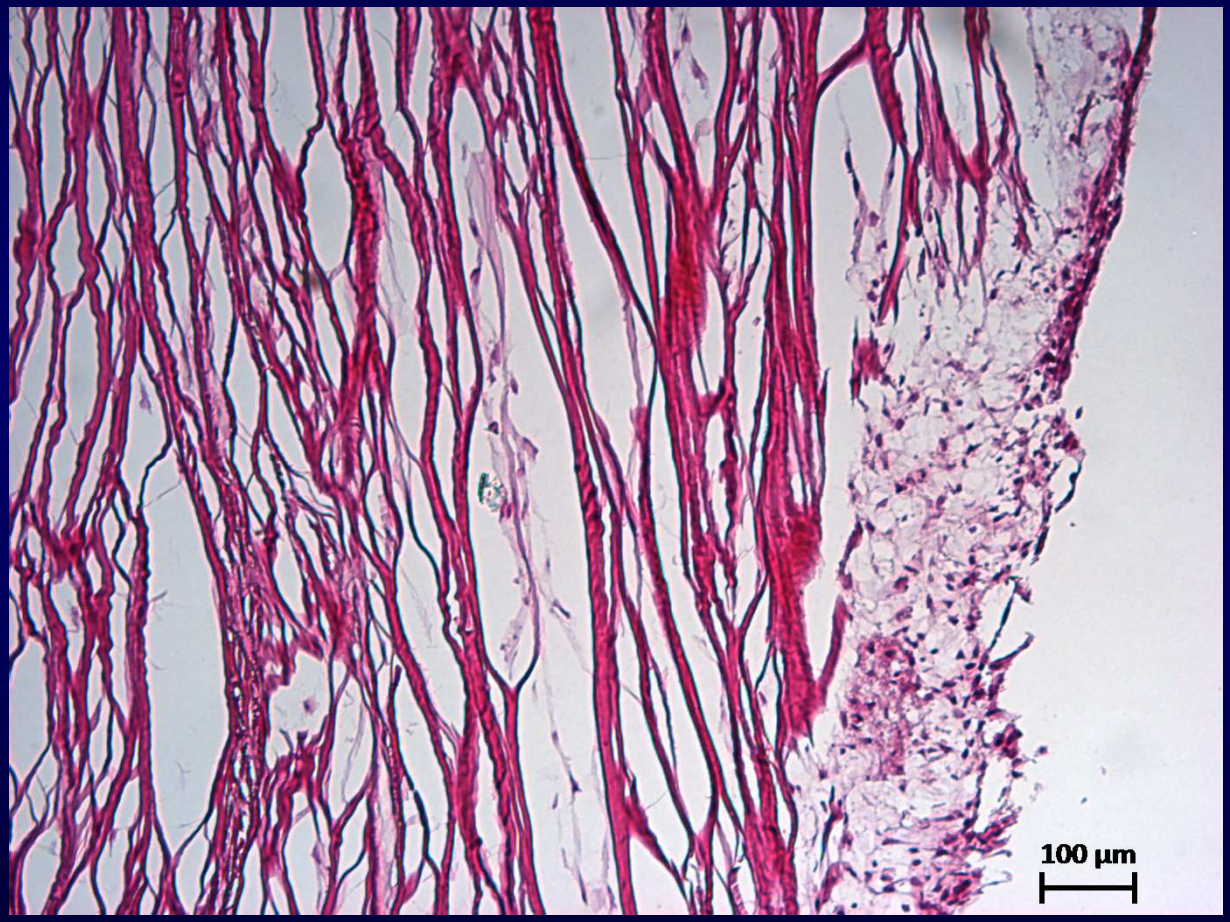
+HEMOXCell® 0.025 g/L



# Axis 3- Impact of HEMOXCell® in MSC 3D culture

## 1- Development of analysis techniques for the meniscal substitute (MENISC-T, TBF)

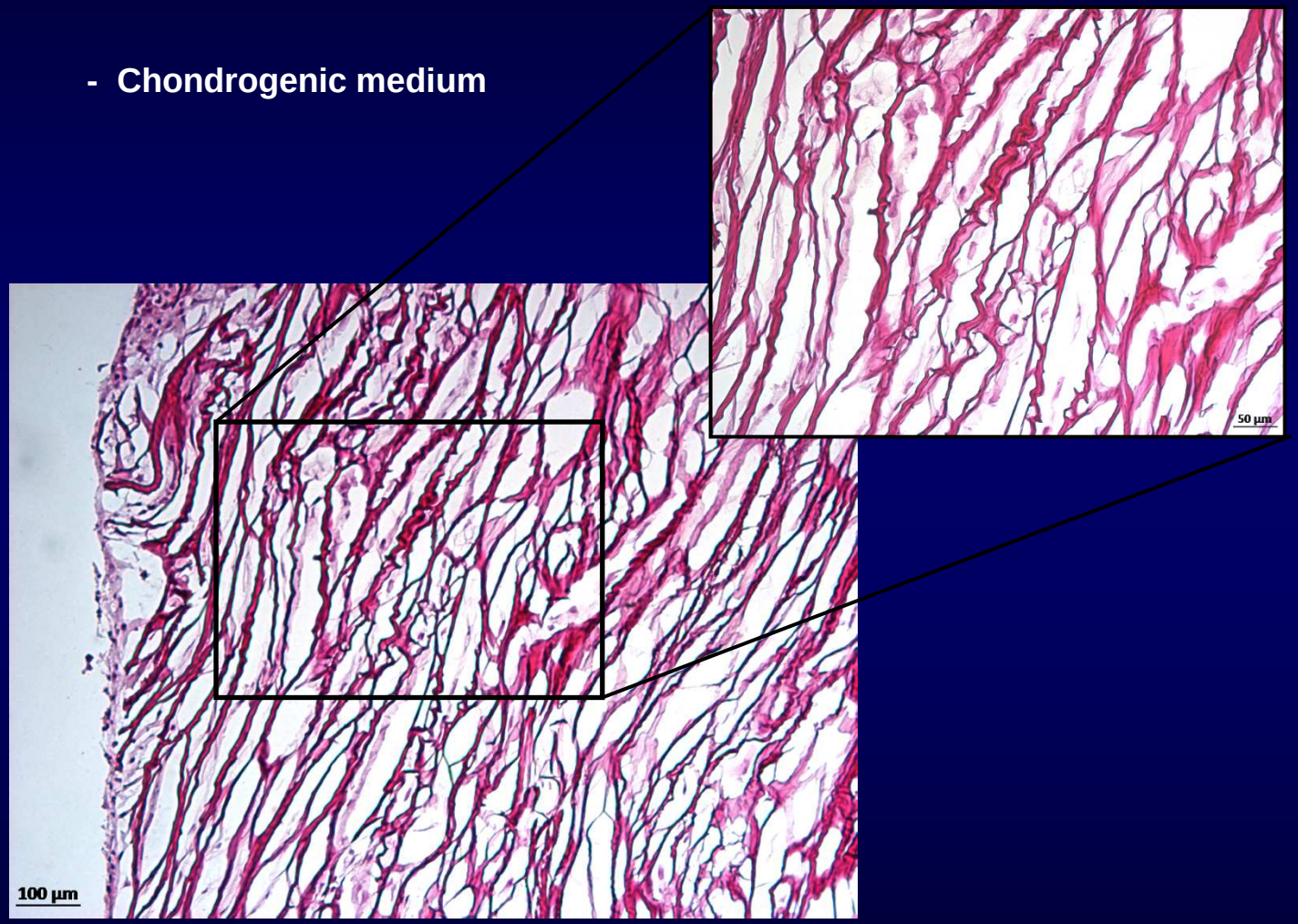
- **Cryostat histological section: H&E staining**
  - **Chondrogenic medium**



# Axis 3- Impact of HEMOXCell® in MSC 3D culture

## 1- Development of analysis techniques for the meniscal substitute (MENISC-T, TBF)

- **Chondrogenic medium**



# Axis 3- Impact of HEMOXCell® in MSC 3D culture

## 1- Development of analysis techniques for the meniscal substitute (MENISC-T, TBF)

- **Chondrogenic medium**

  +HEMOXCell® 0.025 g/L

# Axis 3- Impact of HEMOXCell® in MSC 3D culture

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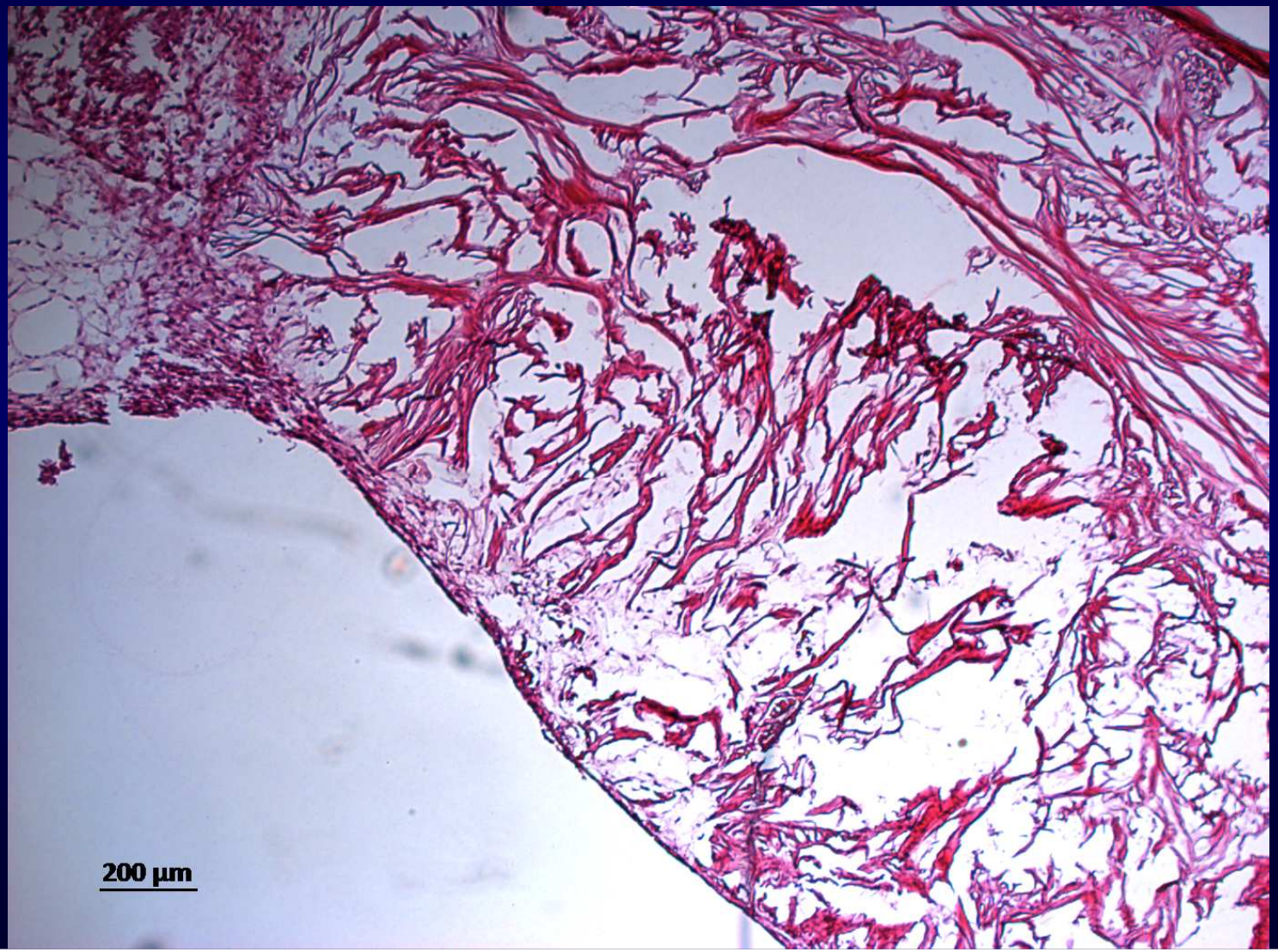
## 1- Development of analysis techniques for the meniscal substitute (MENISC-T, TBF)

- **Chondrogenic medium**

+HEMOXCell® 0.025 g/L

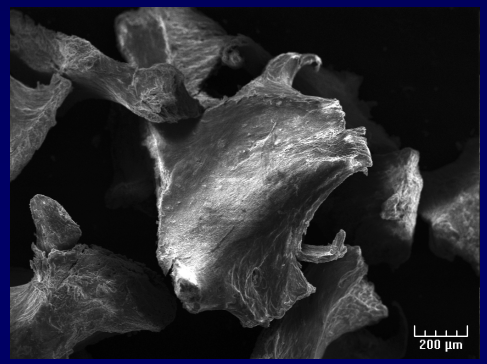


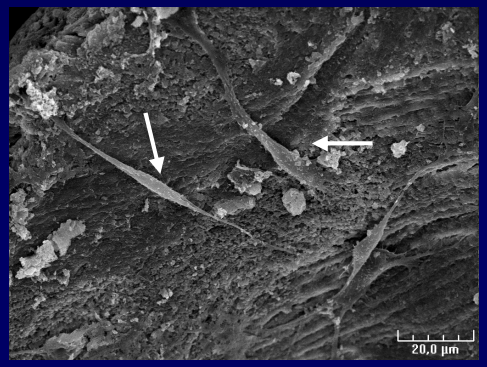
# Axis 3- Impact of HEMOXCell® in MSC 3D culture

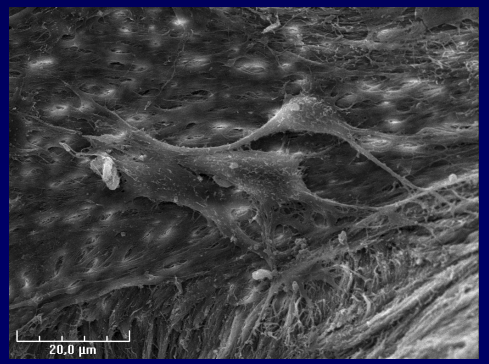
## 1- Development of analysis techniques for the bone substitute (BioBank®)

MSCs cultured on bone powder in standard media

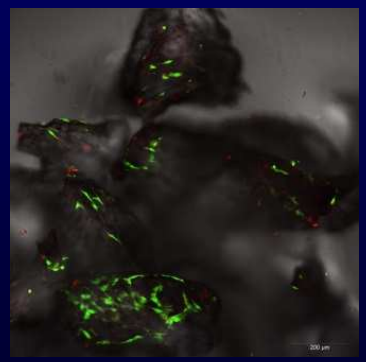
- **Scanning electron microscopy (SEM) photographs (24h)**



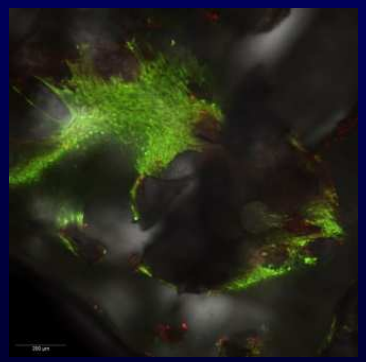




- **Confocal microscopy: Immunolabeling**



2 days



21 days

Live/dead® Viability/cytotoxicity Kit (Invitrogen)

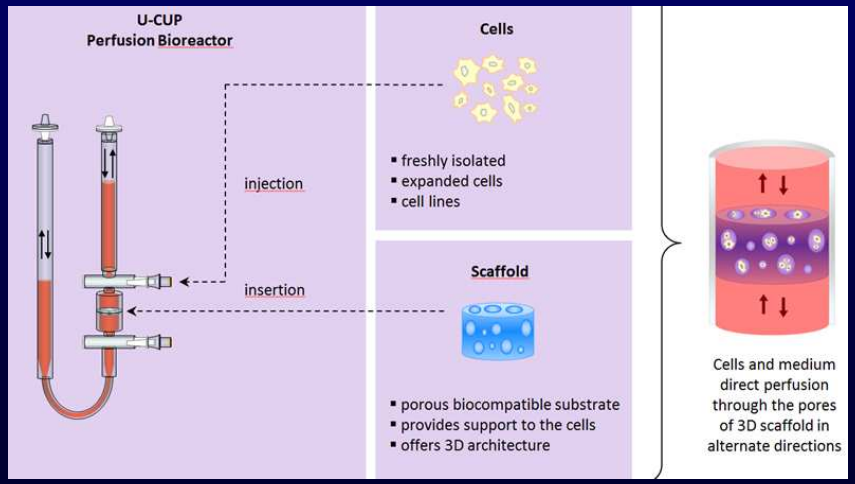
(Green: live cells, red: dead cells)

# Axis 3- Impact of HEMOXCell® in MSC 3D culture

## 3- Development of perfusion culture system

**U-Cup system:**

↑ homogeneous cellularization
↑ nutrient diffusion




CELLEC BIOTEK AG

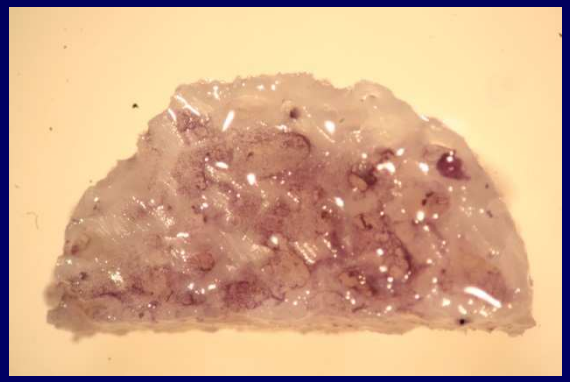
# Axis 3- Impact of HEMOXCell® in MSC 3D culture

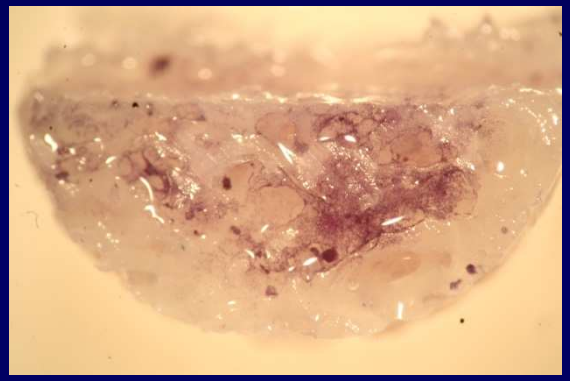
## 3- Development of perfusion culture system

Validation of bone cylinders (8 mm x 4 mm) for the perfusion system U-Cup (CellekBiotek)

MTT solution (living cells in purple)

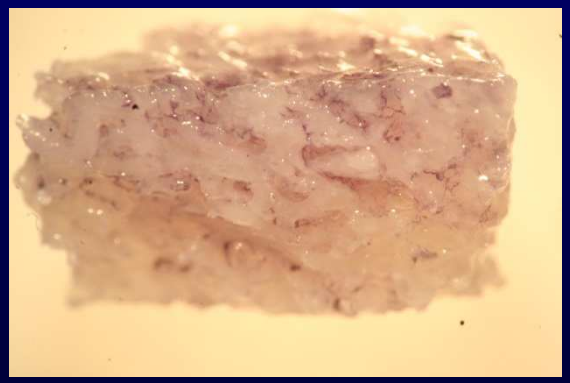
Top





down

cross section



cross section

MTT staining

# Axis 3- Impact of HEMOXCell® in MSC 3D culture

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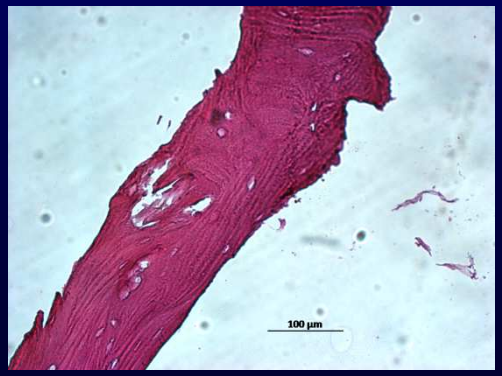
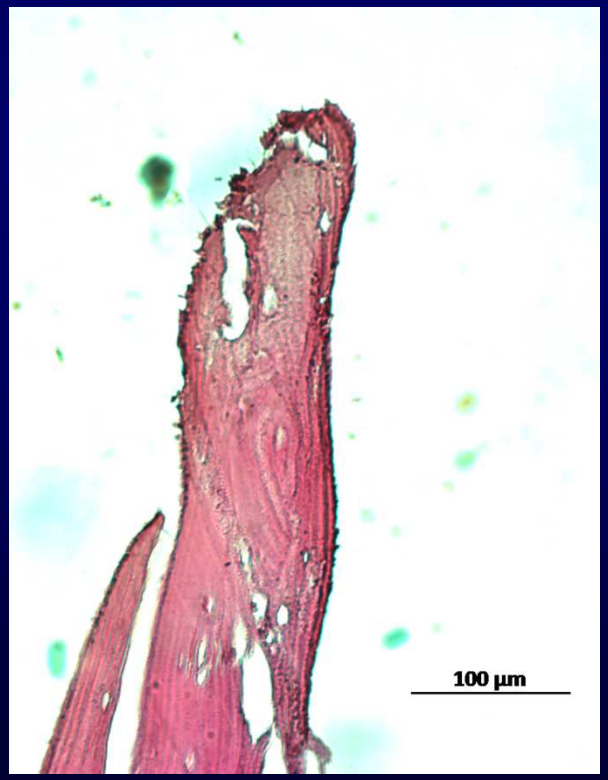
## 4- Development of bone section analysis

Bone decalcification assay :Decalcifier I
Optimal  incubation time of 1h. (7µm sections, HE staining)

# In conclusion

## Axis 1- Characterization of meniscal cell type

1 and 2 - MSCs co-cultured with fibrochondrocytes exhibit a gene expression profile similar to chondrocytes and fibrochondrocytes.

3- The firsts results for fibrochondrocytes biomarkers are promising

4- Comparative 2D profiles analysis

## Axis 2- Impact of HEMOXCell® in MSC 2D culture

0.025 g/L HEMOXCell® was found to be optimal to promote MSC proliferation (+25%) without impacting cell adhesion and characteristics.

## Axis 3- Impact of HEMOXCell® in MSC 3D culture

Meniscal and bone scaffolds are well-suited for cell adherence, viability and proliferation. Most analysing protocols have been developed.

# Perspectives

## Axis 1- Characterization of meniscal cell type

1 and 2 - MSCs co-cultured with fibrochondrocytes exhibit a gene expression profile similar to chondrocytes and fibrochondrocytes.

→ **This observation will be confirmed with fibroblaste samples as controls**

3- The firsts results for fibrochondrocytes biomarkers are promising

→ **Ongoing analysis with Strasbourg**

4- Comparative 2D profiles analysis

→ **Ongoing analysis with PurIProB (Brest)**

## Axis 2- Impact of HEMOXCell® in MSC 2D culture

0.025 g/L HEMOXCell® was found to be optimal to promote MSC proliferation (+25%) without impacting cell adhesion and characteristics.

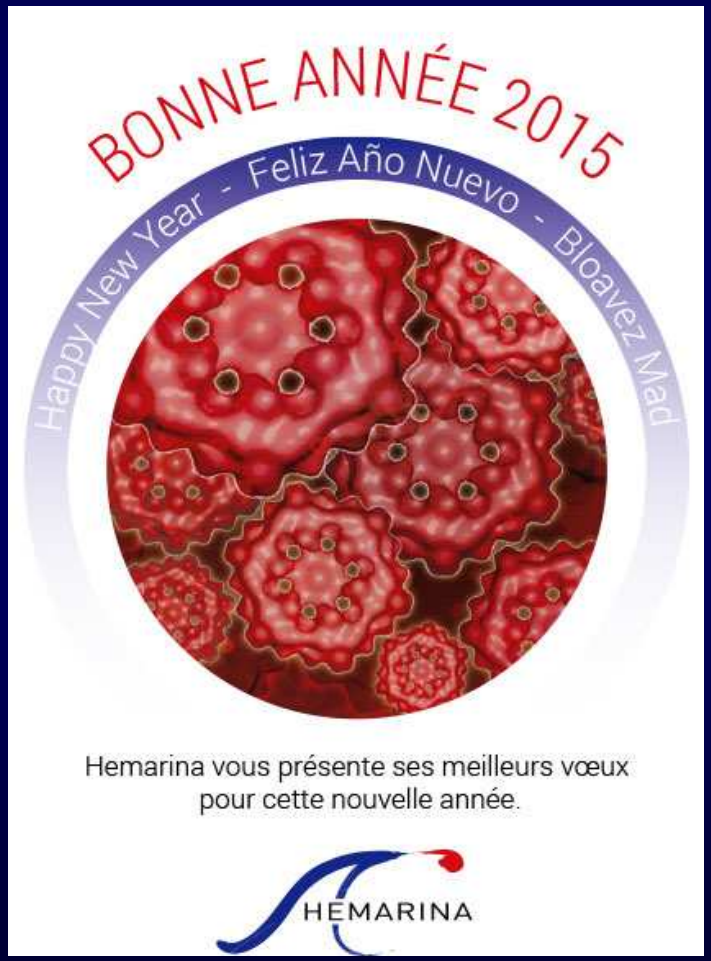
→ **A ROS staining analysis will be preformed (Manuscript in submission)**

## Axis 3- Impact of HEMOXCell® in MSC 3D culture

Meniscal and bone scaffolds are well-suited for cell adherence, viability and proliferation. Most analysing protocols have been developed.

→ **These protocols will be performed on 3D scaffolds after culture under perfusion**

# Thank you

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