Fucoidan promotes early step of cardiac differentiation from human embryonic stem cells and long term maintenance of beating areas.

Sofiane Hamidi, Didier Letourneur, Rachida Aid, Antonio Di Stefano, William Vainchenker, Francoise Norol, Catherine Le Visage

Fucoidan promotes early step of cardiac differentiation from human embryonic stem cells and long term maintenance of beating areas

Sofiane Hamidi, M.Sc.,[1,2,3] Didier Letourneur, Ph.D.,[4] Rachida Aid, M.Sc.[4], Antonio Di Stefano, Ph.D.,[1,2,3] William Vainchenker, M.D., Ph.D.,[1,2,3] Françoise Norol, M.D., Ph.D.[1,5,*] and Catherine Le Visage, Ph.D.[4,*]

[1] INSERM, UMR 1009, Institut Gustave Roussy, Villejuif 94805, France

[2] Université Paris-Sud, UMR 1009, Institut Gustave Roussy, Villejuif, 94805, France

[3] Institut Gustave Roussy, Villejuif, 94805, France

[4] Inserm, U698, Cardiovascular Bioengineering, X Bichat Hospital, Paris, 75018, France; Sorbonne Paris Cité, Université Paris 13, 93430, Villetaneuse, France; Univ Paris Diderot, 75018, Paris, France

[5] AP-HP, Service de Biothérapie, Hospital Pitié Salpétrière, Paris, 75013, France

* FN. and CLV contributed equally to this study

Corresponding author:

Dr Catherine Le Visage

Telephone number: (33) 1.40.25.86.00

Fax number: (33) 1.40.25.86.02

E-mail: catherine.levisage@inserm.fr

ABSTRACT

Somatic stem cells require specific niches and 3D scaffolds provide ways to mimic this microenvironment. Here, we studied a scaffold based on Fucoidan, a sulfated polysaccharide known to influence morphogen gradients during embryonic development, to support human Embryonic Stem Cells (hESCs) differentiation towards the cardiac lineage.

A macroporous (pore 200 µm) Fucoidan scaffold was selected to support hESCs attachment and proliferation. Using a protocol based on the cardiogenic morphogen BMP2 and TGFβ followed by TNFα, an effector of cardiopoietic priming, we examined the cardiac differentiation in the scaffold compared to culture dishes and embryoid bodies (EBs). At day 8, Fucoidan scaffolds supported a significantly higher expression of the 3 genes encoding for transcription factors marking the early step of embryonic cardiac differentiation NKX2.5 ($p<0.05$), MEF2C ($p<0.01$) and GATA4 ($p<0.01$), confirmed by flow cytometry analysis for MEF2C and NKX2.5. Fucoidan scaffolds ability to locally concentrate and slowly release TGFβ and TNFα was confirmed by Luminex technology. We also found that Fucoidan scaffolds supported the late stage of embryonic cardiac differentiation marked by a significantly higher ANF expression ($p<0.001$), although only rare beating areas were observed. We postulated that absence of mechanical stress in the soft hydrogel impaired sarcomere formation, as confirmed by molecular analysis of the cardiac muscle myosin MYH6 and immunohistological staining of sarcomeric α-actinin. Nevertheless, Fucoidan scaffolds contributed to the development of thin filaments connecting beating areas through promotion of smooth muscle cells, thus enabling maintenance of beating areas for up to 6 months.

In conclusion, Fucoidan scaffolds appear as a very promising biomaterial to control cardiac differentiation from hESCs that could be further combined with mechanical stress to promote sarcomere formation at terminal stages of differentiation.

INTRODUCTION

Human embryonic stem cells (hESCs), by their inherent ability of unlimited proliferation and differentiation, are a very promising tool for use in a wide range of applications, including basic biological stem cell research, cell replacement therapies, tissue engineering or toxicology testing. The differentiation strategies are based on the ontology of precursor cells and involve exposure to factors that are known to play key roles in tissue development. Nevertheless, despite extensive research on biochemical and biological signals, hESCs differentiated on standard tissue culture plastic yield progenitors with diminished proliferative potential.[1-3]

The naturally occurring three-dimensional (3D) microenvironments *in vivo* also called niche plays a central role in the regulation of somatic stem cells through cell-cell interactions, cell-matrix contacts and localized soluble factors; the interplay between stem or progenitor cells and their niche creates a dynamic system necessary for maintaining a balance between self-renewal and differentiation of the cells.[4, 5] Significant progress has been made in characterizing such specific microenvironments. Culturing stem cells inside of 3D biomaterial scaffolds provides a way to accurately mimic them, providing required environmental clues and advantages over traditional 2D culture methods. [6, 7,8] The clues are several and include biochemical and mechanical properties; the later ones have been widely investigated since pioneering works by Discher showed that multipotent stem cells might be driven in their differentiation by their tight or loose attachment depending upon the stiffness of the substrate.[9-11] While 2D culture has been used for the majority of hESCs culture experiments, the acknowledgement that surface physical, mechanical, chemical properties can exert specific effects on stem cells has prompted investigations to bioengineer structures that mimic the 3D environment. Indeed, several studies have demonstrated the feasibility of growing embryonic cells in a tridimensional environment. It has been observed that culturing mouse ESCs (mESCs) in scaffolds promotes the expression of multiple genes related to their stemness [12] and controls their self-renewal

and fate.[13, 14] Similarly, hESCs culture within 3D bioengineered scaffolds optimized tissue engineered formation.[15, 16] However, many issues remain, in particular the support and the optimal combination of scaffolds with extracellular matrix molecules to promote differentiation towards a specific lineage.

Scaffolds are based either on natural or on synthetic biomaterials, used alone or in mixture, providing scaffolds with different biochemical and mechanical properties. One of the challenges involves the development of biocompatible materials functioning as synthetic analogs of the extracellular matrix. In this context, we previously described the preparation of a polysaccharide-based scaffold as a novel biomaterial for cardiovascular engineering.[17] Naturally-derived polysaccharides are hydrophilic, biodegradable, and biocompatible. Their biochemical similarity with the extracellular matrix provides mechanical properties that match those of the native tissue, such as strength and flexibility experience.

Here, we tested the influence of a 3D microenvironment based on Fucoidan, a sulfated polysaccharides extracted from brown seaweed, to support hESCs differentiation towards the cardiac lineage. Sulfated polysaccharides are thought to influence embryo development through binding with proteins at several levels of specificity. They have structural motifs capable of differential affinities with growth factors and chemokines and this property may be an important factor to support the space and time shaping of morphogen gradients during embryonic development.[18] Indeed, addition of Fucoidan could provide a mechanism by which specific affinities of sulfated polysaccharides sequences for different growth factors and morphogens form gradients, which give rise to correct formation of the developing embryo.[19] We assumed that Fucoidan could also exert a spacial organization of specific proteins during the process of hES differenciation in 3D scaffold and thus influence the ways of differentiation. Here, we demonstrate that culturing hESCs in a 3D Fucoidan-

scaffold promotes early steps of cardiac development, with others signals required for the late step of differentiation.

MATERIALS AND METHODS

Maintenance of human embryonic stem cells (hESCs)

Undifferentiated hESCs (H9 line, WiCell Research Institute) were grown on a mitotically inactivated mouse embryonic fibroblast feeder layer in Dulbecco modified Eagle/F12 culture medium supplemented with 20% knock-out (KO) serum replacement (KO DMEM medium), 1mM glutamine, 0.1% non-essential amino acids, 0.1% penicillin/streptomycin, 0.1mM of 2-mercaptoethanol (all from Gibco-Invitrogen, France) and 10 ng/mL of human basic fibroblast growth factor (bFGF) (Peprotech, Neuilly-Sur-Seine, France), in plastic dishes. For expansion, hESC colonies were manually detached.

hESCs *in vitro* differentiation

Cardiac differentiation from hESCs was induced in DMEM medium supplemented with 10% KO serum, 1mM of glutamine, 0.1% NEAA, 0.1mM of 2-mercaptoethanol in the presence of SU5402, an inhibitor of FGF pathway at day 0 (10μM; Merckmillipore), the cardiogenic morphogen BMP2 and TGFβ [20] from day 1 to day 3 and then TNFα, an effector of cardiopoietic priming of the endoderm, from day 3 to day 6 (all from Peprotech); differentiated cells were then maintained in 10% serum medium until the appearance of spontaneous contractions (Fig. 1A).[21] The process of differentiation was induced either : i) in 2D conditions by maintaining differentiating cells on standard tissue culture plastic dishes; ii) by slight detachment of hES cells using collagenase IV 1mg/ml (Stem Cell Technologies, France) for 6-7 min at 37°C, cells scrapping and subsequent embryoid bodies formation into a low adherence plate until day 11 or iii) by seeding hESCs after detachment into porous scaffolds (see below)(Fig. 1B). The contracting areas generated during differentiation were identified by light microscopy.

Fabrication of porous polysaccharide scaffolds

Polysaccharide-based scaffolds were synthesized according to a previously described protocol.[22, 23] Scaffolds were prepared using a mixture of pullulan/dextran in water (pullulan, Mw 200000, Hayashibara; dextran Mw 500000 Sigma). In addition, some scaffolds were loaded with bovine gelatin (Sigma) that was solubilized in the pullulan/dextran mixture (20% w/w). Chemical cross-linking of polysaccharides was carried out using the cross-linking agent sodium trimetaphosphate (STMP) (30% (w/v), Sigma) under alkaline conditions. Briefly, 9 mL of the polysaccharide solution were mixed with 1 mL of NaOH 10M and 300 mg of STMP in 1 mL of water were then added to the mixture. Sodium chloride (50% w/w) or sodium carbonate (37% w/w) was added as porogen agents to produce scaffolds with an average pore diameter of 40 μm (microporous) and 200 μm (macroporous), respectively. The mixture was then poured into Petri dishes and incubated at 50°C for 15 min. Hydrogels prepared with sodium carbonate were immersed in a 20% acetic acid solution for 30 minutes. All scaffolds were washed extensively with phosphate buffer saline pH 7.4, freeze-dried then stored at room temperature until use. Macroporous scaffolds were also prepared by incorporating the sulfated polysaccharide Fucoidan (Mw 50000, Sigma) into the pullulan/dextran mixture (9% w/w). For confocal analysis, additional scaffolds were prepared with FITC-labeled dextran. Optical sections were acquired with a Zeiss LSM 510 confocal microscope, FITC-labeled dextran was excited at 488 nm with an argon laser and its fluorescence emission selected by a 505-530 nm bandpass filter. Pore sizes were calculated from confocal data with ImageJ software. 3D visual images of confocal data were created using Amira 3.0 software (Mercury Computer Systems., USA). Results are presented as means ± SEM of three experiments.

Luminex quantification of Fucoidan interactions with cytokines

Scaffolds containing or not Fucoidan were hydrated with DMEM culture medium containing either TNF-α or TGF-β (50ng each). For release studies, scaffolds were placed at 37°C in 500μL of DMEM alone. Medium was replaced every day for 5 days and medium samples (100μL) were collected at day 1, 3 and 5 and stored at -80°C. Released cytokines were evaluated using the xMAP Luminex technology (Multi-analysis profiling). Quantifications were determined using Bio-Plex 200 (Bio-Rad), with culture medium as negative control. Data were analyzed with the Bio-Manager 5.0 software (BioRad). Results were expressed in pg/ml.

Flow cytometry analysis

For all flow cytometry analysis, seeded scaffolds were fixed in 4% paraformaldehyde for 5 minutes then incubated in pullulanase (40 units/mL, Sigma) and dextranase (5 units/mL, Sigma) at 37°C until a complete solubilization of the scaffolds was obtained. After enzyme inactivation by dilution and washing, cells retrieved from the scaffolds were analyzed as cell suspensions.

Cell viability was determined at day 4 and day 12 by a live/dead assay (Sigma). Scaffolds were washed in PBS 1x, incubated at 37°C during 30 minutes in a 25μg/ml calcein AM solution, placed in a 1μg/mL PI solution then incubated at room temperature for 5 minutes. For precise quantification, cells from each sample were distributed in the same volume (400 μL) and the acquisition rate was 1 μL/second for 100 seconds. Seeded viable cells were defined as PI$^-$ events with scatter properties of nucleated cells.

Cardiac progenitors were analyzed at day 4 and 8 of differentiation (n=3 for each conditions). After fixation in 4% PFA and a blocking / permeabilisation step in 1% BSA/ 0.1% TritonX-100 , cells were incubated for 30 minutes with an anti-human NKX2.5 and MEF2c (both from Abcam, France)

or the matching rabbit polyclonal isotype control. An appropriate PE Cy5 conjugated secondary antibody (Invitrogen, France) was used to detect the primary antibodies. Endothelial cells were detected at day 14 using anti-CD31 and anti-CD34 antibodies (both from Becton Dickinson, France). Labeled cells were analyzed using a flow cytometer (BDFACS CANTO, Becton Dickinson, San José, CA, USA).

Molecular analysis of gene expression by conventionnal or quantitative PCR

Total RNA was extracted with RNeasy minikit columns (Quiagen) according to the manufacturer recommendations. RNA quantification was assessed with NanoDrop Nd-1000 UV/Vis (Labtech France, Palaiseau, France). Total amount of obtained mRNA was reverse-transcribed using random primers, super script II reverse transcriptase (Invitrogen) and Rnase out (Rnase inhibitor Invitrogen). The resulting cDNA was amplified using the TaqMan Universal PCR Master Mix (Applied Biosystems, Courtaboeuf, France) containing the specific primers (10 µM)(Table 1) and probe (10 µM). qPCR was run for 35 cycles in standard mode using an 7500 Real Time PCR System (Applied Biosystems). The difference between the threshold cycle (CT) of each gene and the endogenous controls GAPDH and CREBBP was used to determine (ΔCT) gene expression. Relative expression ratios were calculated by correlating values to those of the reference population, a pool included undifferentiated hES cells, cells from embryoid bodies at different time of spontaneous differentiation and cells from human fetal heart. Conventional RT-PCR was performed using Taq polymerase, Taq buffer (Invitrogen), DMSO (D2650 Sigma), dNTP (Invitrogen) on the GeneAmp PCR 2700 thermocycler (Applied biosystems).

Immunofluorescence analysis

Distribution of viable and dead cells inside scaffolds was determined using a live/dead as previously described. Scaffolds were imaged by confocal microscopy (Zeiss) using a 20×/0.5 Plan-Neofluar objective. The 3D stacks were recorded at on a depth of 100μm. The two channels corresponding to green fluorescence and red fluorescence were recorded sequentially at an acquisition rate of 0.5 seconds per image of 1024 × 1024 pixels. We used the following combinations of excitation wave length and emission filter: 489 nm and 500-525 nm band path filter for green fluorescence, 532 nm and 560-675 nm band path filter for red fluorescence.

For immunofluorescence analysis, cardiac cells, embryoid bodies and cellularized scaffolds were fixed in 4% PFA at 14 and 21 days of differentiation and embedded in OCT for cryosections; sections were cut with an ultramicrotome (LM 1850; Leica, Heerbrugg, Switzerland). Cells in 2D culture plates were detached and seeded onto gelatin-coated glass coverslips for 24 hours and fixed using 4% paraformaldehyde. Immunostaining was performed after a blocking / permeabilisation step in 1% BSA/0.5% TritonX-100 for 30 minutes followed by incubation with mouse anti sarcomeric α-actinin antibody (Sigma, France) and Alexa Fluor® 488 Goat Anti-Mouse (Invitrogen). Immunostaining for cardiac troponin was performed with rabbit anti human cardiac troponin I (dilution 1/400, #4002, Cell Signaling Technology) followed by Alexa fluor 488 goat anti rabbit, (dilution1/50, A11008, Invitrogen) and with mouse anti human cardiac troponin T (dilution 1/100, MAB1693, Chemicon) followed by Alexa fluor 594 goat anti mouse, (dilution1/50, A11005, Invitrogen).

Sections were mounted using DAKO fluorescent mounting medium and observed in confocal microscopy (LSM-510 META; Carl Zeiss, Jena, Germany).

Statistical analysis

Statistical analysis was performed using Prism software (GraphPad, La Jolla, CA). Data were expressed as the mean ± SEM and ANOVA, followed by Turkey post test were used for comparisons between samples. Growth factor release data were compared using Mann Whitney tests. A p value of less than 0.05 was considered to be statistically significant.

RESULTS

Fucoidan scaffold improves hESCs survival during the differentiation process

We first examined the optimal conditions of hESCs seeding into a 6mm diameter x 1mm height porous polysaccharide scaffold that could support cell survival. Microporous and macroporous scaffolds, with a mean pore size of 45±1 μm and 195±10 μm respectively, were prepared. In addition, the sulfated polysaccharide Fucoidan was incorporated into macroporous scaffold, with no significant modification of the pore size (175±9 μm). Typical single confocal slices of the FITC-labelled scaffolds are shown on Fig. 2A; the use of FITC-labeled dextran allowed confocal laser scanning microscopy observation of the inner structure of hydrated scaffolds. hESCs infiltrated very quickly within the scaffold porous structure (see supplementary movie 1), under a maximum seeded volume of 15 μL. About 30 hESCs clones could be seeded into a 6mm scaffold. The high degree of transparency of the hydrated scaffold enabled the visualization of cells along the course of the experiment. Microscopic examination promptly showed an increased survival of hESCs in macroporous/Fucoidan scaffold which became evident at day 4 (Fig. 2B). In the macroporous/Fucoidan scaffold condition, adherent cells were observed within the transparent scaffold as large cell clusters 8 days after hESCs seeding. Using propidium iodide, we confirmed that dead cells at day 4 reached 29% and 39% respectively in microporous and macroporous scaffolds compared to 14% in Fucoidan macroporous scaffold (Fig. 2C). At day 12, live cells measured using calcein AM as a viability marker were significantly higher in macroporous and macroporous/Fucoidan versus microporous scaffold at day 12 (77% ± 1% versus 88% ± 3% and 91% ± 2 % respectively, p<0.01). It is worth noting that in absence of fucoidan, viable cell numbers were lower than in fucoidan scafolds.

Whatever the type of scaffolds, some cells were observed to leak out of the scaffold during the time course of differentiation. In order to prevent this cell loss, we incorporated gelatin, an adhesion factor that could improve the adhesion of cells to the surface of the highly hydrated hydrogel, during the scaffold preparation[24]; in presence of gelatin, the total cell number was significantly higher at all time points from day 8 to day 21 ($p<0.05$) (Fig. 2D). We then selected the macroporous/Fucoidan/gelatin scaffold (abbreviated as FucScaffold in Figures), that displayed an optimal architecture and composition to support cell survival, for further experiments.

Fucoidan scaffold slowly releases growth factors

Sulfated polysaccharides are known for their protein affinity. Control and Fucoidan scaffolds were loaded with culture medium supplemented with TGFβ and TNFα. Using protein quantification by Luminex technology, we confirmed that Fucoidan scaffolds could be loaded with either cytokine and could release them all along a follow up of 5 days, while control scaffolds provided a significantly lower growth factor concentration (Fig. 3).

Fucoidan-scaffold enhances early cardiac specification

Using molecular analysis, we first confirmed that Fucoidan scaffolds supported hESCs differentiation towards the 3 germs layers as evidenced by the expression at day 3 of *BRACHYURY* and *HAND 1* (mesoderm), *NESTIN* and *PAX6* (ectoderm) and α-*FP* and *FOXA2* (endoderm) (Fig. 4A). Then, we used a protocol based on the cardiogenic morphogens BMP2 and TGFβ followed by TNFα, an effector of cardiopoietic priming and examined hESCs differentiation towards the cardiac lineage. Beating areas developed from day 15, as observed usually in 2D cultures, and we

confirmed that Fucoidan scaffolds supported a normal molecular program of cardiac differentiation (Fig. 4B).

Subsequently, we evaluated the efficiency of cardiac specification of hESCs seeded in the Fucoidan scaffolds in comparison with differentiating cells on standard cultures dishes and embryoid bodies (EBs) microenvironment. Using qPCR, we examined the expression of genes encoding for 3 transcription factors marking the early step of cardiac differentiation i.e NKX2.5, MEF2C and GATA4. At day 4 of differentiation, the expressions of the transcription factors were similar in the 3 conditions. At day 8, Fucoidan scaffolds supported a higher expression of all 3 genes NKX2.5 ($p<0.05$), MEF2C ($p<0.01$) and GATA4 ($p<0.01$), in comparison with EBs and 2D culture dish (Fig. 5A). This was confirmed at the protein level using flow cytometry analysis (Fig 5B). At day 8, 1 to 3% of cells cultured in Fucoidan scaffolds expressed MEF2C and NKX2.5 while less than 1% of cells cultured in EBs and 2D culture dish conditions expressed these genes ($p<0.05$).

Fucoidan scaffold influences the development of beating areas

The influence of environment on the late cardiac differentiation was examined by quantification of the expression of the genes encoding for the cardiac muscle myosin MYH6 and for atrial natriuretic factor ANF. At day 12, no difference was observed between culture dish, EBs and Fucoidan scaffolds (Fig 6A). At day 21, expression of ANF, an hormone secreted by cardiac muscle cells and involved in the homeostatic control of body water, confirmed that the late stage of differentiation occurred in all culture conditions, and its expression was significantly higher in Fucoidan scaffolds as compared to culture dish and EBs ($p<0.001$, Fig 6A).

In contrast, expression at day 21 of the gene encoding for MH6 was found to be significantly lower in EBs and Fucoidan scaffolds in comparison with culture dish ($p<0.001$)(Fig 6A). Using

immunofluorescent staining, we confirmed that 2D culture dish and EBs supported the expression of sarcomeric α-actinin but Fucoidan scaffolds did not (Fig 6B). The development of myofibrils and sarcomere organization is a major assessment of the terminal stage of differentiation and function of cardiac myocytes. The absence of sarcomeric α-actinin was evidenced by the lower frequency of beating areas in Fucoidan scaffolds. In addition, expression of cardiac troponins cTnT and cTnI at day 21 in culture dish, EBs and Fucoidan scaffolds was confirmed by immunofluorescence (Fig S1).

Given previous observations of Fucoidan effect on matrix remodeling and neoangiogenesis,[25] we also examined markers of endothelial cells and smooth muscle cells at the terminal stage of differentiation. Regarding endothelial lineage, qPCR analysis showed that the expression of genes encoding for VE-cadherin, a glycoprotein specific of the endothelial lineage, was similar in culture dish and in Fucoidan scaffolds and significantly higher in EBs ($p<0.05$) (Fig 6C, left panel). Flow cytometry analysis confirmed that $CD34^+CD31^+$ endothelial cells were present at low frequencies in all 3 conditions (Fig 6B, right panel). Expression of gene encoding for Calponin-1 (CPN1), a calcium binding protein specific of smooth muscle cells, was significantly increased in Fucoidan scaffolds (Fig 6D, left panel) as compared to EBs. This correlates with the obvious development of thin filaments connecting beating areas and the hydrogel (Fig 6D, right panel) and probably support the fact that beating areas formed in Fucoidan scaffolds could be maintained in culture for several months (see supplementary movie) while they died out after few days or less than 3 weeks in culture dishes or EBs.

DISCUSSION

The numerous differences between 2D and 3D environments to control stem cell behavior have prompted development of structures that mimic the 3D properties of natural microenvironment for hESCs differentiation. In this study, we demonstrate that differentiation of hESCs in a 3D environement of a sulfated polysaccharide Fucoidan scaffold promotes the expression of key genes of early cardiac differentiation at the molecular and protein level. The homeobox gene *NKX2-5* (NK2 transcription factor related, locus 5) marks the onset of vertebrate heart development and activates transcription of the myocyte enhancer factor *MEF-2C* to promote cardiomyocyte differentiation and cooperates with zinc finger transcription factors of the GATA family to initiate cardiac gene expression.[26] Thus, the genes enhanced by culture in a Fucoidan scaffold mark the early steps of cardiac differentiation. Probably, the combination of a 3D microenvironment and Fucoidan is of major importance. Although cardiac niche during heart development is largely unknown, it is believed that the three major cell lineages, cardiac, smooth muscle and endothelial cells are found in discrete clusters within the developing right ventricle, the atria and outlow tracts [26, 27,] which are reminiscent of a stem cell niche. Niches include various cells and extracellular matrix (ECM) [28] and ECM of the heart is composed of collagens, fibronectin, elastin, glycoproteins including laminin and proteoglycans.[29] A variety of naturally derived and synthetic biomaterial scaffolds have been investigated as 3D environments for mimicking the heart cellular environment. While synthetic scaffolds can be synthesized to have a greater range of mechanical and chemical properties and often have greater reproducibility, natural biomaterials are often composed of proteins and polysaccharides found in the extracellular matrix. Moreover, sulfated polysaccharides such as Fucoidan may display particularly interesting properties. Indeed, subtle variations in patterns of sulfation can modulate relative affinities for extracellular proteins such as cytokines, growth factors and morphogens.[30, 31] This complex interplay is capable of influencing the diffusion of such molecules through tissue,

concurring to the precise temporal and spatial patterns described in cardiac ECM.[29] Here, we confirm that a Fucoidan scaffold can retain growth factors involved in cardiac differentiation, TGFβ and TNFα. Protection of TGFβ activity by soluble Fucoidan has been also previously reported by Mc Caffrey.[32] Such affinities of Fucoidan for extracellular proteins probably concur to the regulation of growth factor concentration at the immediate contact of hESCs seeded in the scaffold.

As we previously demonstrated, Fucoidan also contribute to ECM elaboration through stimulation of fibroblast proliferation and extracellular matrix deposition; it also modulates connective tissue proteolysis by minimizing human leukocyte elastase activity and protecting elastic fiber network against the enzymatic proteolysis. [33, 34,35, 36]. Here, we confirmed that, at the late stage of cardiac differentiation from hES, Fucoidan promoted the development of thin filaments linking beating areas and scaffold; these attachments probably influence the mechanical environment of differentiated cardiac cells and probably contribute to the very long term maintenance of some beating areas.

Another important factor during *in vivo* tissue development is the formation, inside the complex 3D ECM structure, of an intricate vasculature.[37] Our team contributed to demonstrate that Fucoidan can induce endothelial colony-forming cells (ECFC) to adopt an angiogenic phenotype *in vitro*. Moreover, Fucoidan greatly increases ECFC-mediated angiogenesis in an *in vivo* model of murine hindlimb ischemia.[38, 39] Controlled release of growth factors by chitosan/Fucoidan micro complex-hydrogel and its effect on *in vitro* and *in vivo* vascularization has also been demonstrated by Nakamura et al.[40] Here, we did not find that Fucoidan scaffolds could promote the development of endothelial cells and, rather, observed a low level of VE-cadherin, an endothelial cell-cell adhesion glycoprotein. We believe that these effects are due to the flexible and soft structure of the hydrogel, and that this mechanical factor might have impair the development of contractile areas.

The relatively scarce presence of contractile function in 3D scaffolds was evidenced by microscopic examination of beating areas and confirmed by molecular analysis of the main cardiac contractile protein MYH6. This is probably linked to the absence of mechanical stress in the hydrogel. Indeed, mechanical stimuli have a major influence on a number of fundamental cellular processes, including differentiation, growth, and apoptosis.[11] Many data support the fact that developing cardiac cells are under constant, mechanical stress which can affect the differentiation of stem cells into cardiac myocytes, the development of differentiated cells and their maturation.[41] Mechanical stress-induced sarcomere assembly for cardiac muscle growth in length and width, and microtubules, a major component of the cardiac myocyte cytoskeleton, certainly play a role in the response of cardiac myocytes to mechanical stimulation. One hypothesis is that static stretch promotes MEF2A nuclear translocation and expression of neonatal myosin heavy chain in a calcineurin- and p38-dependent manner.[42, 43] Stretch influence was confirmed in engineered early embryonic cardiac tissue [44] and support the plating of embryoid bodies after 7 days culture, proposed by the pioneering team of cardiac differentiation from hESCs to improve terminal differentiation.[25] In our study, the relative contribution of 3D environment and Fucoidan could not be easily discerned given the increased immediate mortality observed in plain scaffolds that influenced subsequent steps of differentiation. Nevertheless, terminal differentiation occurs in 3D Fucoidan scaffold as demonstrated by immunofluorescence and high expression of the terminal cardiac marker ANF. This can be related to the promotion of early cardiac transcription factors, given that the GATA4/Nkx2.5 complex can bind the ANF promoter and activate its expression.[45] A way to benefit from the early positive effect of Fucoidan hydrogel while promoting mechanical stress would be to fine-tune the scaffold pattern of mechanical tension using nano or microtopography[46] or to guide the cell alignment through the use of external physical stimuli, such as mechanical[47] or electrical stimulation.[48]

In conclusion, the sulfated polysaccharide Fucoidan demonstrates beneficial effects on early steps of cardiac differenciation from hESCs in term of promotion of early cardiac transcription factors and ECM construction and encourage technical developments to improve terminal differentiation of functional myocytes.

Disclosure Statement

No competing financial interests exist.

Figure Legends

Figure 1: Differentiation of hESC towards cardiac lineage

A) For 3D differentiation, hESCs were seeded into porous Fucoidan scaffolds in the presence of an inhibitor of FGF pathway the SU5402 at day 0, the cardiogenic morphogen BMP2 and TGFβ from day 1 to day 3 and then TNFα, an effector of cardiopoietic priming of the endoderm, from day 3 to day 6; differentiated cells were then maintained in 10% serum medium until the appearance of spontaneous contractions. From day 15, viable cells in beating areas could be imaged using a live/dead assay, with beating areas observed as being attached to the scaffold at day 30. **B)** The process of differentiation was induced either in 2 or 3D microenvironments: i) 2D conditions were achieved by maintaining differentiating cells on standard tissue culture plastic dishes; 3D microenvironment was created by slight detachment of hES cells and subsequent ii) embryoid bodies formation into a low adherence plate or iii) hESCs seeding inside Fucoidan scaffolds.

Figure 2: Development of an optimal scaffold composition to support differentiation of hESCs

A) Microscopic view of three different types of porous scaffolds. Porosity was evidenced and measured by confocal microscopy observation of fluorescent FITC-dextran scaffold stacks (top) and 3D reconstructions (bottom). The mean pore sizes were 45µm ± 1 for microporous, 195µm ± 20 for macroporous and 175µm ± 9 for macroporous with Fucoidan. **B)** Macroscopic views of microporous, macroporous, and Fucoidan macroporous scaffold seeded with hESCs at day 4 (magnification 10x). Fucoidan scaffolds supported enhanced cell survival, with clusters of differentiated cells evidenced at day 8. **C)** Representative cytometry analysis of cell viability by a live/dead assay at day 4 and day 12 after labeling with Propidium Iodide and Calcein-AM. For precise quantification, cells from each sample were distributed in the same volume (400 µL) and the acquisition rate was 1 µL/second for

100 seconds. Seeded viable cells were defined as PI⁻ events with scatter properties of nucleated cells. Control dark histogram was obtained with a matching rabbit polyclonal isotype (day 4). At day 12, plots of calcein versus Propidium Iodide fluorescence confirmed that macroporous with fucoidan scaffold supported the higher percent of viable cells. **D)** Gelatin incorporation within scaffold prevented cell loss and significantly increased the total cell number measured in the scaffold from day 8 of differentiation (* p<0.05).

Figure 3: Release of TNFα and TGFβ by control and Fucoidan scaffolds as a function of time.
Scaffolds containing or not Fucoidan were loaded with 50 ng of TNF-α or TGF-β and relase studies were performed in 500μL of DMEM at 37°C. Medium samples were collected at day 1, 3 and 5 and released cytokines were evaluated using the xMAP Luminex technology. Quantifications were determined using Bio-Plex 200 (Bio-Rad), with culture medium as negative control and results were expressed in pg/ml (n=3 experiments, *** p<0.001).

Figure 4: Differentiation of hESC towards cardiac lineage into the Fucoidan scaffolds.
A) Spontaneous specification of hESC cultured within FucScaffold toward the three germ layers was assessed by PCR analysis of *BRACHYURY* and *HAND 1* (mesoderm), *NESTIN* and *PAX6* (ectoderm) and α-*FP* and *FOXA2* (endoderm) at day 3. **B)** Fucoidan scaffolds support a normal program of cardiac differentiation, as assessed by standard PCR analysis.

Figure 5: Early cardiac specification from hESCs in 2D microenvironment, embryoid bodies and Fucoidan scaffolds.

A) qPCR analysis of the 3 cardiac transcription factors *NKX2.5, GATA4* and *MEF2c* at day 4 and 8 (n=3) **B)** NKX2.5 and MEF2c flow cytometry analysis in the three conditions at day 8 of differentiation. Note the significantly increased expression of the specific transcription factors in Fucoidan scaffolds at day 8. * p<0.05, ** p<0.01.

Figure 6: Terminal stage of cardiac, endothelial and smooth muscle differentiation in 2D condition, embryoid bodies and FucScaffolds.

A) qPCR analysis of MYH6 and ANF expression at the end of differentiation in the three conditions at day 12 and 21. **B)** Immunohistological examination after sarcomeric α-actinin staining; 2D microenvironment and EB supported expression of sarcomeric α-actinin (left and middle panel) while its expression was very rare in scaffold (right panel). **C, D)** Identification of endothelial and smooth muscle cells presence in culture dishes, embryoid bodies and Fucoidan scaffolds at day21 using qPCR analysis of VE-cadherin (C, left panel), flow cytometry analysis of CD34$^+$CD31$^+$ endothelial cells (C, right panel). and qPCR analysis of Calponin-1, a calcium binding protein specific of SMC (D, left panel). Thin filaments connecting a beating area and the hydrogel could be seen in Fucoidan scaffolds (D, right panel).* p<0.05, *** p<0.001

Figure S1: Immunostaining for cardiac troponins cTnT and cTnI

Immunohistological examination after cTnT and cTnI staining of cells cultured in 2D culture dish, EBs and Fucoidan scaffold. Scale bar: 50 μm

Supplementary movie 1: cells seeded in a porous scaffold infiltrated quickly inside the porous structure

Supplementary movie 2: beating area observed in the Fucoidan hydrogel after 6 months of culture

References

1. Klump, H., Schiedlmeier, B., and Baum, C. Control of self-renewal and differentiation of hematopoietic stem cells: HOXB4 on the threshold. Ann N Y Acad Sci 1044, 6, 2005.

2. Gomez-Lopez, S., Wiskow, O., Favaro, R., Nicolis, S. K., Price, D. J., Pollard, S. M., and Smith, A. Sox2 and Pax6 maintain the proliferative and developmental potential of gliogenic neural stem cells In vitro. Glia 59, 1588, 2011.

3. Montarras, D., Morgan, J., Collins, C., Relaix, F., Zaffran, S., Cumano, A., Partridge, T., and Buckingham, M. Direct isolation of satellite cells for skeletal muscle regeneration. Science 309, 2064, 2005.

4. Watt, F. M., and Hogan, B. L. Out of Eden: stem cells and their niches. Science 287, 1427, 2000.

5. Spradling, A., Drummond-Barbosa, D., and Kai, T. Stem cells find their niche. Nature 414, 98, 2001.

6. Howard, D., Buttery, L. D., Shakesheff, K. M., and Roberts, S. J. Tissue engineering: strategies, stem cells and scaffolds. J Anat 213, 66, 2008.

7. Burdick, J. A., and Vunjak-Novakovic, G. Engineered microenvironments for controlled stem cell differentiation. Tissue Eng Part A 15, 205, 2009.

8. Votteler, M., Kluger, P. J., Walles, H., and Schenke-Layland, K. Stem cell microenvironments-- unveiling the secret of how stem cell fate is defined. Macromol Biosci 10, 1302, 2010.

9. Engler, A. J., Sen, S., Sweeney, H. L., and Discher, D. E. Matrix elasticity directs stem cell lineage specification. Cell 126, 677, 2006.

10. Krieg, M., Arboleda-Estudillo, Y., Puech, P. H., Kafer, J., Graner, F., Muller, D. J., and Heisenberg, C. P. Tensile forces govern germ-layer organization in zebrafish. Nat Cell Biol 10, 429, 2008.

11. Discher, D. E., Mooney, D. J., and Zandstra, P. W. Growth factors, matrices, and forces combine and control stem cells. Science 324, 1673, 2009.

12. Levenberg, S., Huang, N. F., Lavik, E., Rogers, A. B., Itskovitz-Eldor, J., and Langer, R. Differentiation of human embryonic stem cells on three-dimensional polymer scaffolds. Proc Natl Acad Sci U S A 100, 12741, 2003.

13. Liu, H., Lin, J., and Roy, K. Effect of 3D scaffold and dynamic culture condition on the global gene expression profile of mouse embryonic stem cells. Biomaterials 27, 5978, 2006.

14. Blin, G., Lablack, N., Louis-Tisserand, M., Nicolas, C., Picart, C., and Puceat, M. Nano-scale control of cellular environment to drive embryonic stem cells selfrenewal and fate. Biomaterials 31, 1742, 2010.

15. Li, S. C., Wang, L., Jiang, H., Acevedo, J., Chang, A. C., and Loudon, W. G. Stem cell engineering for treatment of heart diseases: potentials and challenges. Cell Biol Int 33, 255, 2009.

16. Delcroix, G. J., Schiller, P. C., Benoit, J. P., and Montero-Menei, C. N. Adult cell therapy for brain neuronal damages and the role of tissue engineering. Biomaterials 31, 2105, 2010.

17. Le Visage, C., Gournay, O., Benguirat, N., Hamidi, S., Chaussumier, L., Mougenot, N., Flanders, J. A., Isnard, R., Michel, J. B., Hatem, S., Letourneur, D., and Norol, F. Mesenchymal stem cell delivery into rat infarcted myocardium using a porous polysaccharide-based scaffold: a quantitative comparison with endocardial injection. Tissue Eng Part A 18, 35, 2012.

18. Nogami, K., Suzuki, H., Habuchi, H., Ishiguro, N., Iwata, H., and Kimata, K. Distinctive expression patterns of heparan sulfate O-sulfotransferases and regional differences in heparan sulfate structure in chick limb buds. J Biol Chem 279, 8219, 2004.

19. Powell, A. K., Yates, E. A., Fernig, D. G., and Turnbull, J. E. Interactions of heparin/heparan sulfate with proteins: appraisal of structural factors and experimental approaches. Glycobiology 14, 17R, 2004.

20. Klaus, A., and Birchmeier, W. Developmental signaling in myocardial progenitor cells: a comprehensive view of Bmp- and Wnt/beta-catenin signaling. Pediatr Cardiol 30, 609, 2009.

21. Behfar, A., Perez-Terzic, C., Faustino, R. S., Arrell, D. K., Hodgson, D. M., Yamada, S., Puceat, M., Niederlander, N., Alekseev, A. E., Zingman, L. V., and Terzic, A. Cardiopoietic programming of embryonic stem cells for tumor-free heart repair. J Exp Med 204, 405, 2007.

22. Autissier, A., Letourneur, D., and Le Visage, C. Pullulan-based hydrogel for smooth muscle cell culture. J Biomed Mater Res A 82A, 336, 2007.

23. Lavergne, M., Derkaoui, M., Delmau, C., Letourneur, D., Uzan, G., and Le Visage, C. Porous polysaccharide-based scaffolds for human endothelial progenitor cells. Macromol Biosci 12, 901, 2012.

24. Skardal, A., Zhang, J., McCoard, L., Xu, X., Oottamasathien, S., and Prestwich, G. D. Photocrosslinkable hyaluronan-gelatin hydrogels for two-step bioprinting. Tissue Eng Part A 16, 2675, 2010.

25. Kehat, I., Kenyagin-Karsenti, D., Snir, M., Segev, H., Amit, M., Gepstein, A., Livne, E., Binah, O., Itskovitz-Eldor, J., and Gepstein, L. Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. J Clin Invest 108, 407, 2001.

26. Srivastava, D. Making or breaking the heart: from lineage determination to morphogenesis. Cell 126, 1037, 2006.

27. Urbanek, K., Cesselli, D., Rota, M., Nascimbene, A., De Angelis, A., Hosoda, T., Bearzi, C., Boni, A., Bolli, R., Kajstura, J., Anversa, P., and Leri, A. Stem cell niches in the adult mouse heart. Proc Natl Acad Sci U S A 103, 9226, 2006.

28. Daley, W. P., Peters, S. B., and Larsen, M. Extracellular matrix dynamics in development and regenerative medicine. J Cell Sci 121, 255, 2008.

29. Schenke-Layland, K., Nsair, A., Van Handel, B., Angelis, E., Gluck, J. M., Votteler, M., Goldhaber, J. I., Mikkola, H. K., Kahn, M., and Maclellan, W. R. Recapitulation of the embryonic cardiovascular progenitor cell niche. Biomaterials 32, 2748, 2011.

30. Lake, A. C., Vassy, R., Di Benedetto, M., Lavigne, D., Le Visage, C., Perret, G. Y., and Letourneur, D. Low molecular weight Fucoidan increases VEGF165-induced endothelial cell migration by enhancing VEGF165 binding to VEGFR-2 and NRP1. J Biol Chem 281, 37844, 2006.

31. Ashikari-Hada, S., Habuchi, H., Kariya, Y., Itoh, N., Reddi, A. H., and Kimata, K. Characterization of growth factor-binding structures in heparin/heparan sulfate using an octasaccharide library. J Biol Chem 279, 12346, 2004.

32. McCaffrey, T. A., Falcone, D. J., Vicente, D., Du, B., Consigli, S., and Borth, W. Protection of transforming growth factor-beta 1 activity by heparin and Fucoidan. J Cell Physiol 159, 51, 1994.

33. Senni, K., Gueniche, F., Foucault-Bertaud, A., Igondjo-Tchen, S., Fioretti, F., Colliec-Jouault, S., Durand, P., Guezennec, J., Godeau, G., and Letourneur, D. Fucoidan a sulfated polysaccharide from brown algae is a potent modulator of connective tissue proteolysis. Arch Biochem Biophys 445, 56, 2006.

34. Deux, J. F., Meddahi-Pelle, A., Le Blanche, A. F., Feldman, L. J., Colliec-Jouault, S., Bree, F., Boudghene, F., Michel, J. B., and Letourneur, D. Low molecular weight Fucoidan prevents neointimal hyperplasia in rabbit iliac artery in-stent restenosis model. Arterioscler Thromb Vasc Biol 22, 1604, 2002.

35. Religa, P., Kazi, M., Thyberg, J., Gaciong, Z., Swedenborg, J., and Hedin, U. Fucoidan inhibits smooth muscle cell proliferation and reduces mitogen-activated protein kinase activity. Eur J Vasc Endovasc Surg 20, 419, 2000.

36. Freguin-Bouilland, C., Alkhatib, B., David, N., Lallemand, F., Henry, J. P., Godin, M., Thuillez, C., and Plissonnier, D. Low molecular weight Fucoidan prevents neointimal hyperplasia after aortic allografting. Transplantation 83, 1234, 2007.

37. Bautch, V. L. Stem cells and the vasculature. Nat Med 17, 1437, 2011.

38. Zemani, F., Benisvy, D., Galy-Fauroux, I., Lokajczyk, A., Colliec-Jouault, S., Uzan, G., Fischer, A. M., and Boisson-Vidal, C. Low-molecular-weight Fucoidan enhances the proangiogenic phenotype of endothelial progenitor cells. Biochem Pharmacol 70, 1167, 2005.

39. Sarlon, G., Zemani, F., David, L., Duong Van Huyen, J. P., Dizier, B., Grelac, F., Colliec-Jouault, S., Galy-Fauroux, I., Bruneval, P., Fischer, A. M., Emmerich, J., and Boisson-Vidal, C. Therapeutic effect of Fucoidan-stimulated endothelial colony-forming cells in peripheral ischemia. J Thromb Haemost 10, 38, 2012.

40. Nakamura, S., Nambu, M., Ishizuka, T., Hattori, H., Kanatani, Y., Takase, B., Kishimoto, S., Amano, Y., Aoki, H., Kiyosawa, T., Ishihara, M., and Maehara, T. Effect of controlled release of fibroblast growth factor-2 from chitosan/Fucoidan micro complex-hydrogel on in vitro and in vivo vascularization. J Biomed Mater Res A 85, 619, 2008.

41. Jacot, J. G., Martin, J. C., and Hunt, D. L. Mechanobiology of cardiomyocyte development. J Biomech 43, 93, 2010.

42. Russell, B., Curtis, M. W., Koshman, Y. E., and Samarel, A. M. Mechanical stress-induced sarcomere assembly for cardiac muscle growth in length and width. J Mol Cell Cardiol 48, 817, 2010.

43. White, E. Mechanical modulation of cardiac microtubules. Pflugers Arch 462, 177, 2011.

44. Clause, K. C., Tinney, J. P., Liu, L. J., Keller, B. B., and Tobita, K. Engineered early embryonic cardiac tissue increases cardiomyocyte proliferation by cyclic mechanical stretch via p38-MAP kinase phosphorylation. Tissue Eng Part A 15, 1373, 2009.

45. Small, E. M., and Krieg, P. A. Transgenic analysis of the atrialnatriuretic factor (ANF) promoter: Nkx2-5 and GATA-4 binding sites are required for atrial specific expression of ANF. Dev Biol 261, 116, 2003.

46. Bian, W., Liau, B., Badie, N., and Bursac, N. Mesoscopic hydrogel molding to control the 3D geometry of bioartificial muscle tissues. Nat Protoc 4, 1522, 2009.

47. Vader, D., Kabla, A., Weitz, D., and Mahadevan, L. Strain-induced alignment in collagen gels. PLoS One 4, e5902, 2009.

48. Flaibani, M., Boldrin, L., Cimetta, E., Piccoli, M., De Coppi, P., and Elvassore, N. Muscle differentiation and myotubes alignment is influenced by micropatterned surfaces and exogenous electrical stimulation. Tissue Eng Part A 15, 2447, 2009.

A

Day0　　　Day1　　　　　　　　Day6　　　　　　Day21　　Day30

H9 hES cells

FGF deprivation
SU-5402

BMP2, TGFβ, TNFα

Early cardiac
differenciation

Beating areas

B

hESCs

Culture Dish

EBs
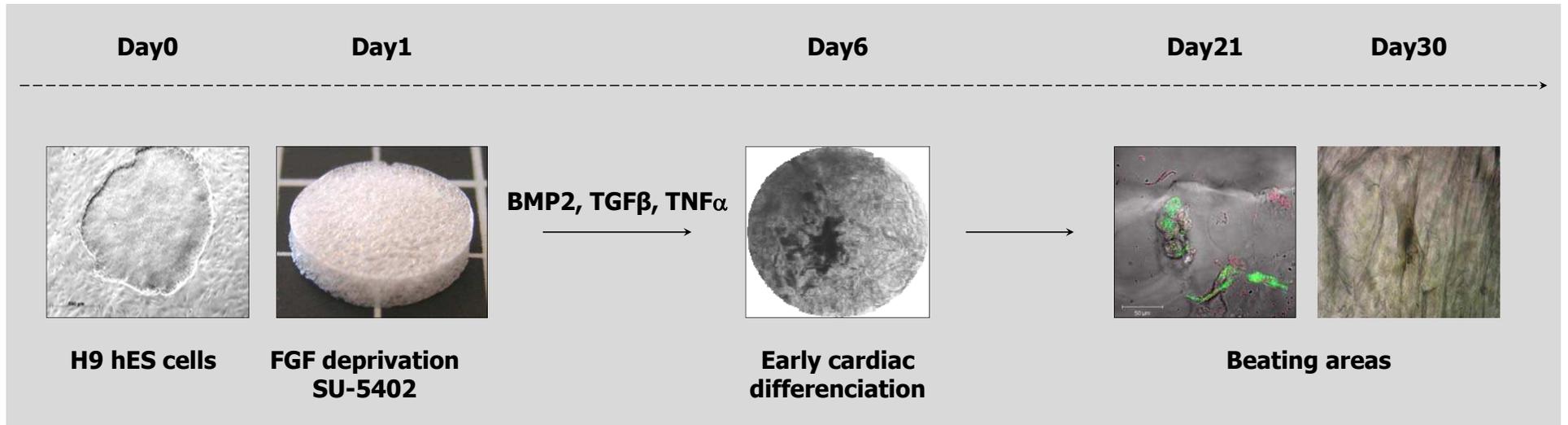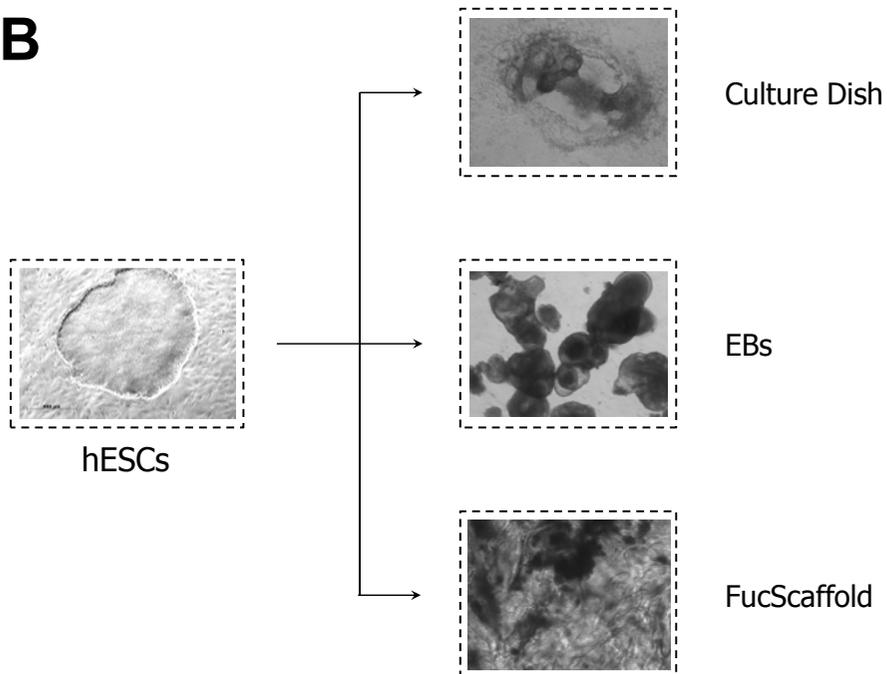
FucScaffold

Figure 1: Differentiation of hESC towards cardiac lineage

Figure 2: Development of an optimal scaffold composition to support differentiation of hESCs

Figure 3: Release of TNFα and TGFβ by control and Fucoidan scaffolds as a function of time.

A
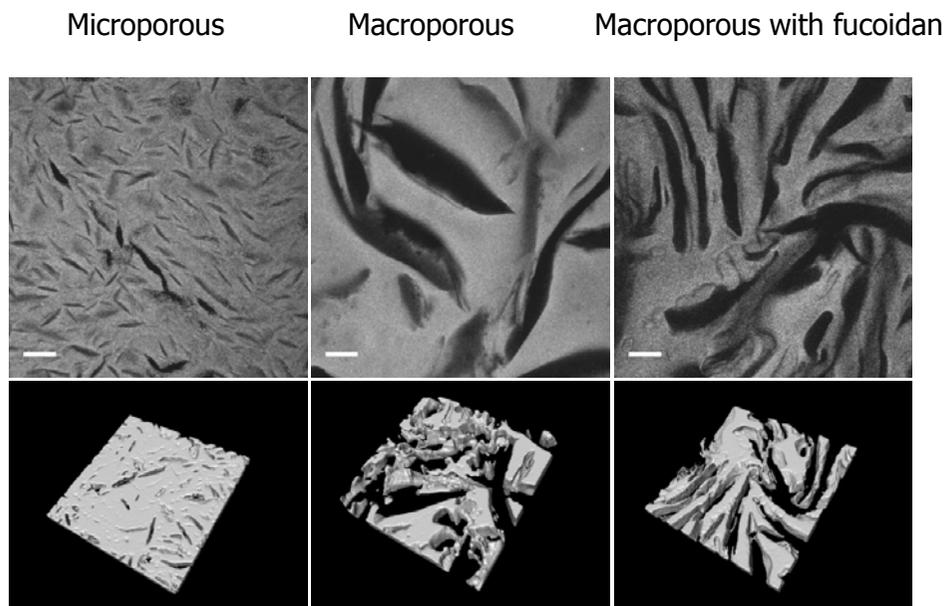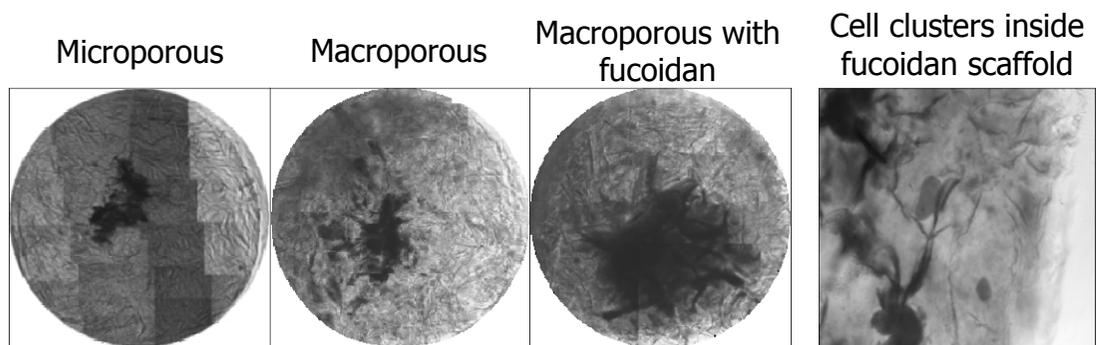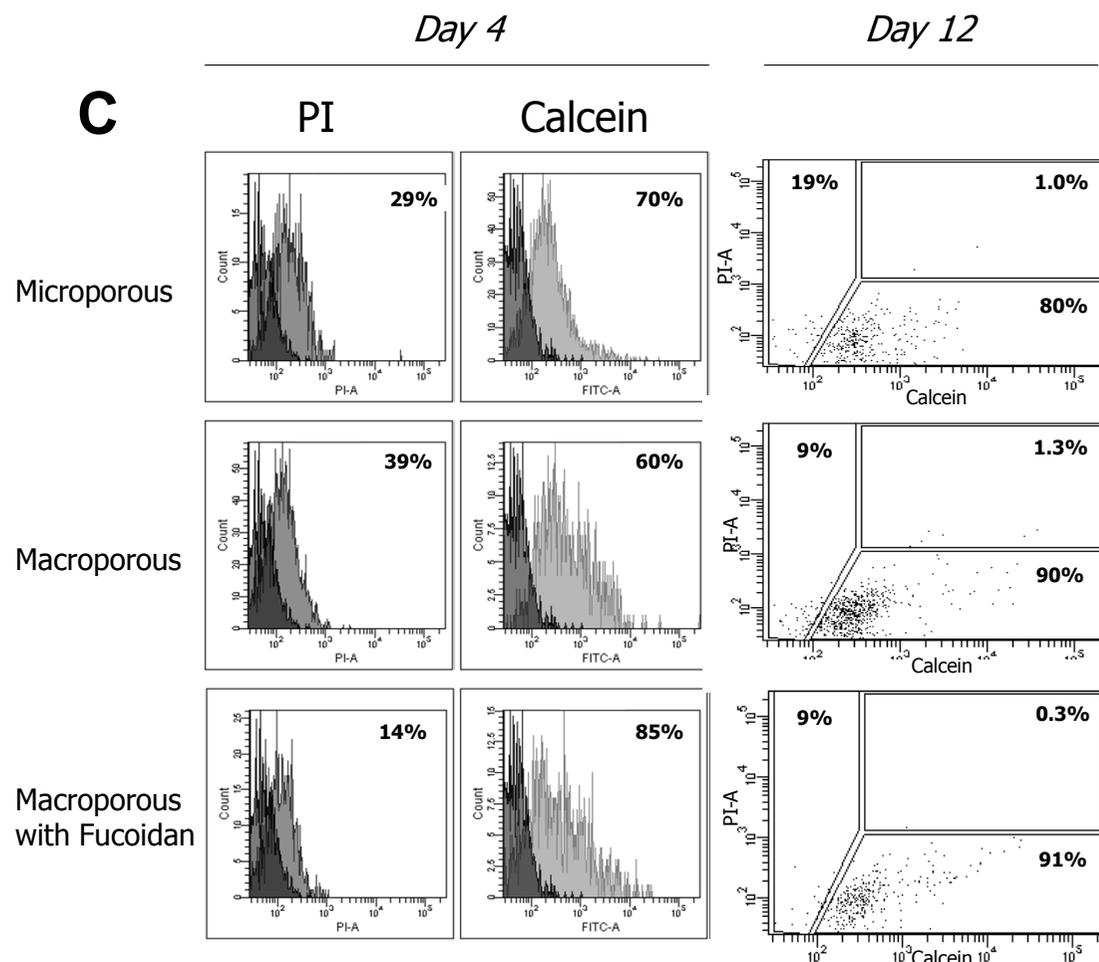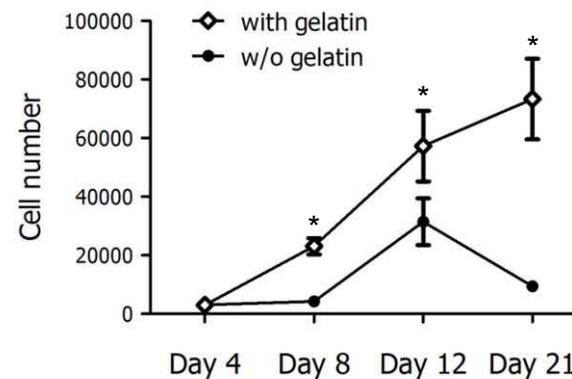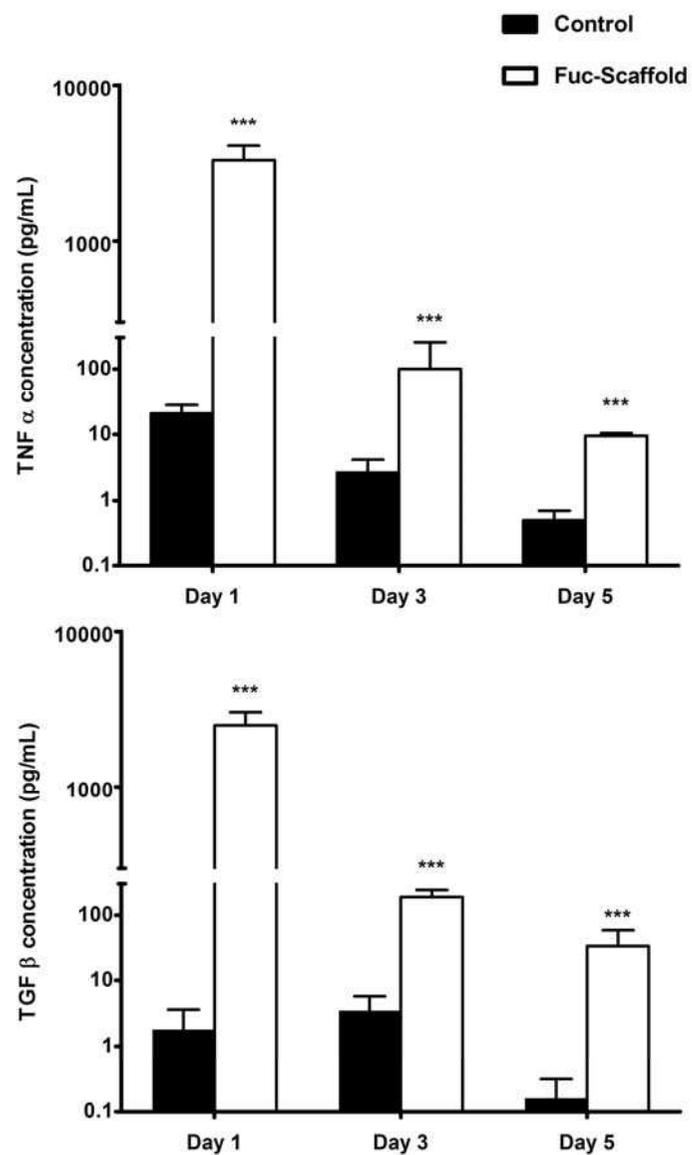
PAX6   α-FP   T-gene   NESTIN   FOXA2   HAND1   GAPDH

B

	D0	D2	D4	D9	D12	D15
NANOG	+	-	-	-	-	-
OCT4	+	+	-	-	-	-
NKX2.5	-	-	+	+	+	+
GATA4	-	-	+	+	+	-
cTNT	-	-	+	+	+	+
αMHC	-	-	-	+	+	+
ANP	-	-	-	+	+	+

Figure 5: Early cardiac specification from hESCs in 2D microenvironment, embryoid bodies and Fucoidan scaffolds
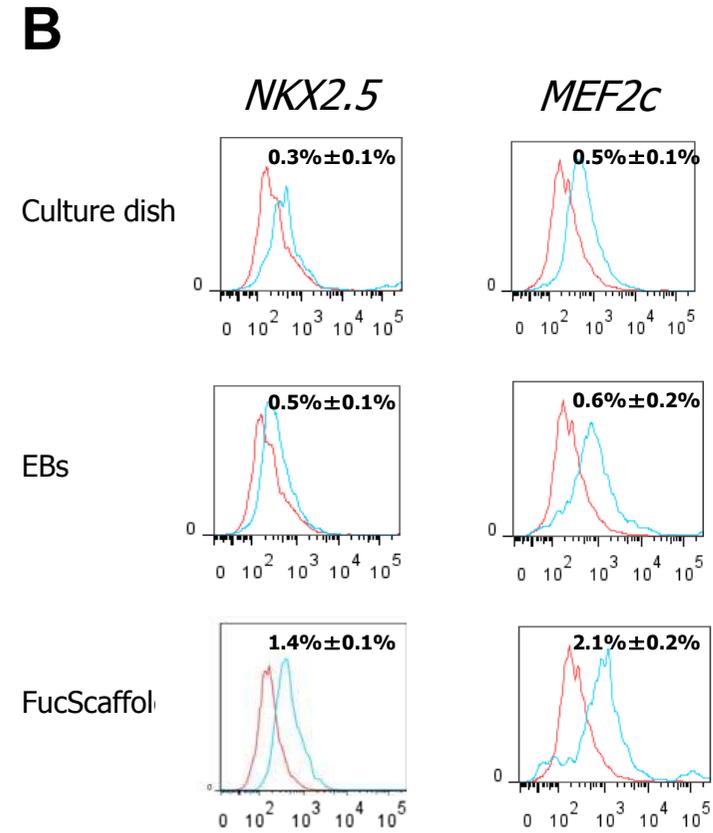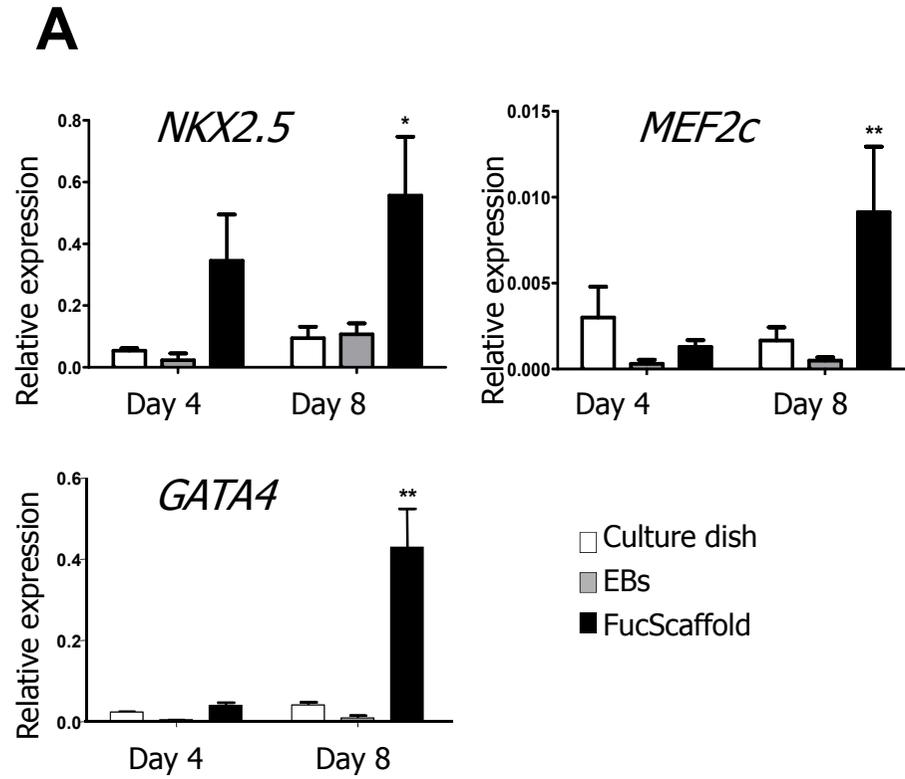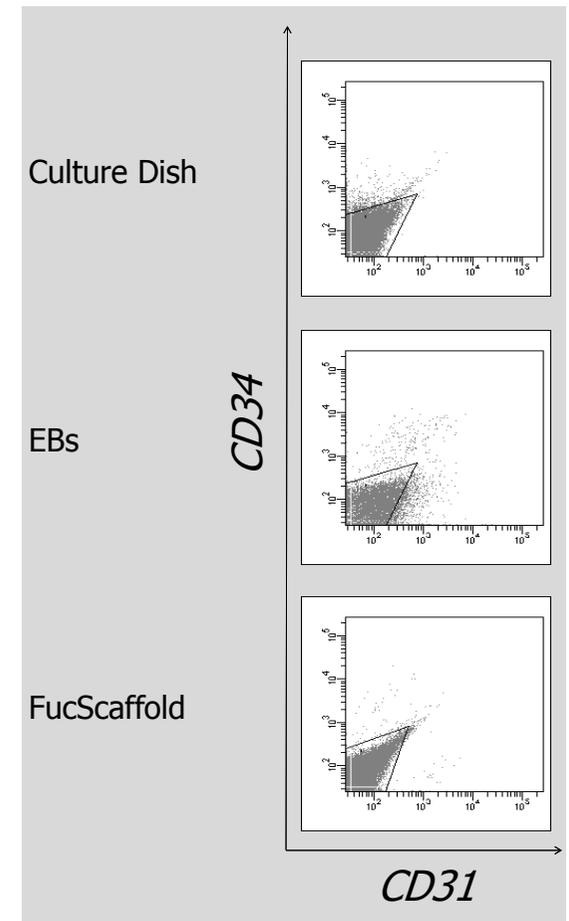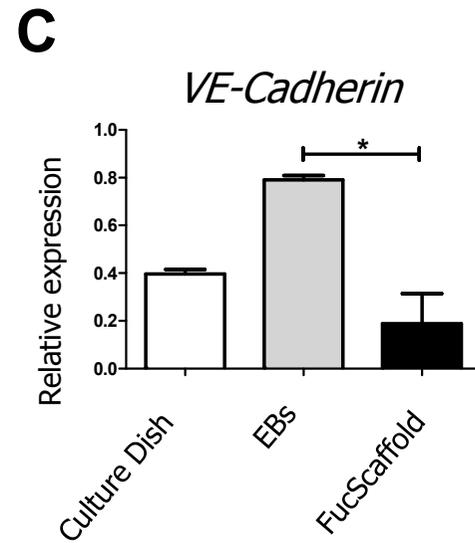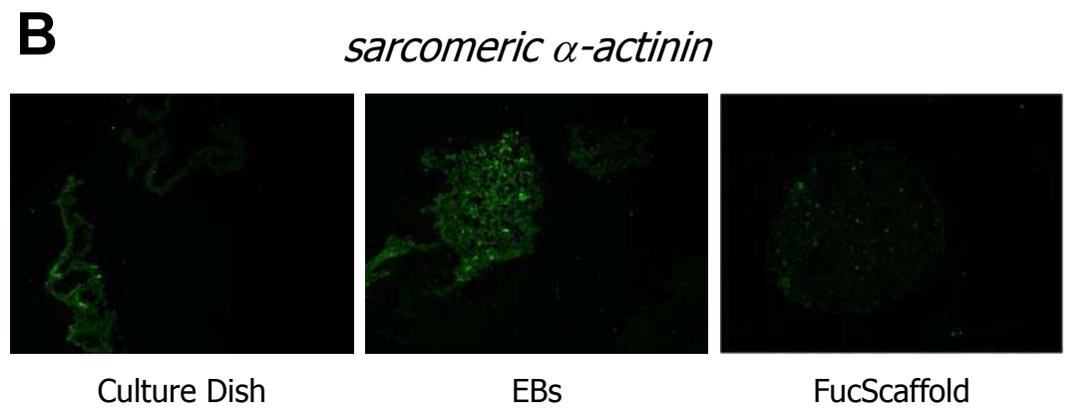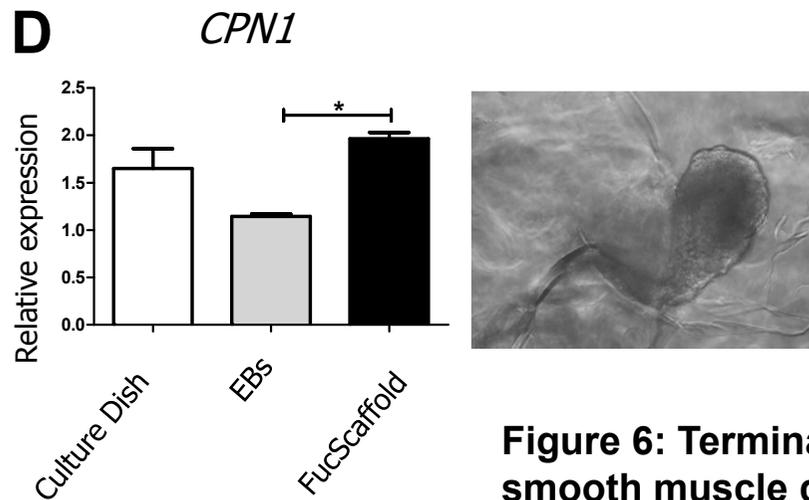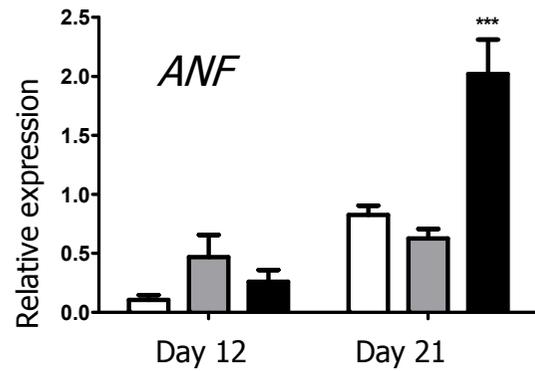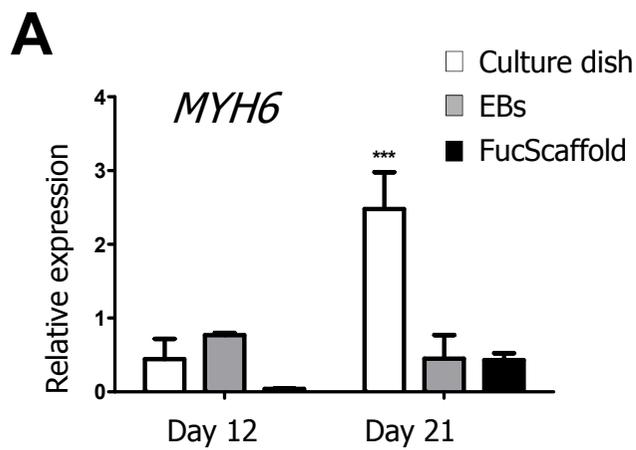
Figure 6: Terminal stage of cardiac, endothelial and smooth muscle differentiation in 2D condition, embryoid bodies and FucScaffolds.

Figure S1: Immunostaining for cardiac troponins cTnT and cTnI