TRACKING TRANSCRIPTION FACTOR ACTIVITY IN 3D NANOSCAFFOLD

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Abstract

Mesenchymal stem cells (MSCs) are multipotent cells present in the stromal fraction of many tissues. Their immunomodulatory properties and differentiation potential have been proven beneficial in number of autoimmune disease therapy models. However a reliable *in vitro* approach to study MSCs differentiation in inflamed tissue and a clear insight into the involved signalling pathways may accelerate their application *in vivo*.

Fibrin nanoscaffolds have been developed for cartilage tissue engineering and provide a natural matrix that can mimic synovium microenvironment. Moreover fibrin hydrogels allow recreating *in vitro* the same stiffness chondrocytes face *in vivo*, leading to improved chondrogenesis differentiation. Besides all these properties, fibrin can be completely degraded by nattokinase, allowing an easy single cell analysis.

The aim of this work is to investigate the role of NF-kB inflammatory pathway in MSCs chondrogenesis performed within fibrin nanoscaffolds. Thus we established NF-kB luciferase reporter line to monitor transcription factor activation. Combining degradable fibrin nanoscaffold technology to reporter line for monitoring transcription factors activity will provide a powerful platform to understand the role of inflammation in different stages of chondrogenesis.

Keywords: Nanoscaffold, fibrin hydrogel, mesenchymal stem cells, inflammation

1. INTRODUCTION

Mesenchymal stem cells (MSCs) are multipotent cells able to differentiate into osteoblasts, chondrocytes and adipocytes. During the last 10 years their biology has raised the interest of many research groups in the field of regenerative medicine [1]. Crosstalk with immune cells and differentiation potential during tissue inflammation are indeed two mechanisms that make them suitable for immunomodulatory therapies [2].

The role of MSCs in inflammation is driven through Toll Like Receptors (TLR), which enable the cells to sense exogenous molecules associated to pathogens and endogenous molecules associated to tissue damage [3]. This signalling cascade can have effect on MSCs differentiation potential and therefore hampering their beneficial properties.

Recently it has been shown that cells can sense stiffness of the material in which they are embedded [4], for this reason we seed MSCs in 3D fibrin nanoscaffold in order to recapitulate MSCs *in vivo* niche. Fibrin is a rapid self-assembly material, composed of fibrinogen and thrombin and it is now intensively studied for cartilage tissue engineering [5]. Moreover changes in fibrinogen content we can tightly regulate hydrogel's stiffness [6].

The aim of the project is to couple MSCs NF-kB reporter line to fibrin hydrogel nanoscaffold in order to establish a platform that can recapitulate *in vivo* MSCs niche. This approach allows us to monitor both MSCs

inflammatory status and differentiation potential, which are the most important aspects for further improving mesenchymal stem cells therapy.

2. RESULTS

To investigate signalling during inflammation we developed a stable NF-κB luciferase reporter line. **Figure 1** shows activation of NF-κB inflammatory pathway after stimulation with a TLR4 activator (LPS).

Afterwards we prepared fibrin nanoscaffolds using 4 different concentration of fibrinogen, which determines changes in stiffness of the material. Structural changes in density of the material related to fibrinogen content are shown (**Figure 2**). We then perform SEM imaging to better characterize hydrogel structure and confirm differences observed already in macroscopic scale (**Figure 3**).

To verify whether hydrogel stiffness affects inflammatory process and differentiation potential of MSCs we seeded MSCs into hydrogel and performed confocal imaging on hydrogel with different stiffness after 7 days of differentiation in chondrogenesis media. Cells were stained with antibodies against a marker of chondrogenesis (SOX9) and anti-firefly luciferase antibody to verify activation of NF-kB pathway during differentiation (**Figure 4**).

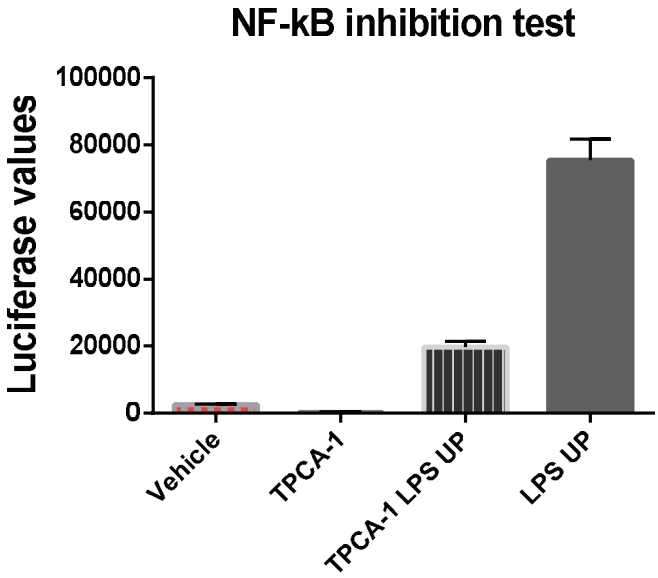

Figure 1 Cultured MSCs NF-κB reporter was treated with inhibitory molecule (TPCA-1 1ug/mL, 6h), TLR4 activator (LPS ultrapure 10ug/mL, 6h) and a combination of both. Firefly luciferase activity was measured using luminescence reader

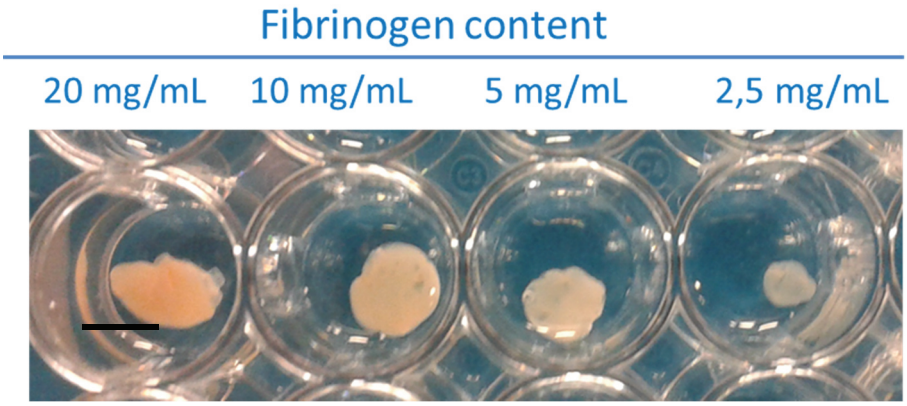

Figure 2 Fibrin nanoscaffold where prepared as stated in methods. Scale bar is 700um

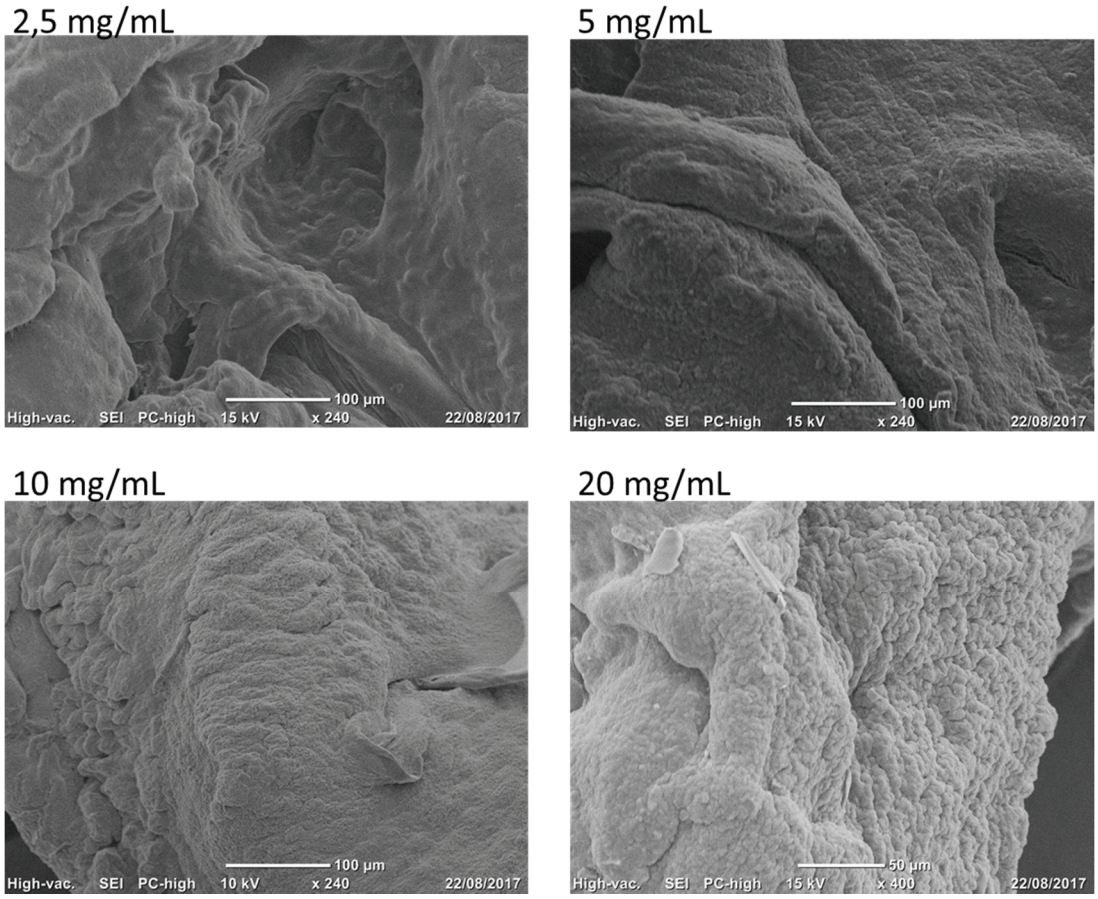

Figure 3 Samples were processed as stated in methods. SEM images provide here a characterization of fibrin nanostructure

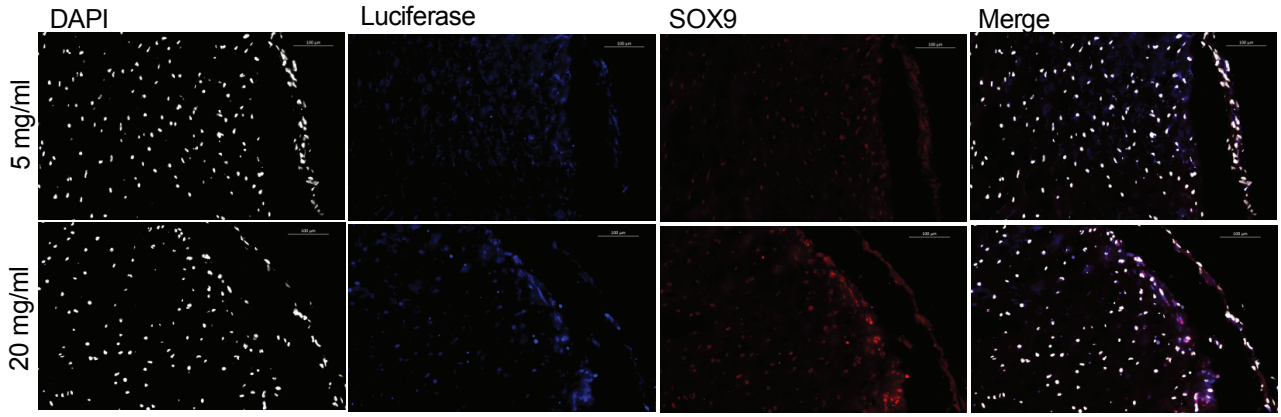

Figure 4 Confocal imaging reveals activation of NF-kB pathway through luciferase reporter and expression of chondrogenesis marker (SOX9) when cells are seeded into 5mg/ml (upper panel) and 20mg/ml (lower panel) fibrin hydrogel

3. DISCUSSION AND CONCLUSION

Developing a suitable platform for correlating MSCs inflammatory profile and differentiation potential will provide a new insight for therapeutic use of stem cells in regenerative medicine. In this work we show that combination of MSCs reporter line to fibrin nanoscaffold can provide easy readout to monitor both

chondrogenesis process and inflammatory status, thus helping to correlate important aspects that make MSCs a promising tool for cellular treatment of inflammatory disorders

4. METHODS

Establish of stable luciferase reporter lines: Cells were seeded in 24 wells plate with 600µl of DMEM without Pen/Strep per well. Cells were transfected adding 2µl of Surentry and 12uL of particles in the media. After 6h transfection media was discarded and complete DMEM was added. After 1 day cells were put in selection (puromycin 0.5µg/ml) in order to obtain a homogeneous population with integrated the vector.

Fibrin hydrogel preparation and cells seeding: Fibrinogen and thrombin were purchased from Baxter. 50uL of 4U/mL thrombin diluted in PBS was mixed with 50uL DMEM media containing $2x10^5$ cells. This solution was mixed 1:1 with fibrinogen 40mg/mL, 20mg/mL, 10mg/mL and 5mg/mL in order to obtain clot with a concentration of 20mg/mL, 10mg/mL, 5mg/mL and 2.5mg/mL respectively.

Luciferase assay: Cells were lysed using one-glo from Promega (lysis and luciferase substrate). After 5 minutes the intensity of fluorescence was measured using illuminometer

Immunofluorescence: Cells in fibrin hydrogel were fixed using PFA 4% (SantaCruz) for 20 minutes, wash three times with PBS, embedded in OCT and cut using cryostat. For immunostaining we used primary antibodies anti-SOX9 and anti-firefly luciferase from Abcam. Cells were washed three times with PBS and stained using AlexaFluor 555, AlexaFluor 488 and DAPI. Imaging was performed using Zeiss LSM 780 confocal microscope.

SEM samples preparation: cells were fixed in glutaraldehyde 3% for 2h and wash with cacodylate buffer. Dehydration was performed using ethanol from 30% to 99.6%. Then samples where transfer for drying and coated with golden/palladium. Image acquisition was performed using SEM Jeol 3000.

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