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ABSTRACT

Aims: Success of functional vascularized tissues repair depends on vascular support system supply and still remains challenging. Our objective was to develop a nanoactive implant enhancing endothelial cell activity, particularly for bone tissue engineering in regenerative medicine field. **Materials & methods:** We developed a new strategy of tridimensional implant based on cell dependent sustained release of vascular endothelial growth factor (VEGF) nanoparticles. These nanoparticles were homogeneously distributed within nanoreservoirs onto the porous scaffold, with quicker reorganization of endothelial cells. Moreover, the activity of this active smart implant on cells was also modulated by addition of osteoblastic cells. **Results & conclusion:** This sophisticated active strategy should potentiate efficiency of current therapeutic implants for bone repair avoiding the need of bone substitutes.

Keywords: VEGF nanoparticles, nanoreservoirs, endothelial cells

INTRODUCTION

Surgical reconstructive procedures frequently require the use of additional tissues (autograft, xenograft, allograft) to restore the physiological functionality of damaged tissues. To overcome the complications associated with these methodologies (site morbidity, limited availability and host tissue reactivity), cell-based tissue engineering strategies using sophisticated biomaterials have been developed and represent a promising part of the regenerative medicine field. In the recent years, a plethora of advanced biomaterials mimicking extracellular matrix were designed based on the micro- and nano-scale environment of tissues, using nanotechnologies as graphene, nanogrooves and carbon nanotubes [1-8]. More recently, and with the development of the third generation materials, there has been increasing interest in developing nanoparticles and nanoreservoirs containing active molecules able to bio-activate scaffolds [9-14].

Bone regeneration is a complex, well-orchestrated physiological process, which occurs during normal healing of fractures, and is involved in continuous remodeling throughout adult life. In the past, a lot of active materials have been developed to improve bone tissue repair *in vivo* [15-18]. The first generation of biomaterials developed involved inert materials without any interaction with another component (prostheses…). Second generation biomaterials (bioactive coating, bio-mimicking materials) were developed to promote biological activity while supporting implant functions, and were either resorbable or bioactive. The third generation biomaterials are a combination between medical device and biological components (bioactive protein coating, endothelialization) and possess both bioresorbable and bioactive properties [19,20].

Applications for bone tissue engineering are various and depend on the severity of injuries to repair. The prosthesis domain aims for replacement after ablation due to severe injuries, the bone substitute domain, targets the substitution of important lesions (until 60 cm^3) to prevent ablation. This domain needs to use materials that mimicking the mechanical properties of the healthy bone. More recently, the domain of regenerative medicine was developed to avoid filling by substitute, and promote the tissue regeneration by materials totally replaced by a functional and biological tissue without clear high mechanical properties. During the last few years, sophisticated biomaterials for bone regenerative medicine were developed in clinic, for example in treatment of non-union fractures or with guided tissue regeneration in the periodontal disease treatment. Those materials can be ~~obtained~~ made bioactive by adding living cells or active growth factors. Among these strategies for bone tissue regeneration, two kinds of active materials can be considered: (i) materials combined with active molecules targeting bone tissue regeneration (BMP-2, BMP-7) [15-18, 21-26] or (ii) active materials indirectly acting on bone repair through combination with angiogenic factors (VEGF, Ang1) [27-32], which have gained increased attention recently.

Indeed, vascularization of biomaterials prior to their implantation is a key feature for tissue repair as establishment of a vascular network provides nutrients, soluble factors, phosphate and calcium necessary for the bone healing process. For healthy tissue repair, the formation of blood vessels in 3D engineered biomaterials still remains challenging today [27-29, 33]. In order to increase vascularization within the implanted site, several active angiogenic materials were developed with majority based on cell activities [34-36] or added factors activity [30-32]. One of the main growth factor involved in angiogenesis is $VEGF_{165}$ (Vascular Endothelial Growth Factor) which acts as an initiator and a modulator of the signaling cascade resulting in proliferation and migration of endothelial cells toward formation of new blood vessels [37, 38]. Moreover, VEGF possesses a pivotal role during bone healing [38], and the sustained release of this growth factor was shown to promote the efficacy of bone regeneration [30]. Not only does VEGF activate proliferation and migration of endothelial cells, but it also recruits mononuclear and macrophage cells, which produce additional angiogenic factors [39]. Consequently, during the last few years, tridimensional scaffolds associated with endothelial cells were developed for the cell-based strategy with the goal to promote biomaterials vascularization [33-36]. Various studies have reported the development of VEGF loaded nanoparticles for bone regeneration, wound healing angiogenesis, or inhibition of the graft shrinkage [40-43]. Most of these models can offer a controlled release of VEGF, depending on various parameters (temperature, pH, …). Herein, via entrapping VEGF-NPs in active nanoreservoirs, we can obtain cell-contact dependent access of NPs, depending solely of cells biological needs, avoiding short lived-effect and instability [44]. Moreover, this strategy of nanoreservoirs with chitosan allows keeping NPs with the biomaterials into the site of damage, and protected them from degradation activity due to their short life span, contrary to a passive release and diffusion *in situ*. Human umbilical vein endothelial cells (HUVECs) are easily extractable and expanded to large numbers. These cells are frequently used for *in vitro* studies

of angiogenesis since they are able to spontaneously organize into capillary-like structures without the help of additional angiogenic growth factors, and can spontaneously anastomose with the host vasculature [29, 45, 46].

Recently, we developed via electrospinning method a thick porous 3D nanofibrous poly(ε-caprolatcone) (PCL) scaffold able to accelerate the regeneration of a robust mineralized bone [22, 23, 47]. Herein, we have developed a double strategy combining both (i) HUVECs, and (ii) VEGF-nanoparticles (VEGF-NPs) active 3D nanofibrous scaffold to improve vascularization in tissue engineering for regenerative medicine applications focusing on bone regeneration. VEGF nanoparticles were used and integrated to the scaffold employing the layer-by-layer technology, enabling an active cell-dependent release of the active molecule [14, 48]. Moreover, mesenchymal cells (human primary osteoblasts) were used to increase the efficiency of the designed nanoactive 3D scaffold, firstly by adhesion of endothelial cells (HUVECs), and then by improving endothelial cells activity.

MATERIALS & METHODS

3D nanofibrous PCL scaffold preparation

PCL 80 kDa, analytical grade, was obtained from Perstorp (Malmö, Sweden). The PCL solution was prepared as previously described [22, 23, 47]: a solution of PCL (23% w/v in dimethylformamide: methylene chloride 1:1 v/v) was electrospun at high voltage (20 kV at the beginning), which was increased during the process (ending at 26 kV). The electrospinning device (EC-DIG electrospinning apparatus; IME Technologies, Eindhoven, Netherlands) delivered the solution at a constant rate of 1.2 mL/h in a 35% humidity and 30 °C atmosphere. The distance between the needle and the collector was set at 16 cm. The electrospun jet was focused in a 25 mm hole within a 2.5 mm-thick poly-(methyl methacrylate) mask plate placed over the conductor. The electrospining process was stopped when the designed material reached a thickness around 10 mm. Pieces of 3mm diameter and 700 um of thickness were then cut for

the experiments. The 3D electrospun nanofibrous membranes were kept in a desiccator at 45°C to remove residual solvents.

Preparation of VEGF-NPs and VEGF-NPs (HA)

Chitosan hydrochloride (CS) (30-400 kDa, HEPPE medical chitosan GmbH (HMC^+), Germany) nanoparticles (NPs) were produced using an ionotropic gelling method. Sodium tripolyphosphate (TPP) (85%, Sigma-Aldrich Co., St Louis, MO, USA) solution (1 mg/mL) was added drop-wise to chitosan hydrochloride solution (1 mg/mL) at a ratio of 1:5 w/w under constant stirring at room temperature. The resultant NPs suspension was left under stirring for 20 minutes to ensure complete particle formation. $hVEGF_{165}$ loaded NPs were obtained using the same method incorporating VEGF (VEGF-NPs) 200 µl (0.05 % w/v) with TPP solution. Chitosan/hyaluronic acid NPs were prepared by the same ionotropic gelling method, with modifications. Hyaluronic acid (HA) (10-20 kDa, Lifecore Biomedical, USA) (0.625 mg/mL) was dissolved in ultrapure water and mixed with TPP solution (1 mg/mL) at a ratio 3.75:1 w/w under constant stirring at room temperature. This poly-anionic phase was then added drop-wise to CS (1 mg/mL) at a ratio of 1:1.80 w/w (HA/TPP: CS) under constant stirring at room temperature. VEGF-NPs (HA) were obtained using the same method incorporating VEGF (VEGF-NPs (HA)) 200 µl (0.05 % w/v) with TPP/HA solution. To obtain dry powders, all were freeze-dried. Samples were frozen at -80 °C, 24h with trehalose at ratio 1:8 (Trehalose: CS) and then freeze-dried for 28 hours (Virtis Advantage, SP Scientific, USA).

Nanoparticles' size characterisation

Particle size analyses of freeze-dried samples were performed using dynamic light scattering (DLS) (Zetasizer Nano series DTS 1060, Malvern Instruments Ltd, UK). 2mg of VEGF-NPs and VEGF-NPs (HA) were dispersed in 2 mL of MES buffer (2-N-morpholino ethane sulfonic acid MES; Sigma-Aldrich Co., 0.04M MES, 0,15M NaCl, pH 5.5), loaded into a cuvette and data recorded at 25 °C (n = 3 suspensions).

PLL-FITC staining of VEGF-NPs

Glass slides were treated with Poly-glutamic acid (Sigma-Aldrich) (500 µm/mL) for 15 minutes, rinsed with PBS for 15 minutes followed by addition with VEGF-NPs (3 mg/mL) for 15 minutes at room temperature. Finally, samples were rinsed in PBS for 15 minutes and then with poly-lysine fluorescein-isothiocyanate (PLL-FITC) for 15 minutes. Glass slides were then observed under an epifluorescent microscope (LEICA DM 4000 B).

Transmission electron microscopy observation For visualisation of VEGF-NPs and VEGF-NPs (HA), freeze-dried sample was dispersed in water and applied to an electronic microscopy copper grid and stained with 2% phosphotungstic acid solution. The NPs were imaged by transmission electron microscopy (TEM) using an FEI CM 120 BioTwin transmission electron microscope (Philips Electron Optics BV, Netherlands) using acceleration voltage 120.0 kV (CM120, Philips Co, The Netherlands).

VEGF-NPs nanoreservoirs formation on 3D PCL scaffold

VEGF-NPs were dissolved at 2 mg/mL in MES buffer (pH 5.5) and chitosan was added at a concentration of 0.5 mg/mL (Protasan UP CL 113, Novamatrix, Sandvika, Norway). The creation of nanoreservoirs containing VEGF-NPs on the nanofibers was carried out using the layer-by-layer technology: briefly the 3D electrospun scaffold was dipped 6 times during 15 minutes alternately in solutions of nanoparticles and BSA gold (Aurion, Binnenhaven, Wageningen, The Netherlands) or BSA (200 ng/mL) (Bovine Serum Albumin, Euromedex, Souffelweyersheim, France). Each bath was followed by a rinsing step with MES buffer.

Scanning electron microscopy observation

To analyse the morphology of the functionalized 3D PCL scaffolds, samples were fixed in paraformaldehyde 4% for 10 minutes at 4 °C, then dehydrated and observed with a scanning electron microscope (SEM Hitachi TM 1000 or FEG Sirion XL; FEI) in conventional mode

(high vacuum) by collecting either secondary electrons or a combination of secondary and retro-diffused electrons.

Cell culture

Human umbilical vein endothelial cells (HUVECs) and human primary osteoblasts (HOBs) were obtained from PromoCell (Heidelberg, Germany). The cells were cultured in specific growth medium (endothelial cell growth medium, osteoblast growth medium, respectively, PromoCell). The cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2. When cells reached confluence, they were harvested with trypsin (Ozyme) and sub-cultured.

HUVECs culture in collagen gel

HUVECs culture medium was mixed with collagen I (2 mg/mL) (Institut de biotechnologie Jacques Boy, Reims, France) and NaOH (0.1M) was added to adjust the pH of the preparation. The cellular suspension of HUVECs, containing or not VEGF-NPs (2 mg/mL), was added and the final solution was seeded on 3D PCL scaffolds or in small Petri dishes, in a humidified atmosphere at 37 °C and 5% CO_2 to polymerise the collagen gel. Cells in collagen gel on scaffolds were then cultured in their corresponding growth medium in a humidified atmosphere at 37 °C and 5% CO_2.

Cell viability

Alamar Blue® (Thermo Fisher Scientific, Waltham, MA, USA) was used to assess cell metabolic activity over time. In this study, 4 x 10^4 human osteoblasts were first seeded on 3D PCL scaffolds containing or not nanoreservoirs of VEGF-NPs. After 7 days, 4 x 10^4 HUVECs were seeded on this pre-coated scaffold, and samples were cultured during a total of 28 days in a proliferative medium consisting of 50% HOB medium/50% HUVEC medium. Analyses were performed at D7, 9, 14, 21, and 28 days by culturing cells with a solution of 10% Alamar Blue®/ Dulbecco's Modified Eagle's Medium without phenol red (Lonza, Levallois-Perret, France) during 4 hours in a humidified atmosphere at 37 °C and 5% CO_2. The same experiments were

performed with HUVECs cells in collagen gel seeded on 3D PCL equipped or not with VEGF-NPs (D3, D7, D14, D21, and D28). Optical density of supernatants was measured at 575 and 590 nm in order to determine the percentage of Alamar Blue® reduction. Statistical analyses were obtained by t-paired student test.

Subcutaneous *in vivo* implantation in nude mice

All procedures regarding animals and tissues were designed in agreement with the recommendations of the European Union (2010/63/EU), and were performed according to authorized investigator Dr N. Jessel (Director of the Osteoarticular and Dental Regenerative Nanomedicine team), holder of a personal license from Prefecture du Bas-Rhin (number 67-315), who oversaw experiments done on mice. All experiments were performed in the Animalerie Centrale de la Faculté de Médecine de Strasbourg with the approval number A 67-482-35 from the Veterinary Public Health Service of the Préfecture du Bas-Rhin, representing the French Ministry of Agriculture, Department of Veterinary Science.

The study involved nude male mice (Crl: NIH-Foxn1^{nu} ; Charles River, L'arbresle, France) 6 weeks of age. The mice were anesthetized with an intra-peritoneal injection of 100 mg/kg of ketamine (VIRBAC Santé Animale; Centravet, Nancy, France) and injection of 10 mg/kg of Xylazine (Rompun® 2%) and animals were placed in a ventral decubitus position on a heating plate, under strict aseptic conditions. After skin incision, 3 mm-long 3D PCL scaffolds, with and without VEGF-NPs nanoreservoirs, which had been seeded with HOBs and HUVECs for 2 weeks (D14), were subcutaneously implanted in the mice. The skin was then sealed with sutures and the mice' behaviour was observed after waking up and during the incubation time. The mice were sacrificed with an intra-peritoneal injection of a lethal dose of ketamine and the samples were extracted after 4 and 8 days of implantation.

Immunofluorescence staining

Samples were fixed with 4% paraformaldehyde solution for 10 minutes at 4 °C and permeabilised with a 0.1% Triton X-100 solution for an hour at room temperature. To specifically label the surface of endothelial cells, samples were incubated overnight at 4 °C with mouse monoclonal anti-CD31 (1/400, Santa Cruz Biotechnology Inc., Dallas, TX, USA), followed by 1 hour incubation at room temperature with the secondary anti-IgG of mouse coupled to Alexa Fluor 488 (Life Technologies). Samples were then incubated for 20 minutes in a solution of Alexa fluor 546-conjugated phalloïdin (1/200, Molecular Probes; Life Technologies, Fisher Scientific, Illkirch, France). After this step, a solution of 200 nM DAPI (Sigma-Aldrich) was added to mark the nuclei of exposed cells. Observation of the samples was carried out using an epifluorescence microscope (Leica DM 4000 B).

RESULTS

VEGF-NPs to design a nanoactive 3D biomaterial

To increase the efficiency of tissue repairing *in vivo*, we developed an active tridimensional biomaterial equipped with VEGF-NPs. Our team previously developed a 3D porous PCL scaffold (Figure 1A) by the electrospinning method, for bone regeneration [22, 23, 47]. As previously described, this 3D-PCL implant (Figure 1B) present a superposition of spaced stratified layers of PCL nanofibers, facilitating macropores formation. This is an interconnected microporous/ macroporous scaffold, constituted of nanofibers (700 □m of thickness; fibers of 689±45 nm in diameter) able to mimic the collagen present in the extracellular matrix of healthy bone tissue, in favor of blood vessel colonization [22, 23, 47]. In this study, to make this 3D nanofibrous scaffold active, we first manufactured VEGF-NPs, and then added these to our 3D biomaterial. The VEGF-NPs are composed of chitosan forming the shell of the nanoparticles, and VEGF encapsulated within the nanoparticles. These VEGF-NPs were obtained by inotropic gelling and freeze-drying method (Figure 1C). These nanoparticles have a mean size of 175.4 ± 4.1 nm for VEGF-NPs and 167.5 ± 10.6 nm for VEGF-NPs containing hyaluronic acid

(VEGF-NPs (HA)) (Figure 1D). These observations, together with TEM observations (Figure 1E and 1F), didn't show significant differences in size between the two types of VEGF-NPs. Then, we functionalized the 3D Scaffold (Figure 2) by depositing VEGF-NPs. To this end, we employed our nanoreservoirs technology using the LbL technique [14]. After having tested different concentrations of VEGF-NPs to form the nanoreservoirs with chitosan solutions, we have found that the greatest condition to obtain a homogeneous deposition of NPs all along nanofibers of the scaffold, and in all the thickness of our 3D nanofibrous PCL was obtained with a concentration of VEGF-NPs of 2mg/mL (data not shown). The LbL $(BSA/VEGF\text{-}NPs)_6$-treated nanofibers (Figure 2B) showed a more homogeneous deposition of VEGF-NPs encapsulated within nanoreservoirs all along the fibers (Figure 2B), confirmed by co-localization with Gold-labeled BSA LbL (Figure 2C), compared to a single adsorption of VEGF-NPs (Figure 2A). Hence, we were able to design a porous tridimensional scaffold mimicking ECM and homogeneously equipped with VEGF-NPs, ready to receive endothelial cells.

VEGF-NPs active 3D Scaffold effect on endothelial cells (GEL)

As endothelial cells require collagen motifs to adhere to a biomaterial, we used collagen gel to increase endothelial cells adhesion to the 3D PCL scaffold. Behavior of endothelial cells in the presence of VEGF-NPs was first analyzed and results were observed by fluorescence staining. In the presence of VEGF-NPs, endothelial cells showed regular linear, tubule-like, cell organization, (Figure 3C), compared to HUVECs cultured without additional VEGF-NPs (Figure 3B). As the endothelial cells organization in the presence of VEGF-NPs was confirmed in the collagen gel, we next analyzed the behavior of endothelial cells on the VEGF-NPs active 3D-PCL scaffold. In this condition, Alamar Blue® test showed no significant difference between cells cultured on VEGF-NPs scaffold and non-functionalized scaffold at each time point (Figure 3A), showing biocompatibility of VEGF-NPs nanofibrous scaffold with

endothelial cells. However, significant difference was found for the non-functionalized scaffold between D3 and D28. This result could be explained by a loss of collagen gel containing endothelial cells, during the incubation time. Endothelial cells reorganization was also observed in this active tridimensional material (Figure 3E). Hence, we showed that our 3D PCL scaffold equipped with VEGF-NPs was active, and biocompatible for rapid endothelial cells activity and reorganization.

Double effect of VEGF-NPs active 3D PCL and HOB on Endothelial cells (HOB)

We sequentially used mesenchymal cells (HOBs) as a coating of VEGF-NPs active 3D PCL, for the adhesion of endothelial cells.

We first investigated the biocompatibility of our 3D VEGF-NPs active nanofibrous material pre-seeded with HOBs during 7 days, and then covered by endothelial cells. Analysis showed an increase in the metabolic activity all along the culture and after seeding of endothelial cells on HOBs (Figure 4A). Interestingly, the metabolic activity was more important when endothelial cells were seeded on a HOB pre-coated VEGF-NPs active scaffold (Figure 4A) compared to cells seeded in collagen gel (Figure 3). At D14, a significant difference occurred with cells cultured on VEGF-NPs biomaterials, showing more significant metabolic activity than cells without VEGF-NPs ($p = 0.018$) (Figure 4A). Moreover, at D28, more tubule-like endothelial structures were observed on the VEGF-NPs 3D PCL scaffold (Figure 4C). These results were observed by indirect immunofluorescence against endothelial specific marker CD31 (Figure 4B, C). Endothelial cells (visible in green) were first able to adhere to the scaffold without any collagen gel (Figure 4B) and second, they were able to form tubule-like and linear structures on this double active implant (Figure 4C). We then subcutaneously implanted our double active scaffold in nude mice to observe its influence on host endothelial cells (Figure SI 1). After 4 days and 8 days *in vivo*, the macroscopic view of the implant revealed that when equipped with VEGF-NPs NRs, it was able to increase the host vasculature recruitment (host

endothelial cells) towards implant by 20% compared to non-fonctionnalized one (Figure SI 1). These results show the potential double activity of our scaffold with regard to endothelial cells. The effect of our implant on tubule-like structures formation was obtained by the addition of VEGF-NPs and improved by the pre-coating with human osteoblast cells HOBs.

DISCUSSION

VEGF-NPs encapsulated in nanoreservoirs on a 3D scaffold to accelerate implant vascularization.

In the last decade, a large variety of active biomaterials have been developed for tissue engineering [1,12-14, 21-26, 49-60]. Focusing on either cartilage or bone tissue regeneration and controlled and active release of growth factor, our team has developed several active nanofibrous materials equipped with active nanoreservoirs of BMP-2 and BMP-7 [14, 21-26]. These natural collagen (Bio-Gide®) or polymeric (PCL) implants showed great potential in regenerative nanomedicine for bone regeneration with primary autologous cells (human mesenchymal stem cells, human primary osteoblasts) [14, 21-26]. The tridimensional electrospun PCL nanofibrous scaffold appeared to be promising due to its biomimicking architecture, with high porosity and enhanced pore interconnectivity, improving cell colonization and infiltration [22, 23]. Particularly for bone tissue engineering and blood supply during the early stages of post-implantation, it is necessary to provide a fast and sufficient blood perfusion within implants for calcium and phosphorus distribution, nutrients and oxygen diffusion, and elimination of wastes. Bone healing can be improved by adjusting the speed of vascularization and during the last 5 years, attempts have been made to increase vascularization in the tissue-engineering field [27-29, 33, 38-39]. Hence, basic strategies using scaffolds focused firstly on the use of cells (HUVEC, EPC) [30-32], and secondly on the use of active molecules (VEGF, Ang1, ...) operating on endothelial cells [34-36] to promote proliferation, migration, sprouting and establishment of functional blood vessel. In our strategy, we chose to

use not only VEGF active molecules (VEGF-NPs), but also endothelial and mesenchymal cells. VEGF plays a pivotal role in bone healing and remodeling. Moreover, if a local excessive dose of VEGF is released over a short time period, this leads to generation of non-functional and leaky blood vessels. To this end, a more sustained release over time is required to promote angiogenesis [30], which is the case in our strategy of nanoreservoirs. Hence, the effects of VEGF on biological processes are dose dependent. Our technology of nanoreservoirs created by LbL allowed a homogeneous distribution of VEGF-NPs all over the scaffold and enabled an active and controlled released of VEGF-NPs, dependent on the cells contact to the nanoreservoirs [14, 48]. In our strategy, HUVECs cells will recognize and adhere to chitosan, whether it is present on NPs or in our nanoreservoirs functionalization. In the case of active nanoreservoirs, cells will degrade by enzymatical reaction nanoreservoirs and will have an access to the NPs containing VEGF. Moreover, encapsulated active molecules represent a major advantage considering the short half-life of the VEGF growth factor (50 min). Our nanoreservoir technology overcomes potential damages that can occur to VEGF by protecting it within nanoreservoirs.

Double effect of VEGF-NPs and mesenchymal cells (HOBs) on endothelial cells organization.

Our nanofibrous scaffold was obtained from polycaprolactone by electrospining. This nanostructured material has already shown its efficacy in terms of bone regeneration with mesenchymal stem cells and primary human osteoblastic cells [14, 21-23]. Moreover, this 3D material, leads to an homogeneous attachment of osteoblastic cells [14, 21-23]. When dotted with chitosan-growth factor nanoreservoirs, 3D PCL present an adhesion of cells specifically in clusters onto the nanoreservoirs, contrary to non-functionalized PCL. The adherence of osteoblastic cells in cluster will also create a cluster of endothelial cells adhesion where they will reorganise, thanks to the angiogenic environment that could be provided by osteoblastic

cells. However, adhesion of endothelial cells requires collagen moieties [61, 62]. Hence, in this study we used a collagen gel to improve endothelial cells adhesion. In these conditions, HUVECs were able to re-organize linearly, in the presence of VEGF-NPs directly in collagen gel or onto the 3D PCL scaffold. However, the use of collagen with 3D PCL scaffold presents technical limitations. Hence, in this study we adapted another alternative to improve endothelial cells adhesion to PCL, with the use of functionalized PCL scaffolds pre-coated with mesenchymal cells. The sequential seeding in our study, with mesenchymal osteoblastic cells prior to endothelial cells lead to a primo-access to the VEGF for mesenchymal cells. These cells will serve as i) anchor to endothelial cells attachment to the biomaterial and ii) secretor of growth factor activation reorganisation of endothelial cells.

In this study we developed an active scaffold able to improve and accelerate endothelial cells organization in tubule-like structures and host vasculature recruitment in the implant after implantation to ameliorate tissue-engineering efficacy. After showing the efficiency of VEGF-NPs on HUVECs behavior, we used HOBs to improve their adhesion on PCL scaffold. Actually, cells of mesenchymal origin, either by their capacity to secrete extracellular matrix (decellularized matrix) or by their own particular nature, were exploited to improve cell attachment on non-collagen materials [48, 63, 64]. Process of angiogenesis and bone formation are thought to be in close interaction in bone healing. Moreover HOBs and mesenchymal stem cells are known to increase the stability of *in vitro* and *in vivo* tubule-like structure formation by endothelial cells [29, 65]. Recently, much attention has been implemented in endothelial cells as mediators of osteogenic differentiation, since the combined use of mesenchymal cells (HOBs, MSCs) significantly enhanced the ability of osteogenic differentiation tissue *in vitro* [22, 23, 66-68]. With the combination of the two strategies in one material (VEGF-NPs and the use of HOBs), we were able to accelerate host vascularization of the implant *in vivo*. These results could be explained by the fact that VEGF was not only available via our NPs for

exogenic endothelial cells added to the scaffold, but also that HOBs and HUVECs used synergistically secrete more angiogenic factors compared to HUVECs alone [69]. Moreover, the access of HOBs to the VEGF-NPs can increase the secretion of angiogenic factors in the implant, resulting in the VEGF molecules acting directly on osteoblasts proliferation and migration [70]. Thus, in our double strategy, Endothelial cells will adhere to the VEGF-NPs material via the previous matrix secretion of HOBs. HOBs, degrading nanoreservoirs by enzymatic reaction, will have an access to the VEGF-NPs, and will secrete angiogenic factor used by HUVECs. Based on the capacity of exogenous endothelial cells to anastomose with host vasculature [29], we subcutaneously implanted our double active implant and observed an enhanced initiation of host vasculature in the implant containing VEGF-NPs compared to the non-functionalized scaffold. Controlled drugs release is an important field for human health. In most cases, the developed materials for regenerative medicine, using VEGF [40-43] lead to a high-dose passive release with overdoses, side effect, and the progression of tumors [71], particularly for regeneration of tissues compromised after irradiation. With our double strategy, VEGF release is sustained and dependent of cells adhesion and reliant on their own needs. Moreover, the growth factor release is localized at the desired site, avoiding thus side effects.

CONCLUSION

In the last few years, new tissue-engineering strategies for bone repair have focused on vascularization to improve the quality of repaired tissues. In this work, our aim was to develop a new strategy of active implant leading to improve endothelial cells organization. This strategy can improve vascularization *in situ* and can enhance the repair potential of tissues *in vivo*. In this study, we developed a 3D biodegradable and polymeric scaffold, highly porous, active for endothelial cells organization and improving the speed of cell reorganization by incorporating VEGF-NPs. This scaffold was active towards endothelial cells not only via the incorporation of VEGF-NPs, but also via incorporation of osteoblast cells as pre-coating on our 3D PCL

nanofibrous scaffold. This work highlights the nanofibrous implant potential, offering an active and sustained release of angiogenic growth factor for regenerative medicine application. The double effect obtained *in vivo* with this living implant could be transfered to the clinic, taking advantage of the therapeutic potential of mesenchymal stem cells. ~~We also think that this new generation of sophisticated implant is adaptable for engineering of other tissues, since it can be equipped with other kinds of cells or active molecules.~~

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Disclosure

The authors report no conflicts of interest in this work.

REFERENCES

1. Dvir T, Timko BP, Kohane DS, Langer R. Nanotechnological strategies for engineering complex tissues. *Nat Nanotechnol*. 6(1), 13–22 (2011).
2. Gusić N, Ivković A, VaFaye J, Vukasović A, Ivković J, Hudetz D, Janković S. Nanobiotechnology and bone regeneration: a mini-review. *Int Orthop*. 38(9), 1877–1884 (2014).

3. Kane R, Ma1 PX. Mimicking the nanostructure of bone matrix to regenerate bone. *Mater Today (Kidlington)*. 16(11), 418–423 (2013).
4. Lim JY, Loiselle AE, Lee JS, Zhang Y, Salvi JD, Donahue HJ. Optimizing the osteogenic potential of adult stem cells for skeletal regeneration. *J. Orthop. Res.* 29(11), 1627–1633 (2011).
5. Metzger W, Schwab B, Miro MM, Grad S, Simpson A, Veith M, *et al.* Induction of osteogenic differentiation by nanostructured alumina surfaces. *J Biomed Nanotechnol*. 10(5), 831–845 (2014).
6. Nayak TR, Andersen H, Makam VS, Khaw C, Bae S, Xu X, *et al.* Graphene for controlled and accelerated osteogenic differentiation of human mesenchymal stem cells. *ACS Nano*. 5(6), 4670–4678 (2011).
7. Watari S, Hayashi K, Wood JA, Russel P, Nealey PF, Murphy CJ, Genetos DC. Modulation of osteogenic differentiation in hMSCs cells by submicron topographically-patterned ridges and grooves. *Biomaterials*. 33(1), 128–136 (2012).
8. Gong T, Xie J, Liao J, Zhang T, Lin S, Lin Y. Nanomaterials and bone regeneration. *Bone Res*. 10(3), 15029 (2015).
9. Zhang S, Uludağ H. Nanoparticulate systems for growth factor delivery. *Pharm. Res.* 26(7), 1561–1580 (2009).
10. Wang X, Wenk E, Zhang X, Meinel L, Vunjak-Novakovic G, Kaplan DL. Growth factor gradients via microsphere delivery in biopolymer scaffolds for osteochondral tissue engineering. *J Control Release*. 134(2), 81–90 (2009).
11. Fioretti F, Mendoza-Palomares C, Helms M, *et al.* Nanostructured Assemblies for Dental Application. *ACS Nano*. 4(6), 3277–3287 (2010).
12. Facca S, Cortez C, Mendoza-Palomares C, Messadeq N, Dierich A, Johnston A, *et al.* Active multilayered capsules for in vivo bone formation. *Proc. Natl. Acad. Sci. U.S.A.*

107(8), 3406–3411 (2010).

13. Facca S, Lahiri D, Fioretti F, Messadeq N, Mainard D, Benkirane-Jessel N, Agarwal A. In vivo osseointegration of nano-designed composite coatings on titanium implants. *ACS Nano*. 5(6), 4790–4799 (2011).
14. Mendoza-Palomares C, Ferrand A, Facca S, Fioretti F, Ladam G, Kuchler-Bopp S, *et al.* Smart hybrid materials equipped by nanoreservoirs of therapeutics. *ACS Nano*. 6(1), 483–490 (2012).
15. Giannoudis PV, Einhorn TA. Bone morphogenetic proteins in musculoskeletal medicine. *Injury*. 40 Suppl 3, S1-3 (2009).
16. Jimi E, Hirata S, Osawa K, Terashita M, Kitamura C, Fukushima H. The Current and Future Therapies of Bone Regeneration to Repair Bone Defects. *International Journal of Dentistry*. 2012, e148261 (2012).
17. Dimitriou R, Jones E, McGonagle D, Giannoudis PV. Bone regeneration: current concepts and future directions. *BMC Medicine*. 9(1), 66 (2011).
18. Nyberg E, Holmes C, Witham T, Grayson WL. Growth factor-eluting technologies for bone tissue engineering. *Drug Deliv Transl Res*. 6(2), 184–194 (2016).
19. Amini AR, Laurencin CT, Nukavarapu SP. Bone Tissue Engineering: Recent Advances and Challenges. *Crit Rev Biomed Eng*. 40(5), 363–408 (2012).
20. Hench LL, Polak JM. Third-generation biomedical materials. *Science*. 295(5557), 1014–1017 (2002).
21. Ferrand A, Eap S, Richert L, *et al.* Osteogenetic Properties of Electrospun Nanofibrous PCL Scaffolds Equipped With Chitosan-Based Nanoreservoirs of Growth Factors. *Macromol Biosci*. 14(1), 45-55 (2014).
22. Eap S, Ferrand A, Palomares CM, Hébraud A, Stoltz J-F, Mainard D, *et al.* Electrospun nanofibrous 3D scaffold for bone tissue engineering. *Biomed Mater Eng*. 22(1–3), 137–141

(2012).

23. Eap S, Keller L, Schiavi J, Huck O, Jacomine L, Fioretti F, *et al.* A living thick nanofibrous implant bifunctionalized with active growth factor and stem cells for bone regeneration. *Int J Nanomedicine*. 10, 1061–1075 (2015).
24. Schiavi J, Keller L, Morand D-N, De Isla N, Huck O, Lutz J-C, *et al.* Active implant combining human stem cell microtissues and growth factors for bone-regenerative nanomedicine. *Nanomedicine (Lond)*. 10(5), 753–763 (2015).
25. Eap S, Ferrand A, Schiavi J, Keller L, Kokten T, Fioretti F, *et al.* Collagen implants equipped with "fish scale"-like nanoreservoirs of growth factors for bone regeneration. *Nanomedicine (Lond)*. 9(8), 1253–1261 (2014).
26. Eap S, Keller L, Ferrand A, Schiavi J, Lahiri D, Lemoine S, *et al.* Nanomechanical Properties of Active Nanofibrous Implants After In Vivo Bone Regeneration. *Nano LIFE*. 4(1), 1450001-1450010 (2013).
27. Kaully T, Kaufman-Francis K, Lesman A, Levenberg S. Vascularization--the conduit to viable engineered tissues. *Tissue Eng Part B Rev*. 15(2), 159–169 (2009).
28. Mercado-Pagán ÁE, Stahl AM, Shanjani Y, Yang Y. Vascularization in bone tissue engineering constructs. *Ann Biomed Eng*. 43(3), 718–729 (2015).
29. Tsigkou O, Pomerantseva I, Spencer JA, Redondo PA, Hart AR, O'Doherty E, *et al.* Engineered vascularized bone grafts. *Proc. Natl. Acad. Sci. U.S.A.* 107(8), 3311–3316 (2010).
30. Wernike E, Montjovent M-O, Liu Y, Wismeijer D, Hunziker EB, Siebenrock KA, *et al.* VEGF incorporated into calcium phosphate ceramics promotes vascularisation and bone formation in vivo. *Eur Cell Mater*. 19, 30–40 (2010).
31. Wang K, Chen X, Pan Y, Cui Y, Zhou X, Kong D, *et al.* Enhanced vascularization in hybrid PCL/gelatin fibrous scaffolds with sustained release of VEGF. *Biomed Res Int*. 2015,

865076-865086 (2015).

32. Lenze U, Pohlig F, Seitz S, Ern C, Milz S, Docheva D, Schieker M. Influence of osteogenic stimulation and VEGF treatment on in vivo bone formation in hMSC-seeded cancellous bone scaffolds. *BMC Musculoskelet Disord*. 15, 350-360 (2014).
33. Baiguera S, Ribatti D. Endothelialization approaches for viable engineered tissues. *Angiogenesis*. 16(1), 1–14 (2013).
34. Sasaki J-I, Hashimoto M, Yamaguchi S, Itoh Y, Yoshimoto I, Matsumoto T, *Imazato S.* Fabrication of Biomimetic Bone Tissue Using Mesenchymal Stem Cell-Derived Three-Dimensional Constructs Incorporating Endothelial Cells. *PLoS ONE*. 10(6), e0129266 (2015).
35. Guerrero J, Catros S, Derkaoui S-M, Lalande C, Siadous R, Bareille R *et al.* Cell interactions between human progenitor-derived endothelial cells and human mesenchymal stem cells in a three-dimensional macroporous polysaccharide-based scaffold promote osteogenesis. *Acta Biomater*. 9(9), 8200–8213 (2013)
36. McFadden TM, Duffy GP, Allen AB, Stevens HY, Schmarzmaier SM, Plesnila N, *et al.* The delayed addition of human mesenchymal stem cells to pre-formed endothelial cell networks results in functional vascularization of a collagen-glycosaminoglycan scaffold in vivo. *Acta Biomater*. 9(12), 9303–9316 (2013).
37. Olsson A-K, Dimberg A, Kreuger J, Claesson-Welsh L. VEGF receptor signalling - in control of vascular function. *Nat. Rev. Mol. Cell Biol.* 7(5), 359–371 (2006).
38. Kleinheinz J, Stratmann U, Joos U, Wiesmann H-P. VEGF-activated angiogenesis during bone regeneration. *J. Oral Maxillofac. Surg.* 63(9), 1310–1316 (2005).
39. Grunewald M, Avraham I, Dor Y, Bachar-Lustig E, Itin A, Jung S, *et al.* VEGF-induced adult neovascularization: recruitment, retention, and role of accessory cells. *Cell*. 124(1), 175–189 (2006).

40. Geng H, Song H, Qi J, Cui D. Sustained release of VEGF from PLGA nanoparticles embedded thermo-sensitive hydrogel in full-thickness porcine bladder acellular matrix. *Nanoscale Res. Lett.* 6(1), 312-320 (2011).

41. Subbiah R, Hwang MP, Van SY, *et al.* Osteogenic/angiogenic dual growth factor delivery microcapsules for regeneration of vascularized bone tissue. *Adv. Healthc. Mater.* 4(13), 1982–1992 (2015).

42. Mohandas A, Anisha BS, Chennazhi KP, Jayakumar R. Chitosan–hyaluronic acid/VEGF loaded fibrin nanoparticles composite sponges for enhancing angiogenesis in wounds. *Colloids Surf. B Biointerfaces*. 127, 105–113 (2015).

43. Jiang X, Xiong Q, Xu G, *et al.* VEGF-Loaded Nanoparticle-Modified BAMAs Enhance Angiogenesis and Inhibit Graft Shrinkage in Tissue-Engineered Bladder. *Ann. Biomed. Eng.* 43(10), 2577–2586 (2015).

44. Mendoza-Palomares C, Ferrand A, Facca S, *et al.* Smart Hybrid Materials Equipped by Nanoreservoirs of Therapeutics. *ACS Nano*. 6(1), 483–490 (2012)

45. Arnaoutova I, Kleinman HK. In vitro angiogenesis: endothelial cell tube formation on gelled basement membrane extract. *Nat Protoc*. 5(4), 628–635 (2010).

46. Dickinson LE, Moura ME, Gerecht S. Guiding endothelial progenitor cell tube formation using patterned fibronectin surfaces. *Soft Matter*. 6(20), 5109–5119 (2010).

47. Eap S, Morand D, Clauss F, *et al.* Nanostructured thick 3D nanofibrous scaffold can induce bone. *Biomed. Mater. Eng.* 25(0), 79–85 (2015).

48. Tang Z, Wang Y, Podsiadlo P, Kotov NA. Biomedical Applications of Layer-by-Layer Assembly: From Biomimetics to Tissue Engineering. *Advanced Materials*. 18(24), 3203–3224 (2006).

49. Chatterjea A, Meijer G, van Blitterswijk C, de Boer J. Clinical Application of Human Mesenchymal Stromal Cells for Bone Tissue Engineering. *Stem Cells International*

[Internet]. (2010). Available from: http://www.hindawi.com/journals/sci/2010/215625/abs/.

50. Peppas NA, Langer R. New challenges in biomaterials. *Science*. 263(5154), 1715–1720 (1994).
51. Hubbell JA. Biomaterials in tissue engineering. *Biotechnology (N.Y.)*. 13(6), 565–576 (1995).
52. Langer R, Tirrell DA. Designing materials for biology and medicine. *Nature*. 428(6982), 487–492 (2004).
53. Griffith LG, Naughton G. Tissue engineering--current challenges and expanding opportunities. *Science*. 295(5557), 1009–1014 (2002).
54. Chaikof EL, Matthew H, Kohn J, Mikos AG, Prestwich GD, Yip CM. Biomaterials and scaffolds in reparative medicine. *Ann. N. Y. Acad. Sci.* 961, 96–105 (2002).
55. Yang C, Hillas PJ, Báez JA, Nokelainen M, Balan J, Tang J, *et al.* The application of recombinant human collagen in tissue engineering. *BioDrugs*. 18(2), 103–119 (2004).
56. Kumar G, Tison CK, Chatterjee K, Pine PS, McDaniel JH, Salit ML, *et al.* The determination of stem cell fate by 3D scaffold structures through the control of cell shape. *Biomaterials*. 32(35), 9188–9196 (2011).
57. Pullela SR, Andres C, Chen W, Xu C, Wang L, Kotov NA. Permselectivity Replication of Artificial Glomerular Basement Membranes in Nanoporous Collagen Multilayers. *J Phys Chem Lett*. 2(16), 2067–2072 (2011).
58. Lee J, Cuddihy MJ, Kotov NA. Three-dimensional cell culture matrices: state of the art. *Tissue Eng Part B Rev*. 14(1), 61–86 (2008).
59. Choi J-Y, Jung H-J, Park B-J, Joung Y-K, Park K-D, Han D-K. Adhesion Behavior of Chondrocyte and Osteoblast on Surface-Modified Biodegradable PLLA Films and Scaffolds. *Polymer Korea*. 36(3), 357–363 (2012).

60. Shah NJ, Hong J, Hyder MN, Hammond PT. Osteophilic multilayer coatings for accelerated bone tissue growth. *Adv. Mater. Weinheim*. 24(11), 1445–1450 (2012).

61. de Mel A, Jell G, Stevens MM, Seifalian AM. Biofunctionalization of biomaterials for accelerated in situ endothelialization: a review. *Biomacromolecules*. 9(11), 2969–2979 (2008).

62. Lin Q, Ding X, Qiu F, Song X, Fu G, Ji J. In situ endothelialization of intravascular stents coated with an anti-CD34 antibody functionalized heparin-collagen multilayer. *Biomaterials*. 31(14), 4017–4025 (2010).

63. Xu Y, Yan M, Gong Y, Chen L, Zhao F, Zhang Z. Response of endothelial cells to decellularized extracellular matrix deposited by bone marrow mesenchymal stem cells. *Int J Clin Exp Med*. 7(12), 4997–5003 (2014).

64. Moroni F, Mirabella T. Decellularized matrices for cardiovascular tissue engineering. *Am J Stem Cells*. 3(1), 1–20 (2014).

65. Pill K, Hofmann S, Redl H, Holnthoner W. Vascularization mediated by mesenchymal stem cells from bone marrow and adipose tissue: a comparison. *Cell Regen (Lond)*. 4, 8 (2015).

66. Pedersen TO, Blois AL, Xing Z, Xue Y, Sun Y, Finne-Wistrand A, *et al.* Endothelial microvascular networks affect gene-expression profiles and osteogenic potential of tissue-engineered constructs. *Stem Cell Res Ther*. 4(3), 52-62 (2013).

67. Grellier M, Bordenave L, Amédée J. Cell-to-cell communication between osteogenic and endothelial lineages: implications for tissue engineering. *Trends Biotechnol.* 27(10), 562–571 (2009).

68. Brennan M, Davaine J-M, Layrolle P. Pre-vascularization of bone tissue-engineered constructs. *Stem Cell Res Ther*. 4(4), 96-99 (2013).

69. Clarkin CE, Emery RJ, Pitsillides AA, Wheeler-Jones CPD. Evaluation of VEGF-mediated signaling in primary human cells reveals a paracrine action for VEGF in osteoblast-

mediated crosstalk to endothelial cells. *J. Cell. Physiol.* 214(2), 537–544 (2008).

70. Athanasopoulos AN, Schneider D, Keiper T, Pendurthi UR, Liegibel UM, Sommer UM, *et al.* Vascular endothelial growth factor (VEGF)-induced up-regulation of CCN1 in osteoblasts mediates proangiogenic activities in endothelial cells and promotes fracture healing. *J. Biol. Chem.* 282(37), 26746–26753 (2007).

71. Lee RJ, Springer ML, Blanco-Bose WE, Shaw R, Ursell PC, Blau HM. VEGF gene delivery to myocardium: deleterious effects of unregulated expression. *Circulation*. 102(8), 898–901 (2000).

Summary points

- Design of a tridimensional active material for regenerative medicine

- We have developed here a VEGF active tridimensional polymeric material containing a homogeneous repartition of nanoparticles with a cell-contact dependant active release.

- The entrapment of VEGF nanoparticles in nanoreservoirs is able to have an effect on endothelial cells behaviour.

-This material offers an active and sustained release of angiogenic growth factor for regenerative medicine application.

- Double strategy to promote endothelial cell reorganization for regenerative medicine

-The adhesion of mesenchymal cells onto the VEGF nanoreservoirs material enables to potentiate its own capacity to act on endothelial cells behaviour.

-The use of mesenchymal cells and VEGF nanoparticles can increase the endothelial cells reorganisation

- This double strategy can promote angiogenesis after implantation and can potentiate the current implants used in regenerative medicine.

Reference annotations

** Mendoza-Palomares C, Ferrand A, Facca S, Fioretti F, Ladam G, Kuchler-Bopp S, *et al.* Smart hybrid materials equipped by nanoreservoirs of therapeutics. *ACS Nano*. 6(1), 483–490 (2012).

We describe in this paper the methodology and the interest of the nanoreservoirs technology built up on nanofibrous materials.

** Eap S, Keller L, Schiavi J, Huck O, Jacomine L, Fioretti F, *et al.* A living thick nanofibrous implant bifunctionalized with active growth factor and stem cells for bone regeneration. *Int J Nanomedicine*. 10, 1061–1075 (2015).

This paper describes the advantage of a tridimensional nanofibrous PCL material with living stem cells and growth factor for bone regenerative medicine.

**Eap S, Ferrand A, Palomares CM, Hébraud A, Stoltz J-F, Mainard D, *et al.* Electrospun nanofibrous 3D scaffold for bone tissue engineering. *Biomed Mater Eng*. 22(1–3), 137–141 (2012).

We describe in this paper the methodology and efficient parameters to assess the design of a thick tridimensional nanofibrous material by electrospinning process.

** Grellier M, Bordenave L, Amédée J. Cell-to-cell communication between osteogenic and endothelial lineages: implications for tissue engineering. *Trends Biotechnol.* 27(10), 562–571 (2009).

This paper describe the importance of reciprocal interactions between osteoblastic and endothelial cells, occurring during bone development and its importance to generate a vascularized construct for bone regeneration.