TISSUE ENGINEERING: Part A
Volume 19, Numbers 7 and 8, 2013

DOI: 10.1089/ten.tea.2010.0741

Vascular Endothelial Growth Factor and Fibroblast Growth Factor-2 Incorporation in Starch-Based Bone Tissue-Engineered Constructs Promote the *In Vivo* Expression of Neovascularization Mediators

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The ideal bone tissue-engineered (TE) construct remains to be found, although daily discoveries significantly contribute to improvements in the field and certainly have valuable long-term outcomes. In this work, different TE elements, aiming at bone TE applications, were assembled and its effect on the expression of several vascularization/angiogenesis mediators analyzed. Starch/polycaprolactone (SPCL) scaffolds, obtained by two different methodologies, were combined with fibrin sealant (Baxter®), human adipose-derived stem cells (hASCs), and growth factors (vascular endothelial growth factor [VEGF] or fibroblast growth factor-2 [FGF-2]), and implanted in vascular endothelial growth factor receptor-2 (*VEGFR2*)-*luc* transgenic mice. The expression of VEGFR2 along the implantation of the designed constructs was followed using a luminescence device (Xenogen®) and after 2 weeks, the explants were retrieved to perform histological analysis and reverse transcriptase–polymerase chain reaction for vascularization (VEGF and VEGFR1) and inflammatory (tumor necrosis factor-alpha, interleukin-4, and interferon-gamma) markers. It was showed that SPCL scaffolds obtained by wet spinning and by fiber bonding constitute an adequate support for hASCs. The assembled TE constructs composed by fibrin sealant, hASCs, VEGF, and FGF-2 induce only a mild inflammatory reaction after 2 weeks of implantation. Additionally, the release of VEGF and FGF-2 from the constructs enhanced the expression of VEGFR2 and other important mediators in neovascularization (VEGF and VEGFR1). These results indicate the potential of VEGF or FGF-2 within a bone TE construct composed by wet-spun SPCL, fibrin sealant, and hASCs in promoting the vascularization of newly formed tissue.

Introduction

The field of tissue engineering has achieved several successes within the recent past.[1,2] Different biomaterials, cells, growth factors, and stimulation conditions, as well as numerous combinations among them have been proposed by several research groups as potential routes to assemble the perfect bone tissue-engineered (TE) construct.[3–8] Despite this, in bone tissue engineering, vascularization remains a fairly large concern, not yet perfectly addressed. Besides the well-known fact that the bone is extremely dependent on a vascular network, which provides nutrients, minerals, and oxygen essential for cell survival,[9] angiogenesis was shown to play a key role, not only in bone growth,[9] but also in bone healing[10] and consequently in bone tissue regeneration. Numerous strategies[4,11–13] have therefore, emerged as a need to achieve the vascularization of bone-engineered constructs within a reasonable time, which contributes to attain functional tissue substitutes.

Noteworthy, works have been showing that endothelial cells, either in single culture or cocultured with primary osteoblasts or stem cells, in 3D structures lead to the formation of vascular-like structures *in vitro*[14,15] and improves vascularization *in vivo*.[11,16–18] Nevertheless, despite the developments in cell isolation and culture technologies, the variability of cell sources, as well as in culture conditions

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among the different studies is still a major issue and might jeopardize some of the conclusions drawn.

A valuable alternative to tackle the vascularization of bone TE constructs relies on the incorporation in the construct of important mediators, such as vascular endothelial growth factor (VEGF)[5,17–20] and fibroblast growth factor (FGF),[21,22] that can be controlled and released from the scaffolding material. In fact, the incorporation of VEGF and subsequent release has been achieved with microspheres,[20] hydrogels,[21,22] and 3D scaffolds.[17] For all these systems, VEGF release showed to promote *in vitro* and *in vivo* vascularization.[17,20] In the same context, FGF-2 showed increased *in vivo* neovascularization after being released from a chitosan/heparinoid hydrogel after subcutaneous implantation.[21,22] A combined approach, VEGF plus FGF incorporated into chitosan hydrogels was also attempted with confirmed release of both growth factors within the first day and with a significant stimulation of human umbilical vein endothelial cells.[23] An uncertain issue is, however, the degradation rate of the carriers and subsequently the release profile and doses of the loaded factors which, if not controlled, might induce an unexpected reaction.[23] Considering this, a different cell-based strategy aiming at targeting not only the vascularization but also the regeneration of a vascularized tissue as bone, has been also proposed.[6,11] The differentiation potential of several mesenchymal stem cells has been taken into consideration when cell-seeded matrices are transplanted into several *in vivo* regeneration models expecting that the undifferentiated cells either undergo a commitment into the lineages of interest[4,18] or significantly contribute to signaling host progenitor cells.[16]

In this work, it was hypothesized that the assembly of a complex TE construct, comprehending a well-studied starch-based scaffold (starch/polycaprolactone [SPCL])[3,8,24] and fibrin glue,[25] human adipose-derived stem cells (hASCs)[26] and key growth factors (VEGF and FGF-2),[27,28] would induce the vascularization of the construct. Taking into consideration the features of the mentioned cells, growth factors, and materials used in the last years of investigation in tissue engineering, it was expected to achieve a deeper knowledge on the effect of each one of those elements on the expression of vascular endothelial growth factor receptor-2 (VEGFR2) and other molecular mediators of neovascularization. In future work, these scaffolds could be used for bone engineering purposes as this material has been shown to be suitable for that.[29,30]

Materials and Methods

Isolation of ASC

Liposuction material was collected from 3 female donors (43–48 years old) and was approved by the local ethics board. Isolation of ASC was performed as described elsewhere.[31] Briefly, adipose tissue was washed with a phosphate-buffered saline to remove most of the blood and tumescence solution. Subsequently, tissue was digested with collagenase (Biochrom) at 37°C for 1 h. To eliminate red blood cells, the isolated fraction was incubated with a erythrocyte lysis buffer for 10 min. Remaining cells were filtered through a 100 μm filter and cultured in expansion medium. During the first 1 to 2 passages, 2D cultures were subjected to an endothelial growth medium (EGM-2®; Lonza) which is already described to support rapid expansion and multipotency of ASCs.[32]

Transfection of the ASCs

To trace the hASCs after implantation, cells were prior transfected with a luciferase plasmid using Lipofectamine™2000 (Invitrogen).[33] Cell transfection was carried out according to the manufacturer's recommendations. Briefly, luciferase DNA (plasmid) and lipofectamine were separately diluted in 50 μL of Ham's F-12 cell culture medium (Sigma-Aldrich), without fetal calf serum (FCS), complemented with 1% L-glutamine, and antibiotics (basal medium), and gently mixed. The two solutions were mixed and incubated for 20 min at room temperature to allow the formation of lipo-complexes. After the incubation period, the mixture was added to the cells in culture and left for 4 h after which the medium was changed to a fresh basal medium. The cells were ready to be used $\sim$20 h after the transfection procedure.

The transfection efficiency is 40%–60%, as has been described in Wolbank *et al.*[33] On the cellular level the expression can be detected for 3 weeks, but the percentage of positive cells decreases over time *in vitro* independent of the chosen culture conditions. After 2 weeks, around 25% of the initial expression remains visible and positive cells remain detectable even after 3 weeks of culture.

Starch-based scaffolds production

Starch-based scaffolds were produced from a blend of starch with poly-ε-caprolactone (30:70%) (SPCL), by two different methodologies described elsewhere: wet-spinning (SPCL-WS)[8] and fiber-bonding (SPCL-FB).[3] Briefly, for the production of SPCL-WS scaffolds, the polymer was dissolved in chloroform at a concentration of 40% (w/v) to obtain a polymer solution with proper viscosity. The polymer solution was loaded into a syringe, placed in a syringe pump (World Precision Instruments), and the solution was subsequently extruded into a methanol coagulation solution. With this methodology, scaffolds composed of fibers of 100 μm diameter and with around 77% porosity are obtained.[8] The fiber mesh structure was formed during the processing by the random movement of the precipitation container. The formed scaffolds were then dried overnight at room temperature to allow any remaining solvents to evaporate. For the fabrication of the SPCL-FB scaffolds, fibermeshes previously obtained by a meltspinning methodology were placed in a glass mould and heated in an oven at 150°C. Immediately after removing the moulds from the oven, the fibers were slightly compressed by a Teflon cylinder and then cooled at -15°C.[3] This procedure creates scaffolds of around 75% diameter with fibers ranging from 120 to 500 μm diameter.[3] All samples were cut into discs of 5 mm diameter and $\sim$1 mm thickness and sterilized by a standard procedure with ethylene oxide.[34]

Assembly of the TE constructs

For the cell tracking experiments, the two types of SPCL scaffolds were seeded with the transfected hASCs in a concentration of 1.33×10^4 cells/scaffold in basal medium supplemented with 10% FCS and 1% antibiotics (penicillin/

streptomycin), and incubated for 24 h at 37°C and 5% CO_2 in a humidified environment. Using hASCs in mice is not contraindicated, as these cells have been shown to be immunoprivileged and have immunosuppressive properties.[31]

The assembling of the TE constructs to implant in the VEGFR2 transgenic mice was performed as follows: each type of SPCL scaffold was mixed with the 2.0 mL two-component FS Tisseel VH (Baxter AG), growth factors (VEGF and FGF-2), and hASCs. The sealer protein component (fibrinogen 75–115 mg/mL) was reconstituted with a fibrinolysis inhibitor solution (Aprotinin 3000 KIU/mL) and spiked either with VEGF (200 ng/mL) or FGF-2 (200 ng/mL). The thrombin component (500 IU/mL) was reconstituted with $CaCl_2$ (40-mmol/mL) and diluted to 4 IU/mL.[35] Scaffolds, cells (1.5×10^4 cells/scaffold/50 μL), and growth factors, which were added to the fibrinogen component, were then mixed with the thrombin component (1:1), in a total volume of 75 μL, at 37°C. The clot was allowed to form for 15 min, at 37°C and 5% CO_2 after which 300 μL of cell culture medium was added. Constructs were kept overnight at 37°C and 5% CO_2.

In vivo *implantation*

Nude mice. All the animal experiments were previously approved by the local ethics authorities. The *in vivo* fate of the *in vitro* transfected hASCs seeded onto SPCL scaffolds was followed in nude mice. Thirteen female Balb/c nu/nu nude mice, with an average weight of 21.6 ± 1.2 g were used; six animals to implant the SPCL-WS scaffolds, six animals to implant the SPCL-FB scaffolds, and one animal as control. All surgical procedures were performed under sterile conditions in a vertical laminar flow hood. Each animal was intraperitoneally (i.p.) anaesthetized with ketamine (60 mg/kg) and xylazine (7.5 mg/kg). Subsequently, the skin of the mice was disinfected with betaisodona and two lateral incisions of ~0.5 cm, containing the subcutis and panniculus carnosus, were performed in the back of the animals. Two caudal-latera- oriented pockets were created in each animal by blunt dissection, where the TE constructs with the transfected ASCs were inserted. After implantation, the panniculus carnosus and the skin of the animals were carefully sutured. The bioluminescence signal, from the *in vivo* luciferase activity that identifies the location of the transfected cells, was quantified (emitted photon counts per second) using the Live Image Software (Xenogen®). Specific areas for the signal detection, considering the original location of the implants and possible migration of the cells from the constructs, were predetermined (Fig. 1A); I and II correspond to the left and right implant sites, III corresponds to the dorsum of the animals, the most probable migration localization. Bioluminescence images were collected immediately after surgery and on days 1, 3, 6, 9, and 13. The luciferase activity was measured 15 min after luciferin subcutaneous injection and normalized to the respective areas for further graphical representation.

Transgenic mice. Thirty-eight FVB/N-Tg(VEGF-r2–luc) Xen mice (VEGFR2-LUC),[36] with an average weight of 33.8 ± 3.6 g were used to assess the effect of the addition of VEGF, FGF-2, hASCs, or fibrin sealant to the SPCL scaffolds for vascularization. These mice carry a transgene

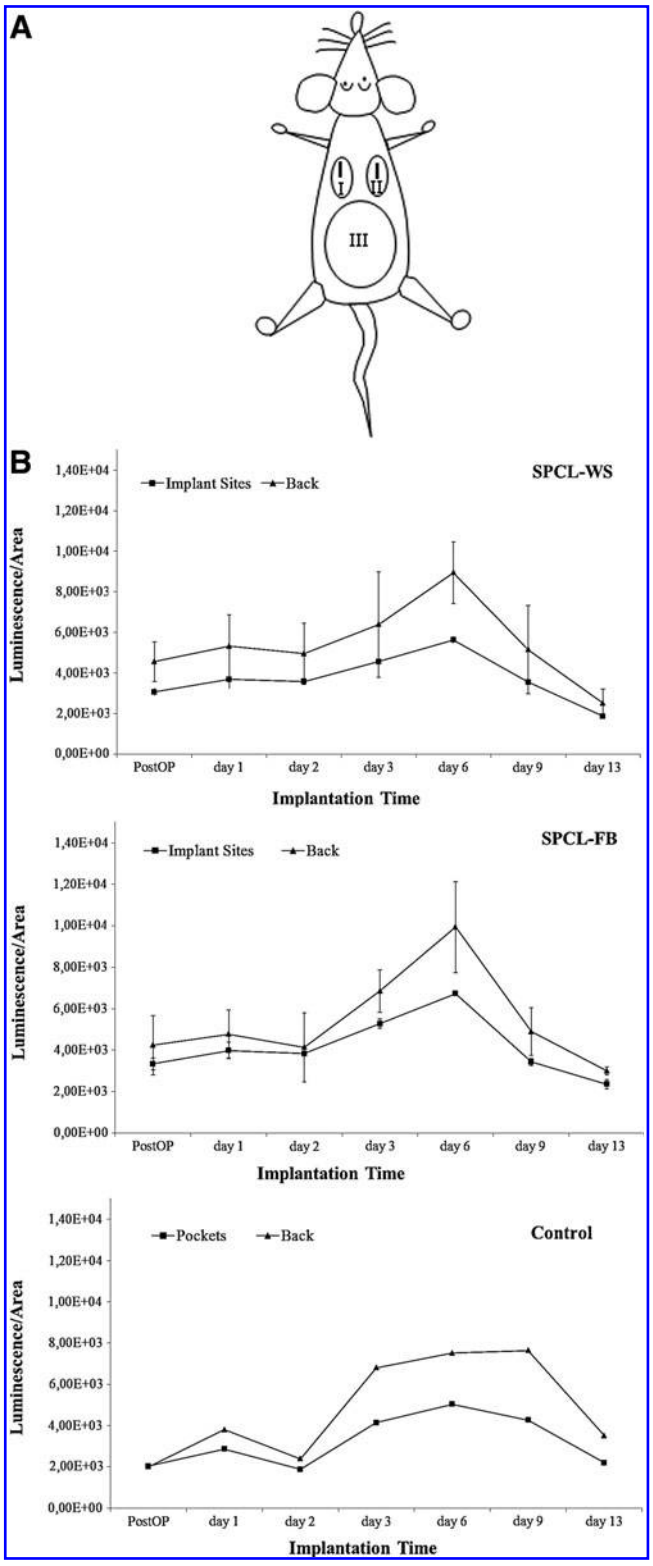

FIG. 1. **(A)** Schematic representation of a nude mouse with the areas considered for the capture of the luminescence signal emitted by the transfected cells seeded on the SPCL-based scaffolds: I and II correspond to the implantation sites; III corresponds to the dorsum (back) of the animals to where the cells eventually migrate. **(B)** Graphical representation of the luminescence signal detected in the different areas, on the SPCL-WS and SPCL-FB implanted nude mice. SPCL, starch/polycaprolactone; SPCL-WS, SPCL-wet spinning; SPCL-FB, SPCL-fiber-bonding.

Table 1. Distribution of the Test Groups for the *In Vivo* Implantation on the Transgenic FVB/N-Tg(VEGF-r2–luc)Xen Mice

Group	*Condition*	
a	Control–subcutaneous pockets without any implant	
b	SPCL-WS	SPCL-FB
c	SPCL-WS + FS	SPCL-FB + FS
d	SPCL-WS + FS + hASCs	SPCL-FB + FS + hASCs
e	SPCL-WS + FS + hASCs + VEGF	SPCL-FB + FS + hASCs + VEGF
f	SPCL-WS + FS + hASCs + FGF	SPCL-FB + FS + hASCs + FGF

SPCL, starch/polycaprolactone; SPCL-FB, SPCL-fiber-bonding; SPCL-WS, SPCL-wet spinning; hASCs, human adipose-derived stem cells; VEGF, vascular endothelial growth factor; FGF, fibroblast growth factor.

that contains a 4.5 kb murine VEGFR2 promoter fragment that drives the expression of a firefly luciferase reporter protein.[36]

Six test groups were established per type of scaffold (Table 1): (a) untreated control to measure endogenous expression of VEFGR2 due to surgical procedure; (b) scaffold group to measure expression of VEFGR2 due to scaffold implantation (SPCL-WS and SPCL-FB); (c) scaffold plus FS to measure the expression of VEGFR2 due to the use of FS (SPCL-WS + FS and SPCL-FB + FS); (d) scaffold plus FS and hASCs group, to measure the expression of VEGFR2 due to the presence of hASCs (SPCL-WS + FS + hASCs and SPCL-FB + FS + hASCs); (e) scaffold plus FS, hASCs and VEGF (200 ng/mL) to measure the expression of VEGFR2 induced by the VEGF delivery (SPCL-WS + FS + hASCs + VEGF and SPCL-FB + FS + hASCs + VEGF); and (f) scaffold plus FS, ASCs and FGF-2 (200 ng/mL), to measure the expression of VEGFR2 induced by the FGF-2 delivery (SPCL-WS + FS + hASCs + FGF-2 and SPCL-FB + FS + hASCs + FGF-2).

Each animal was anaesthetized using 3% isoflurane for induction and maintaining with an i.p. injection of ketamine (60 mg/kg) and xylazine (7.5 mg/kg). Mice were injected subcutaneously with luciferin (150 mg/kg) and imaged with the *in vivo* imaging system (VivoVisions IVISs; Xenogen) to acquire the background image signal corresponding to the presurgical activity, set to 100%. Specific areas for the bioluminescence detection were established (Fig. 2A); I corresponds to the incision area; II and III correspond to the left and right implant sites (pockets). The signal detected at the incision site correlates with the expression of the *VEGFR2* gene with the ongoing inflammatory process as the incision heals. Each animal's dorsum was then shaved and disinfected, and a 1 cm incision at the caudal aspect of the neck was made. For the subcutaneous implantation, a caudal lateral access to each flank was bluntly subcutaneously created through this incision, forming two pockets per animal. Into each pocket, the construct was inserted accordingly to the different test groups. Subsequent measurements in the predetermined areas were referenced to the presurgical baseline and obtained immediately after surgery and on days 3, 6, 9, and 13 after implantation, as well as 15 min after luciferin injection.

Ex vivo *analysis*

At the end of the observation (2 weeks), each animal was i.p. anaesthetized and subsequently sacrificed with an intracardial overdose of ketamine (60 mg/kg) and xylazine (7.5 mg/kg). The scaffolds and surrounding tissue were explanted and, half of the sample was fixed in 3.7% formalin for histological analysis, and the other half was snap-frozen for molecular biology evaluation. Histology was performed according to existing standard protocols for haematoxylin and eosin staining. The angiogenic index of each lesion was determined based on microvessel count (MVC), which is the mean number of microvessels in five areas of highest vascular density at 200× magnification, as described by Maeda *et al.*[37]

Samples for vWF antibody were pretreated with proteinase K (Dako) for 10 min. Then all samples ($n = 6$ histologies were analysed per group) were treated with peroxide (1.5% H_2O_2 in Tris-buffered saline) for 30 min at room temperature to deactivate endogenous peroxidase activity. After rinsing with a TRIS-buffered saline for 10 min, sections were incubated with 2.5% horse serum (Vector Laboratories). Thereafter, tissue sections were incubated with the primary vWF antibody (polyclonal rabbit anti-human; Dako) or primary CD31 antibody (rabbit anti-CD31; Santa Cruz) over night at 4°C and then washed with the TRIS-buffered saline. ImmPRESS anti-rabbit micropolymers (Vector Laboratories) were then added and incubated for 30 min at room temperature. After washing, staining was performed by diaminobenzidine (Sigma Aldrich) for 6 min. The slides were then counterstained with hematoxylin, dehydrated, and mounted permanently with Roti-Histokit II (Carl Roth).

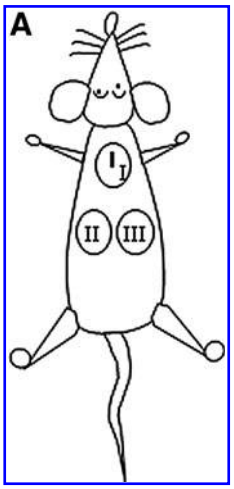

FIG. 2. (A) Schematic representation of a transgenic VEGFR2-LUC mouse with the areas considered for the capture of the luminescence signal emitted by the transfected cells seeded on the SPCL-based TE constructs: I corresponds to incision area; II and III correspond to the left and right implant sites (pockets). **(B)** Graphical representation of the luminescence signal detected in the different areas, on the SPCL-WS assembled constructs. **(C)** Graphical representation of the luminescence signal detected in the different areas, on the SPCL-FB assembled constructs. TE, tissue-engineered; VEGFR2, vascular endothelial growth factor receptor.

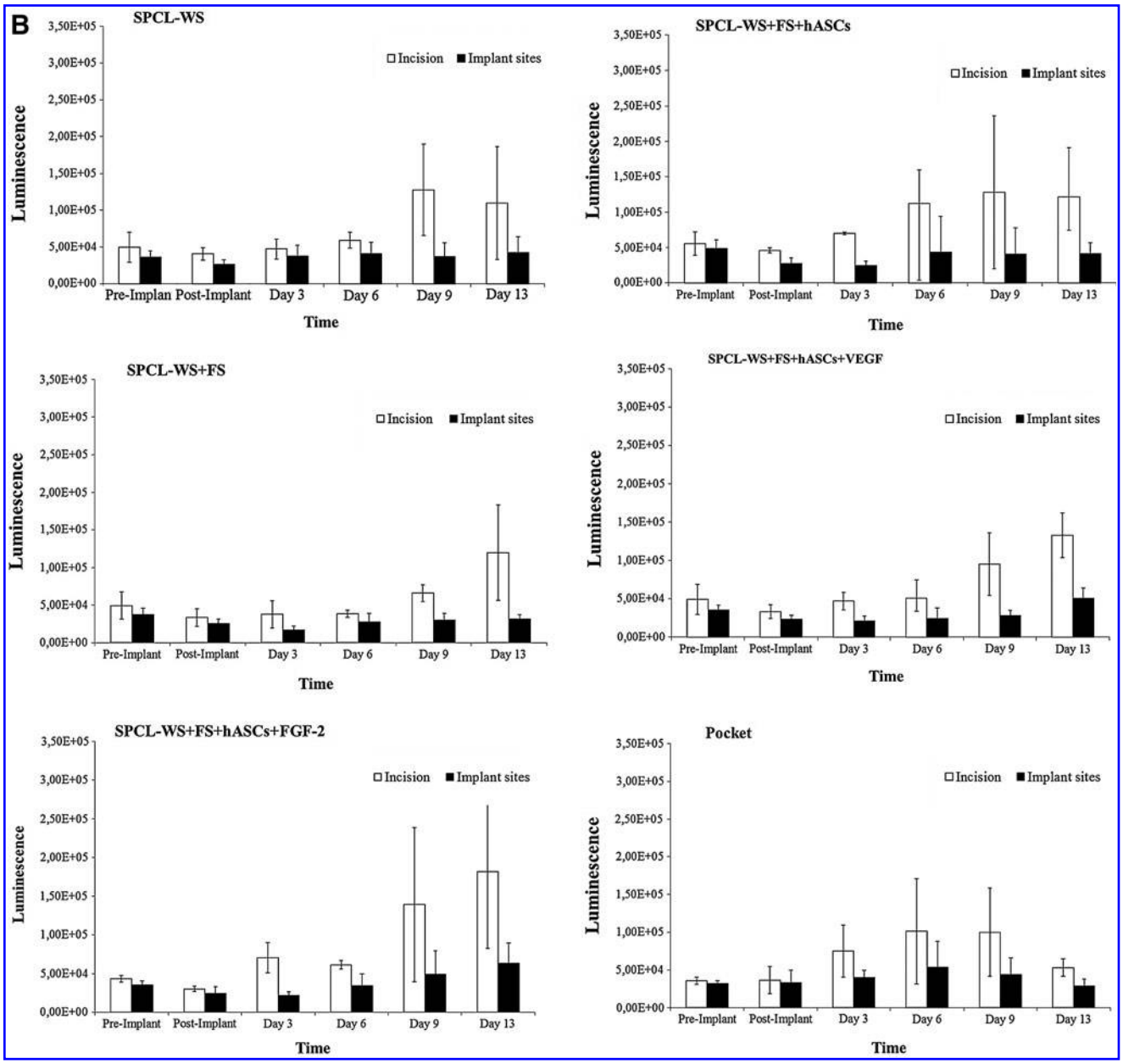

FIG. 2. (Continued).

Molecular biology was evaluated by reverse transcriptase–polymerase chain reaction (RT-PCR) to detect the expression of vascularization and inflammation (Table 2).

Statistical analysis

Mean values and standard deviations are reported for the luminescence signal measurements[38] and represented graphically. Data were analyzed by a single factor analysis of variance test and the significance value was set at $p < 0.05$.

Statistical analysis was performed among the different test groups for the determined time points and, along the total implantation time period, between two followed time points in each tested condition using multiple analysis of variance after having tested for normal distribution. To correct for multiple testing error, a Bonferroni correction was applied. Additionally, the postimplantation time point was compared with the end time point of observation.

Results

In vivo *ASCs tracking*

The luminescence emitted by the transfected cells was detected with the *in vivo* imaging system (VivoVisions IVISs; Xenogen), acquired with the Live Image Software (Xenogen) and recorded as emitted photon counts per second.

Before the *in vivo* implantation of the TE constructs, a SPCL scaffold seeded with the transfected hASC for 24 h, was placed into the dark chamber of the Xenogen equipment to confirm the presence of luminescence-emitting cells on the scaffolds (data not shown).

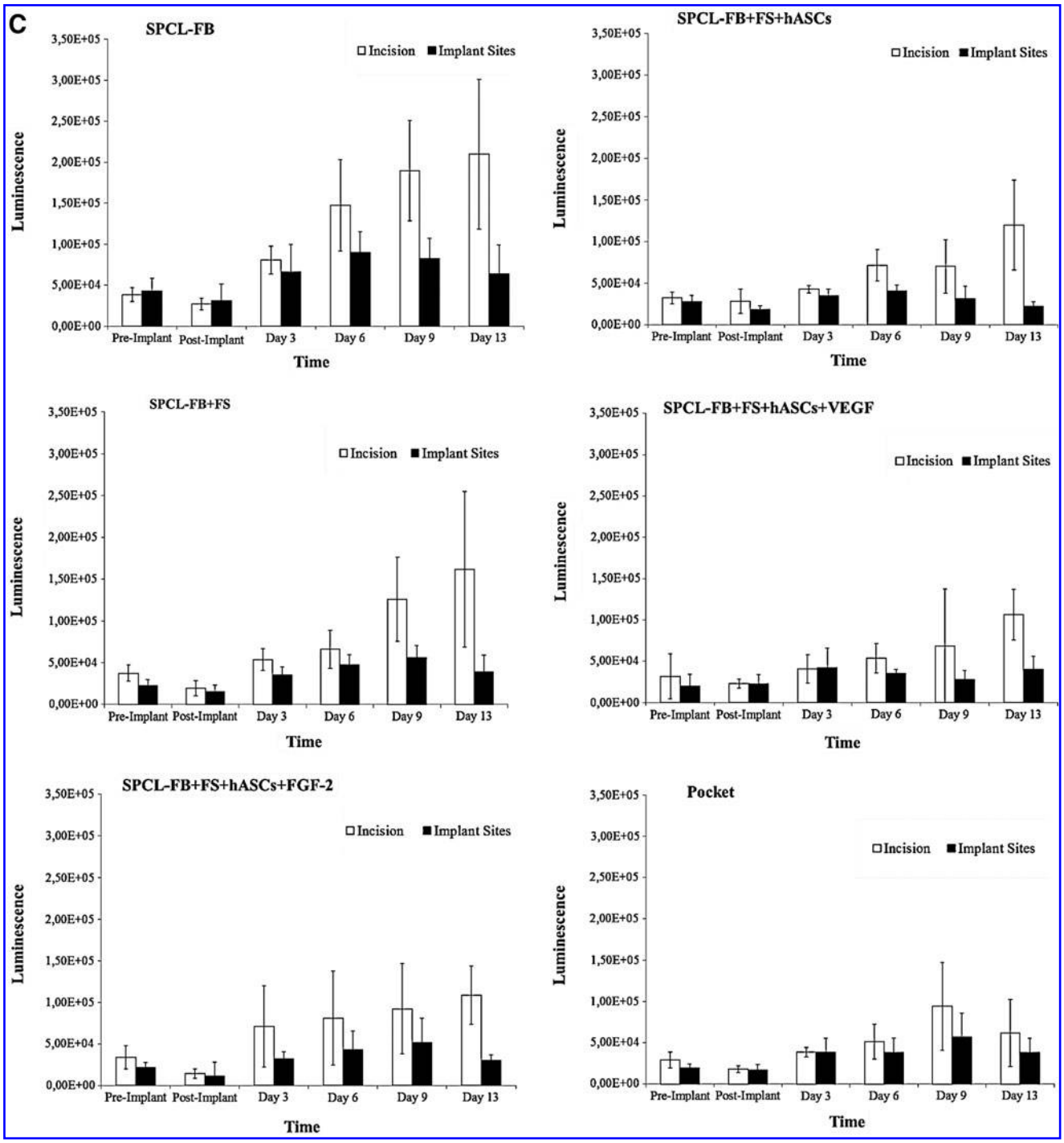

FIG. 2. (Continued).

The signal emitted from the transfected cells was similar in both types of SPCL scaffolds, and the peak of emitted signal was detected on day 6 after implantation (Fig. 1B). For both SCPL-WS and for SPCL-FB scaffolds, the luminescence signal starts to increase from day 2 onward, reaching the maximum value at day 6 and decreases from this day until the end point of the experiment, 13 days (Fig. 1B). Nevertheless, for the control condition, to which two pockets were created without any implantation, the signal was similar to the luminescence emitted by the transfected hASCs seeded on the SPCL-based scaffolds (Fig. 1). The signal at the incision sites was therefore, not taken into consideration for migration purposes.

The transfected cells seemed to migrate from the scaffolds very early, as can be observed by the significantly high signal ($p < 0.05$) detected in the dorsum of the mice implanted with the SPCL-WS and with the SPCL-FB constructs (Fig. 1B). However, the kinetics of the emitted signal, during the

implantation time, were similar for the implant sites and the dorsum of the animals (Fig. 1B).

In vivo *induced neovascularization potential*

VEGFR2 expression. After the assembly of the TE constructs combining the starch-based scaffolds, hASCs, fibrin sealant, and growth factors (VEGF or FGF-2), the constructs were subcutaneously implanted in the back of transgenic VEGFR2-LUC mice. The emitted luminescence was detected with the *in vivo* imaging system (VivoVisions IVISs; Xenogen) and acquired with the Live Image Software (Xenogen), in the predetermined areas of incision and implant sites (Fig. 2A). The luminescence signal identified the expression of the murine *VEGFR2* gene 15 min after the subcutaneous injection of luciferin.

The overall analysis of the luminescence results showed that for all the tested conditions the luminescence signal, at the incision area was higher than at the implantation sites (Fig. 2B, C). Nevertheless, the signal at the incisions was considered to be related to the expected inflammatory process resulting from the surgical procedure. Therefore, the effect of the different constructs on the expression of VEGFR2 was analyzed based on the signal measured at the implantation sites.

The expression of VEGFR2 at the SPCL-WS implantation sites was comparable ($p > 0.05$) to the control (pocket) for all time points except at the preimplantation time (Fig. 2B). At this time point, the animals randomly selected for the implantation of SPCL-WS scaffolds showed a higher VEGFR2 expression ($p < 0.05$) in comparison to the animals selected for controls (pockets). Conversely, after implantation, the two groups of animals showed a similar VEGFR2 expression at the implant sites for all time points. The luminescence signal did not vary along the implantation period.

When fibrin sealant was added to the SPCL-WS scaffolds, a different profile of VEGFR2 expression was observed in comparison to the scaffold alone (Fig. 2B). At day 3 of SPCL-WS + FS implantation, the expression of VEGFR2 was significantly lower ($p < 0.05$), increasing from that day onward to comparable values of the SPCL-WS. Moreover, the VEGFR2 expression for the SPCL-WS + FS decreased ($p < 0.05$) until day 3 and increased from that point reaching a significantly higher ($p < 0.05$) value at day 6 that was comparable to those detected until day 13 ($p > 0.05$).

Higher levels of VEGFR2 expression were detected in the animals where SPCL-FB scaffolds were implanted in comparison to the animals with SPCL-WS before implantation. The VEGFR2 expression at the SPCL-FB implantation sites was higher ($p < 0.05$) than the control for all the time points except postimplantation (Fig. 2C). Nonetheless, a significant increase ($p < 0.05$) of the VEGFR2 expression at the SPCL-FB implantation site was observed from the time point postimplantation up to day 3. The slight diminished expression from day 6 onward was not significant ($p > 0.05$).

The addition of fibrin sealant to the SPCL-FB scaffolds induced a lower VEGFR2 expression ($p < 0.05$) at days 6 and 9 in comparison to SPCL-FB. However, the preimplant expression of VEGFR2 was already lower in the animals for SPCL-FB + FS scaffolds implantation in comparison to the animals for SPCL-FB implantation ($p < 0.05$) (Fig. 2C). Despite this, a significant increased ($p < 0.05$) expression of VEGFR2 was observed from the postimplantation time point to day 3 and 13 of SPCL-FB + FS implantation.

When hASCs were added to the SPCL-WS + FS construct, the luminescence pattern detected at the implantation sites did not change in comparison to the constructs composed of SPCL-WS and fibrin sealant. In addition, along the time of SPCL-WS + FS + hASCs implantation the VEGFR2 expression at the implant sites was similar, significantly decreasing ($p < 0.05$) from pre- to postimplantation (Fig. 2C).

Similarly to what was observed for the SPCL-WS + FS, the addition of hASCs to the SPCL-FB + FS construct did not induced significant differences in the detected luminescence at the implantation sites (Fig. 2C). Along the implantation period, a statistically significant increase of the detected signal was observed until day 3 ($p < 0.05$) (Fig. 2C).

The addition of either VEGF or FGF-2 to the SPCL-WS + FS + hASCs constructs did not change the pattern of VEGFR2 expression as compared to the construct with fibrin sealant and hASCs (Fig. 2B). The expression of VEGFR2 at the SPCL-WS + FS + hASCs + VEGF implantation site significantly increased ($p < 0.05$) from day 9 to 13 reaching a significantly higher value ($p < 0.05$) in comparison to the postimplantation time point. Similar results were obtained along the time of implantation of the SPCL-WS + FS + hASCs + FGF-2 constructs, although the increasing of the luminescence signal was not significant from day 9 to 13 (Fig. 2B).

Table 2. Forward and Reverse Sequences of the Genes Detected by Reverse Transcriptase–Polymerase Chain Reaction

Function	*Gene*	*Sequences*	*Tm (°C)*	*bp*
Vascularization	*VEGF-α*	Forward: CCGAAACCATGAACTTTCT	55.19	
		Reverse: CGTTCGTTTAACTCAAGCTG	56.31	604
	VEGFR1	Forward: GAGGGATAACAGGCAATTC	54.59	
		Reverse: CCCAGCAAGATCGTATAGTC	54.91	960
Inflammation	*IL-4*	Forward: TCATCCTGCTCTTCTTTCTC	54.67	
		Reverse: GATGTGGACTTGGACTCATT	54.82	325
	IFN-γ	Forward: CTACCTTCTTCAGCAACAGC	55.36	
		Reverse: TGTAGACATCTCCTCCCATC	54.92	568
	TNF-α	Forward: GTCTCAGCCTCTTCTCATTC	54.03	
		Reverse: CAGAGTAAAGGGGTCAGAG	54.57	654

VEGFR1, vascular endothelial growth factor receptor-1; TNF-α, tumor necrosis factor-alpha; IL-4, interleukin 4; IFN-γ, interferon-gamma.

In what concerns the incorporation of growth factors (VEGF and FGF-2) into the SPCL-FB + FS + hASCs constructs, like for the SPCL-WS + FS + hASCs constructs, the expression of VEGFR2 did not change, except for the lower signal detected in the animals implanted with the SPCL-FB + FS + hASCs + FGF-2 postimplantation. However, a significant increase ($p < 0.05$) was observed between the implantation day and the end of observation, day 13, for SPCL-FB + FS + hASCs constructs with both growth factors (Fig. 2C).

The addition of the growth factors (VEGF and FGF-2) to the SPCL-WS-based constructs induced a similar profile of VEGFR2 expression in both constructs. From the implantation day until day 3, a decrease on the luminescence signal was observed, after a significant increase ($p < 0.05$) until the end point of the experiment (day 13). The overall data demonstrated that the expression of VEGFR2 was promoted after VEGF or FGF-2 incorporation on SPLC-WS-based constructs.

Inflammation and vascularization. At the end of the experiment (2 weeks), the subcutaneously implanted TE constructs were explanted, along with the surrounding tissue, for histological and molecular biology analysis.

In terms of inflammatory reaction to the implantation of the TE constructs, the histological analysis allowed to observe that for all tested conditions neither the addition of hASCs or fibrin sealant from human origin, nor the release of VEGF or FGF-2 from the implanted constructs to the implantation site, elicited an exuberant inflammatory or rejection response from the transgenic VEGFR2-luc mice (Fig. 3). The observed inflammatory reaction can be considered of moderate intensity and characterized by the presence of some polymorphonuclear neutrophils (PMNs), mononuclear cells (lymphocytes and macrophages), and some foreign body giant cells (Fig. 3). These observations were complemented with the RT-PCR analysis that confirmed the expression of interleukin (IL)-4 and interferon-gamma (IFN-γ), two inflammatory cytokines, for all the tested conditions except when only the SPCL scaffolds were implanted (Fig. 4). In addition, tumor necrosis factor-alpha (TNF-α) expression was detected in all the test groups except the controls in which IL-4 and IFN-γ were also not detected. In fact, in the control animals a residual inflammatory infiltrate, comprising PMNs and mononuclear cells was found as a reaction to the created pockets (Fig. 3).

Concerning the vascularization specific markers, all the tested conditions, including the controls, expressed VEGF. Contrarily, VEGFR1 expression was only detected in the tissues where the SPCL-WS + FS + hASCs + FGF-2, the SPCL-FB + FS + hASCs + VEGF, and the SPCL-FB + FS + hASCs constructs were implanted (Fig. 4). In the groups, SPCL-WS-FS-ASC-VEGF and SPCL-WS-FS-ASC-FGF the MVC was significantly higher than in the pocket (Table 3). These results were confirmed using CD31 and von Willebrand Factor staining. Many endothelial cells, as characterized by these two markers, were seen in SPCL-WS-FS-ASC-VEGF and SPCL-WS-FS-ASC-FGF. These built mature vessels and also microvessels could be identified (Figs. 5 and Fig. 6).

Discussion

Tissue engineering has been facing an impairment on the development of suitable constructs, which is to promote the concomitant ingrowth of new blood vessels as the tissue is healing and remodeling. Considerable steps have been taken toward new advances by coculturing endothelial cells and osteoblasts[11,14,39] or stem cells within 3D matrices[16] or by controlled release relevant growth factors from those structures[21,22] that can simultaneously support cell growth and tissue ingrowth.[4,40] Extensively studied starch-based scaffolds have shown great potential for bone tissue engineering, not only demonstrated by their capacity to support osteogenic differentiation[41] and further bone matrix deposition/mineralization,[12] but also to bear the formation of vascular-like structures both *in vitro*[14,42,43] and *in vivo*.[11] Based on these, the main aim of this work was to assemble an improved bone TE construct, composed by SPCL-based scaffolds, angiogenic growth factors (VEGF and FGF-2), and hASCs, and to assess the effect of each element over the expression of several neovascularization-related markers.

A major concern with TE constructs is the fate and consequent role of the cells composing the construct after transplantation. Cell transfection by lipofection has been shown to be a useful tool to trace cells either in an *in vitro* system[33,44,45] or in animal models.[46,47] An immunosuppressed mouse model was used to implant the two types of SPCL scaffolds seeded with transfected hASCs to conclude about the effect of the processing methodology over the cell fate within the host. It was possible to conclude that the two methodologies used to process the SPCL scaffolds, wet spinning and fiber bonding, did not induce differences, first on the adhesion of the seeded ASCs, and then on their migration after implantation. It was shown that the kinetic profile of the emitted luminescence by the transfected cells seeded on the scaffolds was similar to the SPCL-WS and for the SPCL-FB scaffolds. As the implants were introduced into the subcutaneous pockets of the nude mice, the transfected cells started to migrate from the scaffolds and moved to the dorsum of the animals. This was concluded from the higher luminescence signal observed in the dorsum area in comparison to the implantation areas. This is in accordance with our previous knowledge, showing the release of ASCs from a substrate (unpublished data). The scaffold can thus, be used as a means to implant the cells at the site of interest. The migrating cells can give cues to the surrounding tissue for vascularization and bone formation. Thus, in a clinical setting this may accelerate bone healing by increasing both vascularization and possibly osteogenesis. Nevertheless, the presence of the scaffold at the implant site may interfere with the luminescence signal detected by the software. Therefore, it may be speculated that the luminescence signal detected at the implantation sites was altered by the presence of the SPCL scaffolds. The increased signal observed from day 2 up to day 6 after implantation, and the similar kinetics profile for the implantation sites and for the dorsum of the animals, are indicative of cell proliferation in both sites. The decrease on the emitted signal observed from day 6 onward indicates that the cells are losing the plasmid, which is also in accordance with previous indications (unpublished data).

A VEGFR2-luc transgenic mouse model previously established,[36] using the murine VEGFR2 promoter to direct the expression of the luciferase reporter, was used to assess the expression of VEGFR2 under the studied conditions. It is well reported that VEGFR2 mediates most of the mitogenic, cell survival, and vascular permeability effects of

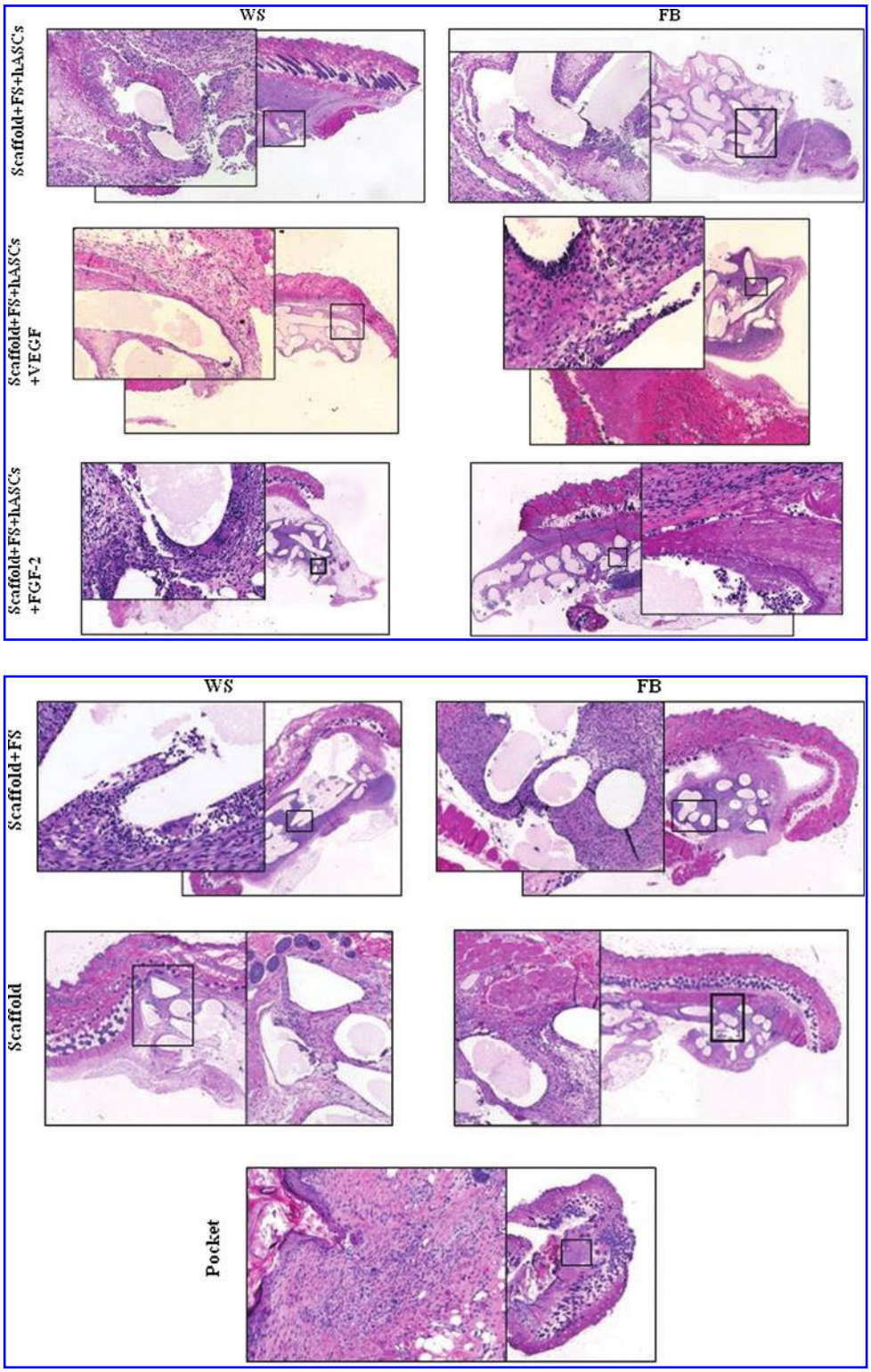

FIG. 3. Micrographs obtained from the TE constructs and surrounding tissue explanted 13 days after implantation in the transgenic VEGFR2-LUC mice. SPCL-WS + FS + ASCs, SPCL-FB + FS + ASCs, SPCL-FB + FS + ASCs + VEGF, SPCL-FB + FS + ASCs + FGF-2 and SPCL-FB + FS with lower magnification of 12.5× and higher magnification of 200×; SPCL-WS + FS + ASCs + VEGF, SPCL-WS + FS + ASCs + FGF-2, SPCL-WS + FS, SPCL-WS, SPCL-FB and Pocket (control) with lower magnification of 12.5× and higher magnification of 100×. ASC, adipose-derived stem cells; FGF, fibroblast growth factor. Color images available online at www.liebertpub.com/tea

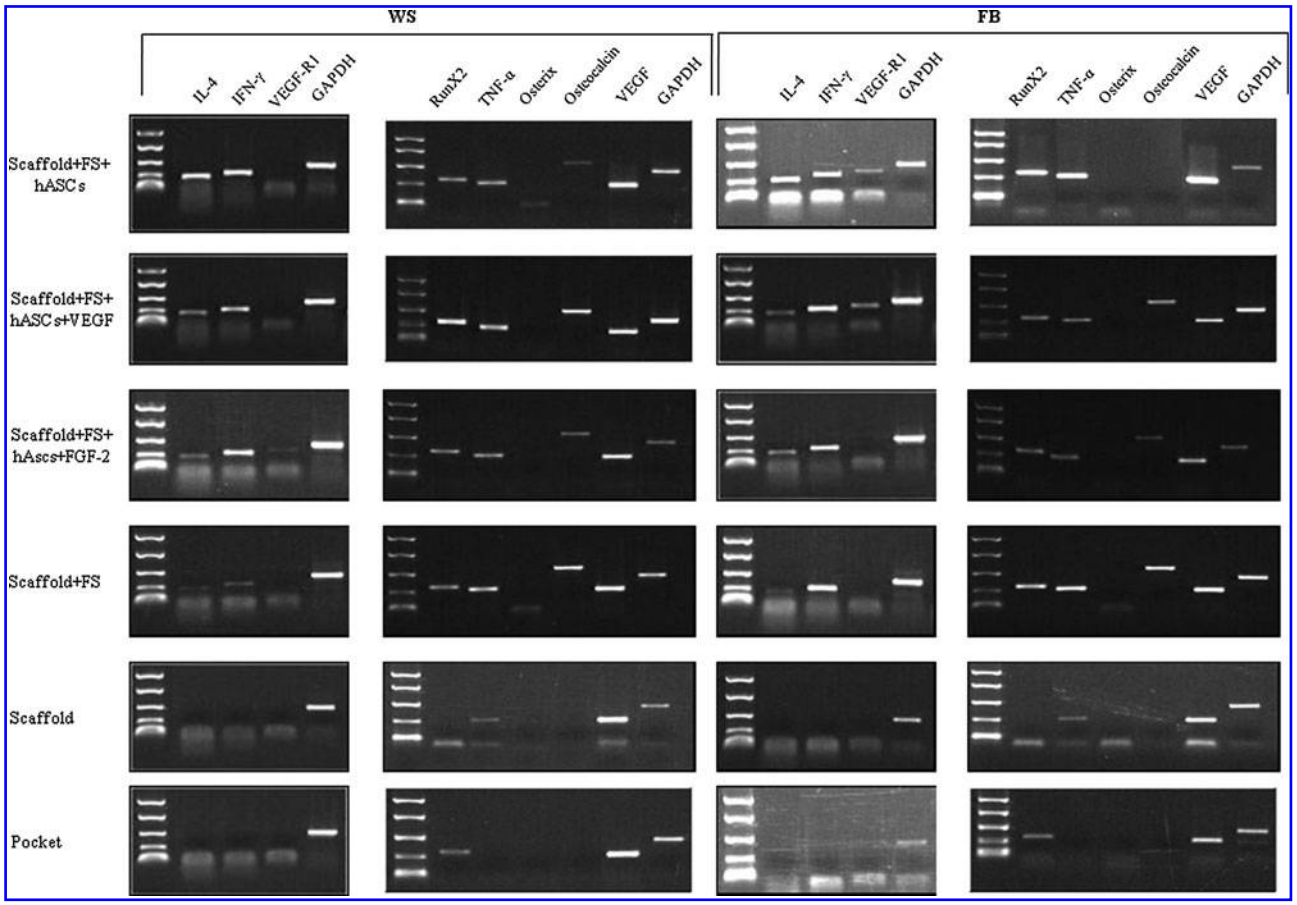

FIG. 4. Electrophoresis gels of the PCR results showing the expression of inflammation, neovascularization, and osteogenic potential specific genes. The gene expression was assessed at the end time point of the experiment (2 weeks), on the implanted TE construct and respective surrounding tissue. PCR, polymerase chain reaction.

VEGF.[48,49] Moreover, as VEGFR2 plays an important role in many aspects of blood vessel growth, an *in vivo* monitoring of the VEGFR2 gene expression, with noninvasive techniques was found useful to achieve its real time function in angiogenesis.[50]

While SPCL scaffolds support the formation of vascular-like structures,[11,42,43] and seeded with bone marrow mesenchymal cells have clearly proved to be suitable for bone TE applications,[7,12,41] the potential of hASCs together with these materials was still to be addressed. The influence of hASCs seeded onto the SPCL scaffolds, as well as the incorporation and subsequent delivery of VEGF and FGF-2, in vascularization/angiogenesis was addressed by monitoring the VEGFR2 expression using the VEGFR2-luc transgenic mouse model and by assessing the induction of healing mediators. As luciferase measurement is a very sensitive method a large spread in an *in vivo* system can be expected. Moreover, it is an individual measurement on each mouse and the position of the mouse may also influence the number of photons measured. Furthermore, the constructs are slowly declining and disappearing from the cells as described in the materials and methods sections. However, thorough statistical analysis was performed and we feel that the difference is important. Proinflammatory cytokines, such as IL-6, IL-8, and TNF-α, and various growth factors, like VEGF, are necessary for healing.[51] Additionally, VEGF is also determinant for progenitor endothelial cells recruitment and for promoting angiogenesis.[52,53] Thereby, it would be important for clinical application as angiogenesis is increased and thus, may ensure survival of transplanted cells and of the whole graft.

Table 3. Microvessel Count in Histological Specimens (Mean ± Standard Deviation)

Group	*Vessel count*
Spcl-ws	55.2 ± 14.5
Spcl-ws-fs-asc	58.4 ± 23.1
Spcl-ws-fs	45.8 ± 25.2
Spcl-ws-fs-asc-vegf	77.9 ± 24.8[a]
Spcl-ws-fs-asc-fgf2	81.3 ± 32.9[a]
Pocket	32.2 ± 19.7

[a] $p < 0.05$.

The herein presented results show that all conditions established with the assembled bone TE constructs induced the expression of the VEGF gene by the tissues hosting the implants, as well as the control tissues where nothing was implanted. Although the control animals had no implant, the creation of the pocket induced an inflammatory reaction, exactly as in the animals where the implants were placed. In both situations, the host tissue had to recover from the injury and restore the damaged tissue and associated vasculature.

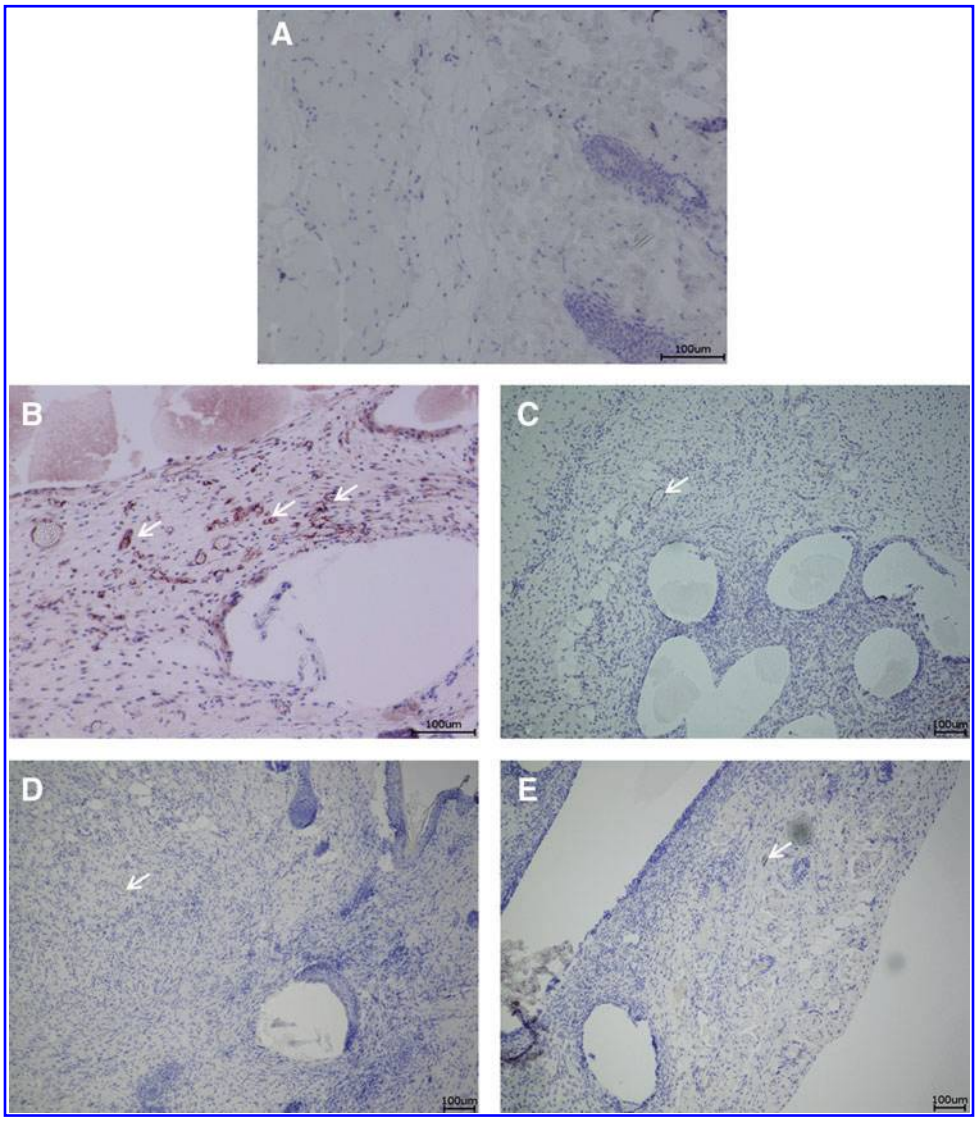

FIG. 5. **(A)** Pocket, **(B)** SPCL-WS, **(C)** SPCL-WS-FS, **(D)** SPCL-WS-FS-ASC-FGF-2, **(E)** SPCL-WS-FS-ASC-VEGF. Staining for CD31 shows endothelial and endothelial precursor cells (arrows). Peroxide coupled CD31 antibody was visualized using diaminobenzidine and is shown as a brown color. The scale bar represents 100 μm. Color images available online at www.liebertpub.com/tea

In this sense, it is possible to substantiate the expression of VEGF in all conditions, including the controls.

At the molecular level it was shown that, when the SPCL scaffolds (SPCL-WS and SPCL-FB) were implanted as simple materials, they did not elicit the expression of the specific markers of inflammation, IL-4 and IFN-γ. In addition, VEGF but not VEGFR1 expression was also detected after SPCL-WS and SPCL-FB implantation. The histological analysis also confirmed a residual inflammatory infiltrate in the vicinity of the SPCL scaffolds. For both short- and long-term subcutaneous implantations of SPCL-WS and SPCL-FB scaffolds, no severe inflammatory reaction has been previously observed.[54] All together, these results demonstrate that SPCL possess a low inflammatory potential very unusual for a biodegradable scaffold, and that the detected VEGFR2 expression in the luminescence experiments are certainly related to the produced VEGF since VEGFR2 is the major receptor for that growth factor.[55] Although it has been reported that inflammatory cells, namely macrophages, express VEGFR1 in response to VEGF stimulation,[55–57] in this study, it was not possible to establish this correlation.

An interesting finding was the lower expression of the VEGFR2 in the SPCL-FB + FS in comparison with the SPCL-FB scaffold. This may be due to the incorporation of fibrin sealant which might be minimizing an eventual physical aggression of the subcutaneous tissue and consequent inflammation with the expression of VEGF receptors. Nevertheless, the histological features showed only a moderate inflammatory reaction for both conditions. Additionally, the molecular analysis showed that the introduction of fibrin sealant, ASCs, and growth factors enhanced the expression of inflammatory mediators (IL-4, TNF-α, IFN-γ) compared with scaffolds alone.

When the hASCs were added, the expression of VEGFR2 showed to be similar to the SCPL-based constructs with fibrin sealant. However, at the molecular level, while the VEGF expression was detected for all the conditions, the expression of VEGFR1 was detected for the SPCL-FB + FS + hASCs construct, but not for the SPCL-WS + FS + hASCs. A possible explanation for this finding may be the differences on the SPCL-FB and SPCL-WS scaffolds, regarding the properties of the fibers. Roughness and fiber's diameter have been shown to influence tissue response[58,59]; thus, the fact

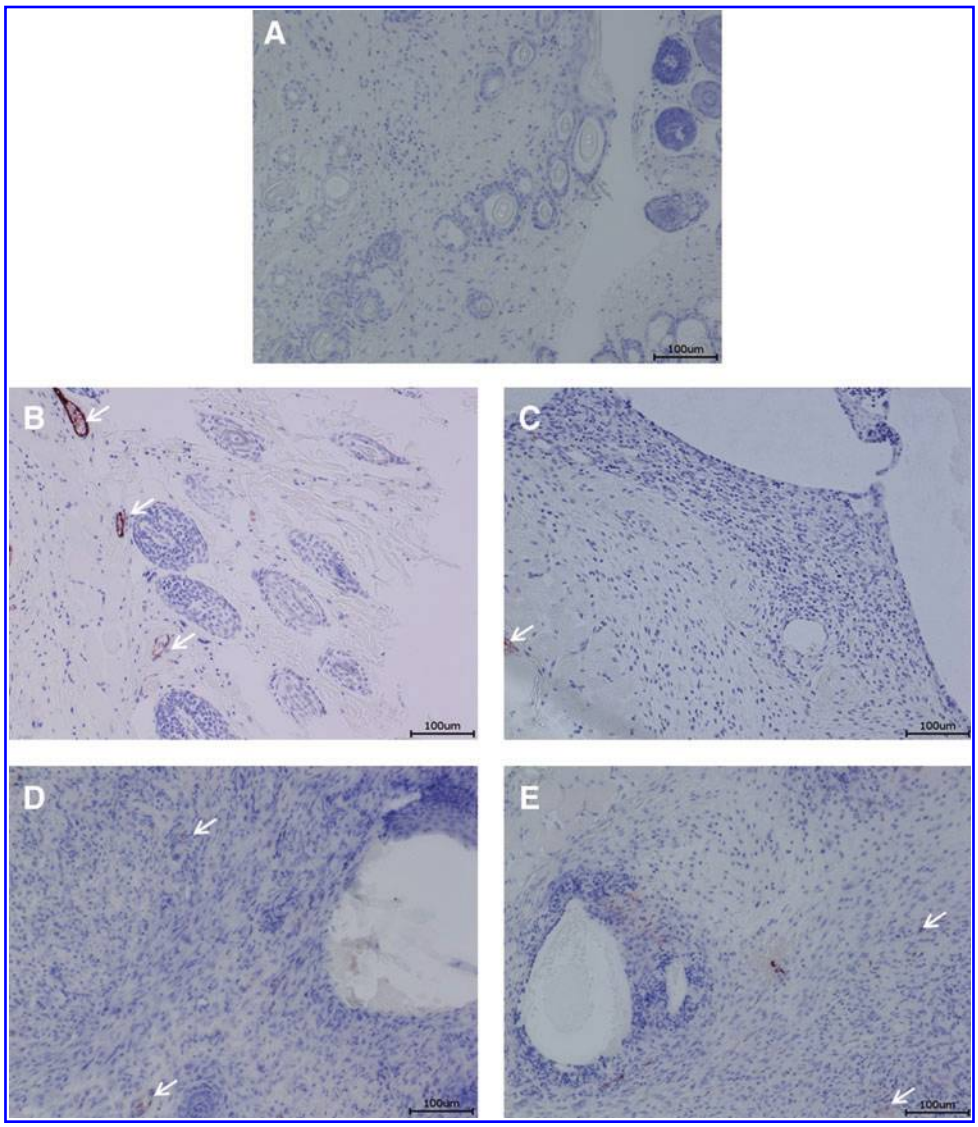

FIG. 6. **(A)** Pocket, **(B)** SPCL-WS, **(C)** SPCL-WS-FS, **(D)** SPCL-WS-FS-ASC-FGF-2, **(E)** SPCL-WS-FS-ASC-VEGF. Staining for von Willebrand Factor shows endothelial and endothelial precursor cells (*arrows*). Peroxide coupled von Willebrand Factor antibody was visualized using diaminobenzidine and is shown as a brown color. The scale bar represents 100 μm. Color images available online at www.liebertpub.com/tea

that SPCL-WS has thinner and smoother fibers might be affecting the VEGFR2 expression in the tissue directly contacting with the scaffold. Moreover, despite the induced expression of the proinflammatory cytokines IL-4, IFN-γ, and TNF-α by the hASCs-assembled constructs, the histological analysis does not corroborate the existence of a persistent acute inflammation.

VEGF and its receptors, among others VEGFR1, are important factors in the establishment, progression, and maturation of new blood vessels.[60–63] During normal wound healing[64] VEGF expression correlates temporally and spatially with the proliferation of new blood vessels.[65] Additionally to VEGF, FGFs are homeostatic factors with function in tissue repair and response to injury in adult organisms.[66]

While the incorporation and subsequent release of both VEGF and FGF-2 from the SPCL-WS did not induce a significant effect over VEGFR2 expression, the expression of this receptor was significantly decreased in the SPCL-FB + FS + hASCs + VEGF and SPCL-FB + FS + hASCs + FGF-2 implantation sites in comparison to unloaded constructs.

The high expression of VEGFR2 at the incision areas in all tested conditions was not an expected result. Nevertheless, it is plausible to detect these high values of luminescence since VEGFR2 is a factor involved on the ongoing inflammatory process and related with healing of the injured region.[64] Additionally, the expression of VEGFR2 translates the vascularization status at the implantation sites.[55]

In summary, we have shown that induction of angiogenic markers occur. Thus, some of our combinations are important inductors. This implies that such combinations should be used rather than others where no induction of these markers was observed. The next step will be to use exactly these constructs in models of bone regeneration to investigate whether they perform better due to their enhanced angiongenic potential than other not equally angiogenic constructs.

Conclusions

Taken together, the presented results show that; (1) the starch-based scaffolds obtained by different processing

methodologies are suitable supports for hASCs; (2) the starch-based scaffolds may be used to assemble a complex TE construct composed by a fibrin sealant, hASCs, and growth factors (VEGF and FGF-2); (3) after 2 weeks of implantation, the assembled TE constructs did not elicit an adverse host reaction, showing a moderate inflammatory response typically observed for implanted devices and; (4) the addition of VEGF and FGF-2 to the TE construct showed to be favorable, at the molecular level, for the expression of neovascularization specific markers.

In summary, the overall results indicate that the combination of SPCL-WS with fibrin sealant, hASCs, VEGF, or FGF-2 seems to be the TE construct with promising features for vascularization of newly formed tissue and thus, may be considered for further studies for bone TE applications.

Acknowledgments

The author Tírcia C. Santos acknowledges the Marie Curie European Program for a short-term scholarship in the Alea Jacta EST project (MEST-CT-2004-008104). This work was developed under the scope of the European Network of Excellence EXPERTISSUES (NMP3-CT-2004-5000283).

Disclosure Statement

No competing financial interests exist.

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Received: December 23, 2010
Accepted: October 16, 2012
Online Publication Date: January 30, 2013