Published in final edited form as:

J Biomed Mater Res A. 2014 September ; 102(9): 3033–3048. doi:10.1002/jbm.a.34969.

Two-stage implantation of the skin and bone integrated pylon (SBIP) seeded with autologous fibroblasts induced into osteoblast differentiation for direct skeletal attachment of limb prostheses

Maxim A. Shevtsov^{a,b}, Oleg V. Galibin^b, Nataliya M. Yudintceva^a, Miralda I. Blinova^a, Grigoriy P. Pinaev^a, Anna A. Ivanova^c, Olga N. Savchenko^c, Dmitriy N. Suslov^b, Igor L. Potokin^d, Emil Pitkin^e, Grigory Raykhtsaum^f, and Mark R. Pitkin^{f,g,*}

^aInstitute of cytology of the Russian Academy of Sciences (RAS), St. Petersburg, 194064, Russian Federation

^bI.P. Pavlov State Medical University, St. Petersburg, 197022, Russian Federation

^cRussian research center of radiology and surgical technology, 197758, St. Petersburg, Russian Federation

^dResearch institute of highly pure biopreparations, 197110, St. Petersburg, Russian Federation

^eThe Wharton School, University of Pennsylvania, Philadelphia, PA, USA

^fPoly-Orth International, Sharon, MA 02067, USA

^gTufts University School of Medicine, Boston, MA 02111, USA

Abstract

Angio- and osteogenesis following the two-stage implantation of the Skin and Bone Integrated Pylon (SBIP) seeded with autologous fibroblasts was evaluated. Two consecutive animal substudies were undertaken: intramedullary subcutaneous implantation (fifteen rabbits) and a two-stage transcutaneous implantation (twelve rabbits). We observed enhanced osseointegrative properties of the intramedullary porous component seeded with fibroblasts induced into osteoblast differentiation, as compared to the untreated porous titanium pylon. The three-phase scintigraphy and subsequent histological analysis showed that the level of osteogenesis was 1.5-fold higher than in the control group, and significantly so ($P < 0.05$). The biocompatibility was further proved by the absence of inflammatory response or encapsulation and sequestration on the histology assay. Treatment of the transcutaneous component with autologous fibroblasts was associated with nearly a 2-fold decrease in the period required for the ingrowth of dermal and subdermal soft tissues into the implant surface, as compared to the untreated porous titanium component. Direct dermal attachment to the transcutaneous implant prevented superficial and deep periprosthetic infections in rabbits *in vivo*.

*Corresponding author: Mark Pitkin, PhD. Tufts University School of Medicine, Physical Medicine and Rehabilitation, 136 Harrison Ave, Boston, MA 02111; 787-297-1204. mpitkin@tuftsmedicalcenter.org.

Keywords

direct skeletal attachment; osseointegration; skin and bone integrated pylon; prosthetic rehabilitation; autologous fibroblasts

Introduction

The first intraosseous transcutaneous implant system for direct skeletal attachment (DSA) of limb prostheses was developed and tested in the 1970s at Rancho Los Amigos Hospital, Downey, CA^{1, 2}. A one-stage implantation of a device for anchoring limb prostheses was performed in three patients who were either above-knee or above-elbow amputees. A stainless steel shaft with a subcutaneous collar made of unpolished carbon was cemented with methylmethacrylate to the intramedullary canal of the bone in the amputee's residuum and then passed through the skin. All three implants had to be removed within 6 months due to the poor bone-implant bond and chronic infection.

Two decades later, a system called Osseointegrated Prostheses for the Rehabilitation of Amputees (OPRA) was introduced by Dr. Per-Ingvar Branemark, who successfully exploited titanium's propensity for osseointegration³. Dr. Branemark's implantation procedure has two steps. First, a threaded cylinder called the *fixture* is screwed into the marrow canal of the residuum bone. About six months later, at the second stage of implantation, a construct called the *abutment* is coupled with the fixture. The abutment penetrates the residuum skin, and its outer part, capped by the abutment screw, is used for attaching the leg prosthesis.

The idea behind the two-stage implantation is that the first stage creates mechanical conditions for better osseointegration. The fixture remains inside the hosting bone marrow canal. The developing osseointegration is protected by the bone walls from accidental external disruptions, loads and moments, and by the residuum skin closure from infection. Once the osseointegration is achieved, the bone-implant interface is able to safely resist external loads and moments. The OPRA system has been applied to more than 100 patients in Europe and in Australia⁴⁻⁶. A similar two-stage system called Endo-Exo Femoral Prosthesis (EEFP) was applied in 54 patients in Germany⁷ and 24 patients in the Netherlands⁵.

A drawback of the two-stage approach is the need for a second invasive surgery in order to transcutaneously implant the external component of the device for DSA. Also, the second (transcutaneous) component in the OPRA and EEFT systems has a smooth surface, which requires constant care to deal with infection⁸.

During the last decade, several new DSA systems were developed that experimented with various treatments of the implant surface or using a one-stage implantation technique. Each of them was addressing various issues in the device-bone and device-skin interface with different design features, as reviewed in⁹. A system called Intraosseous Transcutaneous Amputation Prosthesis (ITAP), University College, London, UK¹⁰, has a perforated flange below the epithelium to increase dermal attachment¹⁰. In studies conducted with the goal of

improving the soft tissue seal around the ITAP, increased fibroblast adhesion was demonstrated to the silanized fibronectin (SiFn) titanium alloy, and on fibronectin adsorbed onto hydroxyapatite (HAFn)¹¹. The ITAP system was reported to be applied to one transhumeral amputee¹², to the human thumb and index finger in one human patient, and to four dogs¹³. The design features of the other one-stage implant system called Percutaneous Osseointegrated Prostheses (POP) system, IMDS Co-Innovative, Logan, UT¹⁴ include a double tempered stem and a porous-coated subcutaneous collar intended to achieve skin-implant integration. It was reported that the collar prevented superficial and deep tissue infections in all animals (14/14, 100%) at the 9-month endpoint, while animals with the smooth Ti implant construct had a 25% (2/8) infection rate¹⁵.

A concept for an intimate tissues-implant interface was further developed by the authors of this paper after introducing the porous composite DSA system called the Skin and Bone Integrated Pylon (SBIP) with minimal enforcing elements^{16, 17}. The frame maintains the needed strength, and is selectively perforated for total porosity of the construct to allow for complete ingrowth of bone and soft tissues^{18, 19, 20}. Total porosity¹⁸ distinguishes the SBIP implantation system from other systems, which use porosity in a limited fashion as a relatively thin layer of coating^{21–24}. The SBIP system allows for various modifications that still have the patented combination of four critical characteristics: pore size, porosity, volume fraction, and particle size¹⁹.

The SBIP system was initially designed for one-stage implantation, which demonstrated a safe interface between the hosting bone and skin in rats, rabbits and cats^{17, 20, 25–27}. That technique eliminates the need for the second surgery, but is still vulnerable with regards to the external disruptions of the outer portion of the device, which may negatively affect the process of osseointegration.

The authors believe that in future clinical applications, the proper balance between the pros and cons of the one-stage and two-stage techniques should be found on an individual basis for each level of amputation where the use of DSA is considered. Thus, in DSA in a particular patient, a multidisciplinary team should have several options in selecting the implantation technique as well as the available pre-treatment of the device.

In planning the current *in-vivo* animal study we wanted to investigate the SBIP system with an option for a two-stage (TS) implantation (SBIP-TS). We further wanted to verify the hypothesis that seeding the SBIP-TS with fibroblasts induced into the osteoblasts inside the collagenic gel will be advantageous for the tissue-device bond.

In this article we present the results of a study on the two-stage implantation of the SBIP-TS kit seeded with autologous fibroblasts into rabbits. The transcutaneous component of the kit had two modifications: porous and solid. The study demonstrated the advantage of the porous component seeded with fibroblasts induced into osteoblast differentiation inside the collagenic gel.

Materials and Methods

Study design

We aimed to assess the influence of the autologous fibroblasts on the biointegrative properties of the two-stage SBIP-TS system. Two consecutive substudies were undertaken: intramedullary subcutaneous implantation and the two-stage transcutaneous implantation. All experiments were performed after the protocol of the study was approved by the ethics committee of I.P. Pavlov State Medical University of St. Petersburg (St. Petersburg, Russia) in compliance with the US Department of Health and Human Services Guide for the Care and Use of Laboratory Animals (1996).

Substudy I: intramedullary subcutaneous implantation

The first study examined intramedullary subcutaneous implantations only. The purpose was to assess the effectiveness of the fibroblast treatment of the intramedullary component of the SBIP-TS kit on the implant-bone interface. In addition, we wanted to learn if an induction of the fibroblasts into osteoblast differentiation would be more beneficial for further osseointegration. We quantitatively assessed the parameters of angio- and osteogenesis in the pores of the pylon with the use of three-phase scintigraphy and further evaluated the pylon with histological analysis. The outcomes of substudy I would then inform which modification and treatment of the intramedullary component of the SBIP-TS kit should be used in substudy II with the two-stage implantation.

Fifteen rabbits were divided into five groups (3 animals each): **(I-1)** control group with a sham-operated limb, **(I-2)** group with a porous titanium composite, **(I-3)** group with a porous titanium pylon seeded with autologous fibroblasts, **(I-4)** group with a porous titanium pylon seeded with autologous fibroblasts induced into osteoblast differentiation, and **(I-5)** group with a non-porous solid titanium implant. After above-knee amputation, we implanted the intramedullary component into the rabbits

The processes of angio- and osteogenesis were assessed with the help of three-phase scintigraphy 1, 2, 4 and 8 weeks after the implantation. Additionally, we performed radiography every month after the operation. At 8 weeks, one animal from each group was sacrificed and assessed on a histological assay. For groups II–IV we selected those animals with most increased (over the period of observation) three-phase scintigraphy parameters of angio- and osteogenesis (i.e., PI, BPR, and DR). Increase of the BPR index corresponds to neoangiogenesis and the increase of the DR index reflects the osteogenesis inside the pores of the pylon. At the same time, the increase of these indices could be an indicator of the inflammatory process due to superficial or deep periprosthetic infection, which would be detected by histological assays. This was an additional argument for conducting histological evaluation for the animals with the highest PI, BPR, and DR indices.

The follow-up period was 9 months after implantation. The results of the assessment of osseointegration in groups **I-2 – I-V**, would support the decision as to which intramedullary component to use in the second substudy with the two-stage implantation. Our initial hypothesis was that the induction of the fibroblasts into osteoblast differentiation would

facilitate the ingrowth of the hosting bone cells into pores of the implant. This hypothesis was confirmed and is described in the Results section.

Substudy II: two-stage transcutaneous implantation

In the second substudy we evaluated the role of fibroblasts on the biointegration of the transcutaneous component attached to the intramedullary component at the level of the derma. We assessed clinically the time period for the reliable attachment of the dermal tissues to the pylon; further this tissue contact was evaluated with the help of the histological assay. The intramedullary porous composite component seeded with the fibroblasts induced into osteoblast differentiation was selected for substudy II based on the outcomes of substudy I. 6–8 weeks after the implantation of the intramedullary component, we attached the transcutaneous part of the SBIP-TS device pretreated with fibroblast in collagen gel, as described in the Obtaining the fibroblasts and seeding the titanium pylon section.

Twelve rabbits were divided into three groups (4 animals each): **(II-1)** non-porous solid titanium transcutaneous component, **(II-2)** porous titanium transcutaneous component, and **(II-3)** porous titanium transcutaneous component seeded with autologous fibroblasts in collagen gel. After 2 months one animal per group was randomly selected for histological assessment. The follow-up period was 5 months after implantation.

Development of Composite Porous Pylons for Two-Step implantation

The design of both components of the pylon SBIP-TS for two-stage implantation consisted of a threaded insert surrounded by a porous cladding (Poly-Orth International, Sharon, MA). The inserts were sintered with powders sieved to (–80+200) mesh (ADMA Products Group, Hudson, OH) in the molds fabricated by Payne Engineering & Fabrication Co., Canton, MA, from boron nitride (Momenite, Columbus, OH). The titanium alloy Ti6Al4V ELI was used as the material for the insert and for the powders. The sintering was conducted in a vacuum for 4 hours at 1090 °C, which was above the beta transus temperature of 996 °C. Porosity of the sintered portion had an average range of 45–50%. The pore size was in the range of 60–120 μm ¹⁹.

The first (intramedullary) component of the SBIP-TS kit (Figure 1-1) has a solid perforated inserts in a shape of a threaded rod 3 mm in diameter. The insert for the second (transcutaneous) component (Fig. 1–2), was a hollow cylinder with inside and outside threads. The inside diameter of the cylinder was 3 mm and it was screwed into the tip of the first component through a skin incision during the second implantation. The outside diameter of the cylinder was 5 mm.

In Figure 1–3, are depicted both components assembled into the SBIP-TS pylon. Inserts are sintered with titanium powder and a porous cladding is subsequently created.

Obtaining the fibroblasts and seeding the titanium pylon

Dermal fibroblasts were established from cells that migrated from rabbit skin fragments that were obtained as has been described elsewhere²⁸. Briefly, the skin fragment was cut into small, 0.1×0.1 cm pieces. The pieces were placed in a Petri dish with DMEM cell medium,

supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, antibiotics (100 U/ml penicillin G, 0.1 mg/ml streptomycin) and incubated at 37°C in a CO₂ incubator, in 5% carbon dioxide at 90% relative humidity. In 3–4 weeks, a confluent fibroblast cell monolayer was developed on a plate surface. After 2–3 passages for increasing the number of cells, the fibroblasts were used for seeding the porous titanium implant. Before application, the viability of fibroblasts was determined by 0.4% trypan blue exclusion.

For intraosseous (intramedullary) and transcutaneous components of the SBIP system the fibroblasts were managed in different ways. For seeding the intraosseous part of the SBIP, fibroblasts were pretreated *in vitro* for induction of the differentiation into osteoblasts. For this, the cells were grown in a 90% α MEM culture medium supplemented with 10% FBS, 10 mM β -glycerophosphate sodium (Sigma, USA), 10⁻⁸ M dexamethazone (Sigma, USA), 50 μ g/ml sodium ascorbate (ICN, USA). The obtained cells were assessed for osteoblast markers, including alkaline phosphatase and osteocalcin.

For seeding the transcutaneous part of the implant with fibroblasts (1 $\times$ 10⁵ cells/ml) we applied collagen gel with cells (Figure 2, A). The implants were cultivated in the gel for 7 days according to a routine technique²⁸. Before inserting the implants with fibroblasts in animals, the cellular interaction with titanium composite was extracted from collagen gel, fixed with 4% paraformaldehyde (PA), and then evaluated with a scanning electron microscope (SEM) (GSM-35.7, Japan).

For determining the optimal time for generating the cellular monolayer on the titanium samples we cultivated pylons with fibroblasts for 7, 10, and 15 days and then assessed with a SEM the cellular density of the samples.

Animals and the amputation technique

Male New Zealand rabbits, weighing 3.5–4 kg, were obtained from the Nursery of Laboratory Animals “Rappolovo” of the Russian Academy of Medical Sciences (RAMN) (St. Petersburg, Russia). Animals were housed in cages under pathogen-free conditions, controlled temperature and humidity, and a 12 hour light-dark cycle, with food and water *ad libitum*.

For anesthesia we used intravenous injection of ketamine (10–50 mg/kg) and xylazine (1–3 mg/kg) mixture, which was administered directly into the marginal ear vein after placement of a catheter. A light sedative agent fentanyl/droperidol (0.2 mg/kg, intramuscular injection) was used prior to anesthesia to avoid stress in the rabbit. For tranquilization and muscle relaxation during intubation of the rabbits, benzodiazepine (diazepam, 1 mg/kg, intravenously) was used. During surgery, heart rate (at 130–325 beats/min) and respiratory rate (at 32–60 per min) were monitored, as well as blood pressure (at 90–130/90–60 mm Hg) and body temperature (at level 38.5–39.6°C).

In all animals we performed above knee amputation²⁹. We marked with a marking pen the anterior and posterior skin flaps at the level of bony resection (4 cm above knee joint). The skin and subcutaneous tissues were incised down to the fascia provided that the anterior flap was 2.5 cm longer than the posterior flap. The greater saphenous vein was ligated and

divided on the medial aspect of the thigh. Subcutaneous tissue and fascia were divided in line with the skin and reflected proximally. Femoral vessels and nerves were identified deep to the sartorius muscle. Posteriorly, the sciatic nerve was identified deep to the hamstrings on the adductor magnus. Nerves were gently pulled down from their muscular bed approximately 2 cm, ligated with nonabsorbable monofilament sutures, transected with a surgical knife and allowed to retract back to the muscle mass. Before the incision, we flushed 0.25% bupivacaine to the epineural space 5–7 cm proximally. Deep femoral vessels were divided, the artery and vein were sutured and ligated individually. Then we cut the bone with a circular saw. Muscle flaps were approximated (myoplasty). The quadriceps and hamstrings muscles were myodesed to each other in covering the bony end of the femur. Drains were inserted and the wound was closed with interrupted absorbable sutures (Vicryl 4.0). After the surgery, the wound was worked-up twice per day with antiseptic Chemi Spray™ (chlortetracycline (hydrochloride), gentian Violet) (INVESA, Spain).

During the early postoperative period all animals were given antibiotic Bicillin-3 (Benzathine Benzylpenicillin) (Sintez, Russia) 300.000 EU, 1 intramuscular 1 injection per 3 days (for a total of 4 injections). Also rabbits received Catosal™ (10% Butaphospan, Cyanocobalamin) (Bayer HealthCare LLC, USA) 1 ml subcutaneously for 14 days.

After the attachment of the transcutaneous component to the intramedullary implant the animals (in all groups) were allowed to freely ambulate and move in the cage. During their movement inside the cage animals could lean on the implant. From the first day after the surgery the animals were monitored twice a day (including weekends and vacations) by the designated veterinarians.

Intraosseous implantation

During the first substudy, we implanted intraosseous components of the SBIP-TS kit with different treatments (see **Study Design**). The best “implant-treatment” combination discovered in the first substudy was used in the second substudy. The intraosseous components were implanted immediately after the above-knee amputation. The component was press fit intramedullary into the rabbit's residual femoral bone and the soft tissues and skin were closed over the residuum. The femoral canal was reamed for achieving the tight press-fit of the pylon.

The rabbits' radiographs were taken prior to the surgery, one month after the implantation of the intraosseous component, and then monthly for up to five months. Radiography was performed by the same operator with the same equipment (46 kV, 200 mA, 32 ms, Trophy N800 HF, Fujifilm 24*30 cm² IP cassette type C, 1 m film-focus distance). For radiography, rabbits were sedated by an intramuscular injection of xylazine (1–3 mg/kg) and ketamine (10–50 mg/kg) mixture.

Two-stage implantation of the titanium components

In the substudy II, approximately 6–8 weeks after the implantation of the intraosseous component, a skin opening was created and the second (transcutaneous) porous composite titanium component of the SBIP-TS kit (Fig. 1–2) was attached to the intramedullary

component (Fig. 1–3). The transcutaneous component was seeded with autologous fibroblasts without induction into differentiation (with 7–10 days of *in vitro* cultivation prior to implantation procedure). The formation of a cellular monolayer on the implant prior to the operation was controlled with scanning electronic microscopy (SEM).

Three-phase bone scintigraphy

For evaluation of the angiogenic and osteogenic processes in the region of the porous titanium implants we used a 3-phase bone scintigraphy³⁰ on rabbits (n=15). By setting the regions of interest (ROI) on the segment with the porous part of the pylon and on a symmetric segment on the contralateral intact limb, we calculated the perfusion index (PI), the uptake ratio of the blood-pool image (BPR), and the uptake ratio of the delayed image (DR). PI reflects the regional blood flow in the of bone; the blood-pool image reflects the level of hyperemia; accumulation of ^{99m}Tc-MDP in the delayed image reflects the activity of bone metabolism³¹.

Before the scintigraphy, rabbits were anesthetized by intravenous injection of ketamine (10–50 mg/kg) and xylazine (1–3 mg/kg). Immediately after the bolus injection of 111 MBq ^{99m}Tc-methylene diphosphonate (MDP) into the right marginal ear vein followed by flushing with 10 ml saline, the first-pass radionuclide angiographic data were obtained with 128×128 matrices in the anterior view every 2 s for 2 min. The blood-pool image (BPI) was obtained at 3 min after injection for 3 min, with 256×256 matrices. A delayed image was obtained 3 h later in the same position, with 256×256 matrices. All data were obtained using a large field-of-view gamma camera (SPECT Infinia GE, USA) equipped with a low-energy, high-resolution, parallel-hole collimator.

For analysis on the blood-pool image and delayed image, we set manually the region of interest (ROI) on the segment of porous titanium pylon and set a symmetric ROI on the contralateral intact area as the control. The uptake ratio of the blood-pool image (BPR) and the uptake ratio of delayed image (DR) were calculated by dividing the count density of the porous titanium segment by that in the contralateral normal area in each image. The perfusion index (PI) was obtained by ^{99m}Tc-MDP angiography. We set the region of interest (ROI) on the segment with porous titanium and symmetrically on the contralateral area to calculate PI. The time-activity curve of each ROI was generated, and the PI was determined by dividing the peak count of the arterial phase of titanium region by that of the contralateral normal area.

Histological analysis

To evaluate the bone growth development of the porous titanium components implanted into the rabbit femur, as well as the interaction between soft tissues -- including skin -- with the pylon, we performed histological analyses at Alizée Pathology, LLC (Thurmont, MD, USA). At the designated time point, one animal per group was randomly selected and sacrificed. Samples of the implanted femur sections were collected and fixed in 10% formalin. Femur samples were trimmed and embedded in methyl methacrylate (MMA). The blocks were bisected: one half to generate one longitudinal level to assess the skin/implant interface and the other half was cut to generate transverse cross section levels (bone/implant

interface). All slides were stained with hematoxylin and eosin (H&E). After the histological processing the resulting slides were evaluated via light microscopy.

Bacteriological assay

If a clinically chronic non-healing wound was identified around transcutaneous component the tissue samples were collected. After anesthesia with intravenous injection of ketamine (10–50 mg/kg) and xylazine (1–3 mg/kg) mixture, smears were collected from the wound base with a 3 or 7 mm curette. Samples were taken from two sites from each wound: from the leading edge of the wound; and the opposing leading edge. Each specimen was placed in non-bacteriostatic saline, immediately transported to the laboratory for culture-based analyses. Samples were then serially diluted and plated on non-selective media including sheep blood agar, MacConkey and chocolate agar plates and incubated overnight at 37°C in 6% CO₂. Smears were stained by Gram's staining method.

Organisms were identified by using standard biochemical tests³². Antimicrobial Sensitivity Test (AMST) was done by Kirby-Bauer disc diffusion method³³

Statistical analysis

The Kruskal-Wallis test³⁴, which is the non-parametric analog to the one-way ANOVA test, was run to detect the presence of differences among the three treatment and two control three-phase scintigraphy groups. We opted for the non-parametric version because of the low number of observations in each group (n=3). When the null hypothesis of no difference between groups was rejected, the non-parametric Mann-Whitney test was then run for pairwise comparisons, to determine if the group with the largest parameter (among PI, BPR, DR) was statistically significantly larger than all of the other groups. A one-way alternative was used, since the two-way test with 3 subjects in each group has power equal to 0. The open source software package R (<http://www.r-project.org/>), version 2.15.2 was used for analysis. Statistical significance was determined at the alpha = 0.05 level.

Results

Interface of the porous titanium composite with the autologous fibroblasts

Analysis of cellular density on the 7th, 10th, and 15th days of incubation of the autologous fibroblasts showed that this parameter continued to increase dramatically up to the 15th day, when we could observe multilayer coatings which in some samples closed the pores of the pylon (*data not shown*). From the SEM imaging data we could deduce that between 7 to 10 days was optimal time for autologous fibroblasts' seeding the porous titanium implants; in this time the confluent monolayer could be generated while the pores of the pylon were preserved for ingrowth of the hosting bone. The fibroblast could manage to interact with the implant, migrate into the pores, proliferate and form the confluent monolayer. Multiple cellular contacts (bundles) between adjacent titanium pores are shown in Figure 2 with the cells presented not only on the surface (Fig. 2-A), but also in the depth of the pylon (Fig. 2-B–D). Due to migration through the pores, the fibroblast cells were observed near the core of solid insert of the pylon.

With fibroblasts induced into the osteoblast differentiation, development of the osteoblast-like cells in *in vitro* conditions before treatment of the implants is shown in Figure 3. The osteoblast-like cells formed the confluent monolayer over the titanium surface that morphologically was similar to the cellular monolayer generated by the non-induced fibroblasts (*data not shown*). The optimal period for formation of the cellular monolayer also constituted 7–10 days. Further incubation of the pylon with cells resulted in the formation of the multilayer cellular coatings which closed the titanium pores.

After above-knee amputation and insertion of the intramedullary component all animals recovered rapidly and freely ambulated after the surgery. This ambulation did not cause the breakage of the implant or any discomfort to the animal.

Two-stage implantation of the porous titanium pylon seeded with autologous fibroblasts

After 6–8 weeks, depending on the operating facility schedule, we performed the second stage of the implantation of the transcutaneous part of the pylon (Figure 4). From the first series of *in vivo* experiments we had selected the intramedullary component pretreated with fibroblasts induced into the osteoblast differentiation because it demonstrated the increased parameters of the angio- and osteogenesis on three-phase scintigraphy. This intramedullary component was implanted into all rabbits for the second stage. The animals were taken for the second surgery in a random order. All animals recovered from the procedure without complications.

In group **II-1** with the transcutaneous non-porous component (solid titanium threaded cylinder) we did not observe the attachment of dermal or subdermal tissues to the implant. Four-five weeks after the implantation we observed the phenomenon of marsupialization – the formation of fibrous tissue around the pylon (*data not shown*). In all four animals in this group there were infectious complications. A subsequent bacteriological analysis of the samples revealed *S. aureus* and *E.coli*. This led to osteomyelitis and the loss of the intramedullary implant in all 4 animals 12–13 weeks after the surgery.

In group **II-2**, with the porous transcutaneous pylon without fibroblasts, visually we could observe the attachment of the derma to the porous component after 6–7 weeks in all rabbits. Clinically, no signs of infectious complications were observed, and this was further confirmed by the bacteriological examination.

In group **II-3**, where the porous titanium pylon was seeded with autologous fibroblasts, the time for attachment of the derma to the component was 3–4 weeks, significantly less than in group **II-2** (Figure 5-A). The follow-up period for animals from groups **II-2** and **II-3** constituted up to 5 months. At the end of the follow-up period there were no signs of infection in any of the animals.

According to the radiograph's examination, there were no complications in the femur bone (i.e., bone ulceration, bone thinning, etc.) as a consequence of the insertion of the titanium intramedullary component (Fig. 5-A). We observed significant quantities of subperiosteal bone formed on the distal end (femur osteotomy site) of the titanium pylon. In some cases we observed soft tissue fibrosis and osteophytosis. From the serial radiographs it was clear

that there was no axial displacement of the titanium pylon, which indicates that the implant inside femur was well fixated due to the process of osseointegration. After the attachment of the transcutaneous component to the intramedullary component we did not observe any signs of osteomyelitis in groups **II-2** or **II-3** of the animals with porous titanium component (with or without fibroblasts) (Fig. 5-B). In group **II-1** with the non-porous solid titanium transcutaneous component, after 10–12 weeks the early signs of the osteomyelitis (i.e., periosteal reaction, osteolysis) were revealed in all animals (n=4).

Three-phase scintigraphy data analysis

Three-phase scintigraphy was applied to assess the osseointegrative properties of the intramedullary component of the pylon, both when treated and when untreated with autologous fibroblasts (groups **I-2** – **I-5**). Three-phase scintigraphy was performed at 1, 2, 4, and 8 weeks after implantation of the titanium pylon's intramedullary component. Each time that scintigraphy was performed, three parameters were measured and analyzed: perfusion index (PI); an index of the uptake ratio of the blood-pool image (BPR); and an index of the uptake of the delayed image (DR). There were three animals in each of the groups **I-2** – **I-5**. In Table 1 are presented, for each group and time of measurement, the mean PI, BPR and DR measurements, together with their standard deviations.

In Table 1 are also the p-values for the test of equality among the 5 groups. Each cell in the bottom row of Table 1 corresponds to a specific parameter being tested at a specific number of weeks after implantation of the intramedullary component of the SBIP-TS system into the femur. That is, these Kruskal-Wallis tests were run for each of the respective parameters (PI, BPR, DR) during each scintigraphy measurement during weeks (1, 2, 4, 8). The perfusion index (PI) differed significantly among all groups in all weeks; the uptake ratio of the blood pool image (BPR) was different among the groups in weeks 2, 4, and 8; and the uptake ratio of the delayed image (DR) differed significantly among the groups in week 4 and 8. With each subsequent week of observations, more parameters exhibit differences.

For those parameter-week combinations in which differences among the groups were observed, we wished to determine whether one group was statistically significantly larger than all others. It was found that the **I-4** group had a PI index higher than all others in weeks 1, 4, 8; it had a BPR index higher than all others in weeks 2, 4 and 8; and it had a DR index higher than all others in weeks 4 and 8.

By week 4, and remaining so in week 8, the PI index for group **I-4** was statistically significantly higher than for all other groups. Likewise, by week 4, and remaining so in week 8, the BPR index for group **I-4** was statistically significantly higher than for all other groups. The DR index for group **I-4** was statistically significantly higher than for all other groups by week 4; it remained highest in week 8, a standard deviation above the next highest group. We therefore conclude that **I-4** attains the highest parameter values among the 5 groups, and does so by the 4th week. See week 4 in Table 2 where group **I-4** was compared, pairwise, with all other groups. The cells in Table 2 with N/A are such because the Kruskal-Wallis test did not detect any difference among the five groups; hence pairwise comparisons were unnecessary.

As an example of the **I-4** group's significant improvement over the others, its median PI index was nearly 31% higher than the next highest median PI index in week 4 and 26% higher in week 8. It was 362% and 405% higher than in control group **I-1** in weeks 4 and 8, respectively.

At the 8th week the PI was strongly elevated in other groups, too. The rise of the arterial perfusion over the entire period of observation is illustrated for animal #2 from the **I-2** group when we observed the increase of the time-activity curve of the ROI over the region of the porous titanium in comparison to the control non-operated limb (Figure 6).

The uptake ratio of the blood-pool image (BPR) rose consistently for groups **I-2**, **I-3**, and **I-4** 4th week. Going from week 1 to 2, 2 to 4, and 4 to 8, the median I-2 BPR rose by 34%, 52%, and 57%, respectively. Similarly, the median I-3 BPR rose by 73%, 59%, 40%. And the median I-4 BPR rose by 76%, 97%, 28%.

The uptake ratio of the delayed image (DR) also reflected the same tendency in the noticeable elevation of the DR parameters in groups **I-2**, **I-3**, and **I-4** over control sham-operated rabbits or the group **I-5** with non-porous solid titanium component (Figure 7) with a prevalence of the DR index in the group with porous pylon seeded with fibroblasts induced into osteoblast differentiation (Table 1; Fig. 7). Four weeks after implantation, the median DR for the groups **I-2**, **I-3**, and **I-4** were 2.61, 2.89 and 3.85, respectively, and experienced increases of 24%, 35%, and 28%, respectively between the 4th and 8th weeks to 3.24, 3.91, and 4.92. The most representative animals are presented in Figure 8, showing the prevalence of all scintigraphy parameters in animal #3 from group **I-3** (Fig. 8-C).

Histological analysis

The histological slides were evaluated for (i) inflammation (presence of heterophils/neutrophils, eosinophils, lymphocytes, plasma cells, macrophages, and multinucleated cells); (ii) tissue response, and (iii) general healing (fibrosis, pseudobursa, osteogenesis). The femoral amputation line showed optimal bone repair characterized by bridging of the gap between the femoral cortex and the endosteal portion of the porous titanium pin by mature trabecular bone (Figure 9). There was also direct bone apposition along the titanium and filling of the porous pin across the width of the pin at its parieto-cortical end. The pin was in direct contact with the inner cortex along one edge of the femur where bone apposition as well as infiltration of the porous pin by newly formed bone trabeculae were extensive (Fig. 9-B). Along the opposite medullary line there was a 1.7 mm wide gap in the medulla between the inner cortex and the pin. The gap was occupied by fibrous connective tissue of variable density with irregularly spaced and sparse bone trabeculae. Some of these trabeculae came into contact with and infiltrated the spaces of the titanium pin. The outer cortex showed a layer of remodeled callus that was fully continuous with the trabecular bone entrapping the pin at the amputation line. There was no appreciable inflammatory response along the titanium pin.

The rate of bone to implant contact was intermediate to high (~36% of implant surface), which was in part due to infiltration of bone within the pores of the titanium shaft. The highest values of bone implant contact were recorded along the distal edge of the porous pin

which indicated excellent osteointegration. The sequential sections across the femur bone shaft showed no evidence of instability (no granulation tissue or encapsulation or scar tissue around the pin). There was remodeling of the shaft characterized by endosteal thickening where the shaft was closest to the pin (appeared to be the antero-lateral side). Direct apposition of new bone to the titanium pin was observed, albeit along small areas and inconsistently from level to level. There was no inflammatory response along the pin.

The transcutaneous portion of the titanium pylon was in direct contact with the fibrous connective tissue of the amputation stump (Figure 10). We assessed the ingrowth of the soft tissues as well as the skin inside the pores of the SBIP-TS system. We observed the presence of the viable soft tissues as well as derma inside the pores of the pylon (Fig. 10B). There was a dermo-epidermal and the direct attachment of the connective tissue attachment to the porous component. Overall, the skin-device contact zone showed little active inflammation and good healing.

Discussion

The results of this study support the initial hypothesis that the two-stage implantation procedure of the SBIP-TS system enhanced with fibroblasts is feasible, safe and could be an alternative to similar prosthetic technologies used in pre-clinical and clinical applications^{4, 35}, and to the SBIP modifications previously reported^{16, 17}.

Inclusion of the SBIP-TS kit in the SBIP system being evaluated in the current study gives an additional option for planning further studies. For example, in the experiments with biological treatment, as in the current study, the intraosseous and transcutaneous components can be processed with different agents for different periods of time. On the other hand, the one-stage implantation may be advantageous, when the two plastic surgeries on the residuum are not recommended and for studies in prosthetic neurocontrol via DSA³⁶

In recent years, numerous *in vivo* studies have demonstrated the benefits of cell seeding technology in the development of tissue-engineered grafts, as this approach promotes improved long-term graft function. The development of tissue-engineered vascular grafts proved to be an applicable alternative to current synthetic grafts^{37,38}, and cell seeding promoted the graft's potency and longevity^{38–42}. The role of the fibroblasts in different processes, include parenchymal cell regulation due to growth factor production, vascularization due to the stimulation of neoangiogenesis, connective tissue formation, etc^{43–46}. It was shown that fibroblasts in certain microenvironments can undergo osteoblastic, chondrogenic, adipogenic and hepatogenic differentiation^{47, 48}. Consequently, preliminary seeding of the titanium pylon by autologous fibroblasts could significantly increase the effectiveness of the implantation due to the fibroblasts' multitudinous functions (Figure 11).

For our experiments we applied fibroblasts derived from the rabbit's skin. Previously it was reported that fibroblasts may be isolated using tissue culture adherence from many tissue sites (e.g., placenta, muscle, adipose tissue, etc)^{49, 50}. Although niche-specific differences may exist between stromal cells, their differentiation and functional properties did not

differ⁴⁷. Earlier we demonstrated that fibroblasts cultured on the titanium implants can subsequently generate a cellular monolayer²⁸. For facilitation of cell attachment to the matrix of the scaffold we used collagenic gel. Sagnella et al. reported that biological glues can mimic the extracellular matrix (ECM) and encourage more cells to stably adhere to the scaffold⁵¹. Though fibrin or fibronectin is the most commonly described agent for adhesive coating^{39, 42} we observed a better fibroblast cell attachment in case of collagen gel²⁸. Collagen gel also provides natural three-dimensional (3D) matrices that are regarded as a more physiological model for developing cell–matrix interactions than the traditional two-dimensional (2D) tissue culture^{52–54}.

For the intramedullary and transcutaneous components the fibroblasts were treated in two different ways. For seeding the intramedullary component of the SBIP system we generated a subset of the fibroblasts with osteoblast-like properties (Fig. 3). Fibroblasts induced into osteoblast differentiation presented the characteristic properties of the latter, including production of osteocalcin and alkaline phosphatase. The treatment of the pylon with osteoblast-like cells facilitated the ingrowth of the host cells into the pores of the pylon (Figure 12). The scintigraphy analysis demonstrated the significant increase of the uptake ratio of the delayed image (DR), which reflects the activity of bone metabolism (Table 1)³¹. Thus the DR index for the treated animals was 1.5 times higher than in the animals with intramedullary component unseeded with fibroblasts, and 3.8 times higher than in the animals with a sham-operated limb ($P<0.05$). The application of the autologous cells also corresponded to elevated values of the PI and BPR indexes (Table 1) that reflects the processes of angiogenesis and arterial blood perfusion in the region of the porous titanium implant.

The importance of the increase of the PI, BPR and DR indices was demonstrated previously by Kawano et al., who assessed the three-phase scintigraphy for predicting the outcomes of the distraction osteogenesis in patients⁵⁵. Thus, the insignificant increase of the DR index was associated with poor clinical prognosis due to insufficient fracture consolidation⁵⁵. The data obtained in the current study from the three-phase scintigraphy is further confirmed by the histological assay (Figs. 8, 10). The intramedullary component showed good stability due to the osseointegration with the hosting bone. The porous titanium pylon was associated with significant bone ingrowth along the bone-implant contact surface as well as formation of the novel blood vessels inside the pores. The absence of inflammatory response or encapsulation and sequestration was demonstrated.

The transcutaneous component of the SBIP system was treated with autologous fibroblasts not induced into the specific cell type differentiation. Application of cell seeding technology was beneficial for the attachment of the dermal and subdermal soft tissues to the porous transcutaneous part of the SBIP. Such attachment could prevent the marsupialization phenomenon and decrease the risks of periprosthetic infections. The period for generating sufficient connections between the porous component and the soft tissues was nearly two times smaller in the group with the transcutaneous component which was seeded with cells — up to 3–4 weeks (in comparison to 6–7 weeks in the group with untreated pylons). As was confirmed by the histological assay, dermal tissues were attached to the porous component, thus preventing the generation of the fibrous capsule around the transcutaneous

component (Fig. 10-B). Fibroblasts probably acted as a source of growth factors such as fibroblast growth factor (FGF), keratinocyte growth factor (KGF) and others that could facilitate the dermal tissues' ingrowth into the pores of the SBIP⁵⁶.

In the current study we demonstrated the efficacy of the two-stage implantation of the SBIP system combined with the autologous fibroblasts. The low risk of the periprosthetic infections due to the direct dermal attachment to the implant, together with the good stability of the intramedullary component due to the improved osseointegrative properties can make this modification of the SBIP system a valuable tool for pre-clinical and clinical applications.

The authors recognize the limitations of the study which include the small number of animals in each group; the fact that the animals were not supplied with the prosthesis and that the overall length of the affected limb together with the outside portion of the transcutaneous SBIP-TS component was less than the length of the unaffected limbs. The duration of the postoperative period was less than one year and that did not allow for evaluation of the dynamics of the hosting bone thinning observed in⁵⁷. The authors plan to address these limitations in their future work.

Conclusions

The pylon kit SBIP-TS designed for a two-stage (TS) implantation demonstrated high functionality in the animal studies. Induction of fibroblasts into osteoblast differentiation improved osseointegration of the intramedullary component. The level of angiogenesis and osteogenesis in the pores of the pylon as well as infection free skin attachment could be increased by the application of the fibroblasts in the collagen gel.

Acknowledgments

We thank Olga G. Genbach, Nelly V. Koroleva, Tatyana V. Zakoldaeva, Irina V. Kononova, Yulia I. Shevchuk, Vladimir V. Sotnikov for animal research assistance; Pavel P. Klein for the illustrations preparation. The study was supported in part by Award Number R44HD057492 from the Eunice Kennedy Shriver National Institute of Child Health & Human Development, NIH.

References

1. Mooney V, Predecki PK, Renning J, et al. Skeletal extension of limb prosthetic attachment--problems in tissue reaction. *Journal of Biomedical Materials Research*. 1971; 5(6):143–159.
2. Mooney V, Schwartz SA, Roth AM, et al. Percutaneous implant devices. *Annals of biomedical engineering*. 1977; 5(1):34–46. [PubMed: 851262]
3. Branemark R, Branemark PI, Rydevik B, et al. Osseointegration in skeletal reconstruction and rehabilitation: a review. *J Rehabil Res Dev*. 2001; 38(2):175–81. [PubMed: 11392650]
4. Hagberg K, Brånemark R. One hundred patients treated with osseointegrated transfemoral amputation prostheses--rehabilitation perspective. *Journal of rehabilitation research and development*. 2009; 46(3):331. [PubMed: 19675986]
5. van de Meent, H.; Frölke, JP.; Palm, B. Preliminary results of the first 24 patients in The Netherlands. 4th Int. Conf. Advances in Orthopaedic Osseointegration; San Francisco: UCSF; Feb 10–12. 2012 p. 31

6. Al Muderis, M.; B. B; Kumar, A., et al. Early results of the osseointegration group of Australia accelerated protocol. 4th Int. Conf. Advances in Orthopaedic Osseointegration; San Francisco: UCSF; Feb 10–12. 2012 p. 32
7. Aschoff HH, Juhnke DL. Evaluation of 10 Years Experience with Endo-Exo Femur Prostheses - Background, Data and Results. *Z Orthop Unfall*. 2012; 150:607–614. [PubMed: 23171987]
8. Aschoff HH, Kennon RE, Keggi JM, et al. Transcutaneous, distal femoral, intramedullary attachment for above-the-knee prostheses: an endo-exo device. *The Journal of Bone & Joint Surgery*. 2010; 92(Supplement_2):180–186. [PubMed: 21123601]
9. Pitkin M. Design features of the implants for direct skeletal attachment of limb prostheses. *Journal of Biomedical Materials Research Part A*. 2013; 00A:000–000. online DOI: 10.1002/jbm.a.34606: <http://onlinelibrary.wiley.com/doi/10.1002/jbm.a.34606/abstract;jsessionid=E7EADFC8D11AC1347A767A49A713C1A8.d03t03?deniedAccessCustomisedMessage=&userIsAuthenticated=false>.
10. Pendegrass CJ, Goodship AE, Blunn GW. Development of a soft tissue seal around bone-anchored transcutaneous amputation prostheses. *Biomaterials*. 2006; 27(23):4183–91. [PubMed: 16618500]
11. Chimutengwende-Gordon M, Pendegrass C, Blunn G. Enhancing the soft tissue seal around intraosseous transcutaneous amputation prostheses using silanized fibronectin titanium alloy. *Biomed Mater*. 2011; 6(2):025008. [PubMed: 21358026]
12. Kang NV, Pendegrass C, Marks L, et al. Osseocutaneous integration of an intraosseous transcutaneous amputation prosthesis implant used for reconstruction of a transhumeral amputee: case report. *J Hand Surg Am*. 2010; 35(7):1130–4. [PubMed: 20541327]
13. Fitzpatrick N, Smith TJ, Pendegrass CJ, et al. Intraosseous transcutaneous amputation prosthesis (ITAP) for limb salvage in 4 dogs. *Vet Surg*. 2011; 40(8):909–25. [PubMed: 22092391]
14. Shelton TJ, Peter Beck J, Bloebaum RD, et al. Percutaneous osseointegrated prostheses for amputees: Limb compensation in a 12-month ovine model. *Journal of Biomechanics*. 2011; 44:2601–6. [PubMed: 21920525]
15. Jeyapalina S, Beck JP, Bachus KN, et al. Efficacy of a porous-structured titanium subdermal barrier for preventing infection in percutaneous osseointegrated prostheses. *J Orthop Res*. 2012; 30(8):1304–11. [PubMed: 22294380]
16. Pitkin M, Raykhtsaum G, Pilling J, et al. Porous composite prosthetic pylon for integration with skin and bone. *J Rehabil Res Dev*. 2007; 44(5):723–738. [PubMed: 17943684]
17. Pitkin M, Raykhtsaum G, Pilling J, et al. Mathematical modeling and mechanical and histopathological testing of porous prosthetic pylon for direct skeletal attachment. *J Rehabil Res Dev*. 2009; 46(3):315–30. [PubMed: 19675985]
18. Pitkin M, Pilling J, Raykhtsaum G. Mechanical properties of totally permeable titanium composite pylon for direct skeletal attachment. *Journal of Biomedical Materials Research Part B: Applied Biomaterials*. 2012; 100B(4):993–9.
19. Pitkin, M.; Raykhtsaum, G. Skin Integrated Device. US Patent # 8257435. 2012.
20. Farrell B, Prilutsky B, Ritter J, et al. Effects of pore size, implantation time and nano-surface properties on rat skin ingrowth into percutaneous porous titanium implants. *J Biomed Mat Res Part A*. 2013; (in press)
21. Goransson A, Gretzer C, Johansson A, et al. Inflammatory response to a titanium surface with potential bioactive properties: an in vitro study. *Clin Implant Dent Relat Res*. 2006; 8(4):210–7. [PubMed: 17100746]
22. Jimbo, R.; Ono, D.; Hirakawa, Y., et al. Clinical implant dentistry and related research. 2009. Accelerated Photo-Induced Hydrophilicity Promotes Osseointegration: An Animal Study.
23. Del Fabbro M. Clinical and Radiographic Evaluation of Branemark Implants with an Anodized Surface following Seven-to-Eight Years of Functional Loading. *International Journal of Dentistry*. 2013; 2013
24. Benchrit S, Byrd WC, Altarawneh S, et al. Development and Applications of Porous Tantalum Trabecular Metal-Enhanced Titanium Dental Implants. *Clinical implant dentistry and related research*. 2013
25. Pitkin M, Raykhtsaum G, Galibin O, et al. Skin and bone integrated prosthetic pylon: a pilot animal study. *J Rehabil Res Dev*. 2006; 43(4):573–580. [PubMed: 17123195]

26. Pitkin M, Raykhtsaum G, Pilling J, et al. Porous composite prosthetic pylon for integration with skin and bone. *J Rehabil Res Dev.* 2007; 44(5):723–38. [PubMed: 17943684]
27. Farrell, B.; Pitkin, M.; Popat, K., et al. Effect of pore size, implantation time and nano-surface properties on rat skin ingrowth into porous titanium. 4th Int. Conf. Advances in Orthopaedic Osseointegration; San Francisco: UCSF; Feb 10–12. 2012 p. 13
28. Blinova M, Yudintzeva N, Nikolaenko N, et al. Cell Cultivation on Porous Titanium Implants with Various Structures. *Cell and Tissue Biology.* 2010; 4(6):572–579. [PubMed: 21949585]
29. Eidt, J.; Kalapatapu, V. Lower extremity amputation: Techniques and Results. In: Cronenwett, editor. *Rutherford's Vascular Surgery.* 7th ed.. Vol. Vol. 2. Saunders; Philadelphia, PA: 2010.
30. Wheelless, CR. Wheelless' textbook of orthopaedics. 2011. DataTrace Internet Pub: Available at: http://www.wheelsonline.com/ortho/triphasic_bone_scan
31. Nutton RW, Fitzgerald RH Jr, Kelly PJ. Early dynamic bone-imaging as an indicator of osseous blood flow and factors affecting the uptake of 99mTc hydroxymethylene diphosphonate in healing bone. *J Bone Joint Surg Am.* 1985; 67(5):763–70. [PubMed: 3997929]
32. Mackie, TJ.; McCartney, JE.; Cruickshank, R. Mackie & McCartney's Handbook of bacteriology; a guide to the laboratory diagnosis and control of infection. 10th ed.. E. & S. Livingstone; Edinburgh: 1960.
33. Winn, WC.; Koneman, EW. Koneman's color atlas and textbook of diagnostic microbiology. Lippincott Williams & Wilkins; 2006.
34. Siegel, S.; Castellan, NJ. Nonparametric statistics for the behavioral sciences. 2nd ed.. McGraw-Hill; New York: 1988.
35. Aschoff HH, McGough RL. The Endo-Exo Femoral Prosthesis: a new rehabilitation concept following above knee amputation. *Journal of Bone & Joint Surgery, British Volume.* 2012; 94(SUPP XXXIX):77–77.
36. Pitkin M, Cassidy C, Muppavarapu R, et al. Recording of Electric Signal Passing Through a Pylon in Direct Skeletal Attachment of Leg Prostheses with Neuromuscular Control. *IEEE transactions on bio-medical engineering.* 2012; 59(5):1349–53. [PubMed: 22345523]
37. Shin'oka T, Matsumura G, Hibino N, et al. Midterm clinical result of tissue-engineered vascular autografts seeded with autologous bone marrow cells. *The Journal of thoracic and cardiovascular surgery.* 2005; 129(6):1330–1338. [PubMed: 15942574]
38. L'Heureux N, Dusserre N, Marini A, et al. Technology insight: the evolution of tissue-engineered vascular grafts--from research to clinical practice. *Nat Clin Pract Cardiovasc Med.* 2007; 4(7): 389–95. [PubMed: 17589429]
39. Pawlowski KJ, Rittgers SE, Schmidt SP, et al. Endothelial cell seeding of polymeric vascular grafts. *Front Biosci.* 2004; 9:1412–21. [PubMed: 14977556]
40. Hashi CK, Zhu Y, Yang GY, et al. Antithrombogenic property of bone marrow mesenchymal stem cells in nanofibrous vascular grafts. *Proc Natl Acad Sci U S A.* 2007; 104(29):11915–20. [PubMed: 17615237]
41. Cho S-W, Lim SH, Kim I-K, et al. Small-Diameter Blood Vessels Engineered With Bone Marrowâ€Derived Cells. *Annals of surgery.* 2005; 241(3):506. [PubMed: 15729075]
42. Salacinski HJ, Tiwari A, Hamilton G, et al. Cellular engineering of vascular bypass grafts: role of chemical coatings for enhancing endothelial cell attachment. *Med Biol Eng Comput.* 2001; 39(6): 609–18. [PubMed: 11804165]
43. Blanpain C, Horsley V, Fuchs E. Epithelial stem cells: turning over new leaves. *Cell.* 2007; 128(3): 445–58. [PubMed: 17289566]
44. Orimo A, Gupta PB, Sgroi DC, et al. Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. *Cell.* 2005; 121(3):335–48. [PubMed: 15882617]
45. Chang HY, Chi JT, Dudoit S, et al. Diversity, topographic differentiation, and positional memory in human fibroblasts. *Proc Natl Acad Sci U S A.* 2002; 99(20):12877–82. [PubMed: 12297622]
46. McGettrick HM, Smith E, Filer A, et al. Fibroblasts from different sites may promote or inhibit recruitment of flowing lymphocytes by endothelial cells. *Eur J Immunol.* 2009; 39(1):113–25. [PubMed: 19130557]

47. Haniffa MA, Wang XN, Holtick U, et al. Adult human fibroblasts are potent immunoregulatory cells and functionally equivalent to mesenchymal stem cells. *J Immunol.* 2007; 179(3):1595–604. [PubMed: 17641026]
48. Lysy PA, Smets F, Sibille C, et al. Human skin fibroblasts: From mesodermal to hepatocyte-like differentiation. *Hepatology.* 2007; 46(5):1574–85. [PubMed: 17969047]
49. da Silva Meirelles L, Chagastelles PC, Nardi NB. Mesenchymal stem cells reside in virtually all post natal organs and tissues. *J Cell Sci.* 2006; 119(Pt 11):2204–13. [PubMed: 16684817]
50. Bieback K, Kluter H. Mesenchymal stromal cells from umbilical cord blood. *Curr Stem Cell Res Ther.* 2007; 2(4):310–23. [PubMed: 18220915]
51. Sagnella S, Anderson E, Sanabria N, et al. Human endothelial cell interaction with biomimetic surfactant polymers containing Peptide ligands from the heparin binding domain of fibronectin. *Tissue Eng.* 2005; 11(1–2):226–36. [PubMed: 15738677]
52. Sun T, Jackson S, Haycock JW, et al. Culture of skin cells in 3D rather than 2D improves their ability to survive exposure to cytotoxic agents. *J Biotechnol.* 2006; 122(3):372–81. [PubMed: 16446003]
53. Cukierman E, Pankov R, Yamada KM. Cell interactions with three-dimensional matrices. *Curr Opin Cell Biol.* 2002; 14(5):633–9. [PubMed: 12231360]
54. Hakkinen KM, Harunaga JS, Doyle AD, et al. Direct comparisons of the morphology, migration, cell adhesions, and actin cytoskeleton of fibroblasts in four different three-dimensional extracellular matrices. *Tissue Eng Part A.* 2011; 17(5–6):713–24. [PubMed: 20929283]
55. Kawano M, Taki J, Tsuchiya H, et al. Predicting the outcome of distraction osteogenesis by 3-phase bone scintigraphy. *J Nucl Med.* 2003; 44(3):369–74. [PubMed: 12621002]
56. Rheinwald JG, Green H. Epidermal growth factor and the multiplication of cultured human epidermal keratinocytes. *Nature.* 1977; 265(5593):421–4. [PubMed: 299924]
57. Nebergall A, Bragdon C, Antonellis A, et al. Stable fixation of an osseointegrated implant system for above-the-knee amputees. *Acta orthopaedica.* 2012; 83(2):121–128. [PubMed: 22489885]

Figure 1.
(1)- intramedullary component; (2)- transcutaneous component; (3)- SBIP-TS assembly of both components.

Figure 2.

(A) - Light microscopy photo of the porous titanium pylon in the collagen gel with autologous fibroblasts (shown by white solid arrows). Scale bar – 1 cm.

(B–D) - Scanning electronic microscopy of the porous titanium pylon seeded with autologous fibroblasts: (B) – control, non-treated pylon; (C, D) – pylon with fibroblasts on the 7th day of cultivation at $\times 600$ and $\times 1500$ magnification, respectively. The red solid arrows point to the cells inside the pylon.

Figure 3. Light and confocal microscopy of the autologous fibroblasts (Control) and cells induced into the osteoblast differentiation (Experiment) to assess the presence of the alkaline phosphatase (AP) (*purple color*) and osteocalcin (*green color*). Scale bar – 7.5 μm.

Figure 4.

(A)- intraoperative photograph of the intramedullary component of the pylon 8 weeks after implantation; (B) the procedure of attachment of the transcutaneous component to the pylon; (C, D) two views of the transcutaneous component after 2 months of operation.

Figure 5.

(A) - Radiography of the intramedullary component of the SBIP system 8 weeks after implantation. Red arrows point to the callus formation around the pylon. (B) - Radiography of the SBIP system after implantation of the transcutaneous component.

Figure 6.

Perfusion scintigramms for the animal #2 from the II group in 1, 2 weeks and 1 and 2 months after the implantation of the intramedullary component into the femur. Region of interest (ROI) was set on the segment of porous titanium pylon (*blue color*) and symmetric ROI was set on contralateral normal area as control (*yellow color*). Increased perfusion in the region of the porous pylon is shown starting from the 2 week. After 2 months of implantation there was nearly 2.5-fold increase the perfusion in the region of the porous pylon in comparison to the control non-operated limb.

Figure 7.

Box plot graphics of the index of uptake of the delayed image (DR) in groups I–V on the 1, 2, 4 and 8th weeks after implantation of the intramedullary component of the SBIP system. Boxes have the median observation in the center, and maximum and minimum observations, respectively at the upper and lower whisker boundaries. Each box corresponds to 3 observations.

Figure 8.

Three-phase scintigraphy for the control sham-operated animal #1 (A), animal #2 with the porous pylon (B), and animal #3 with porous pylon seeded with fibroblasts which were induced into osteoblast differentiation (C). Presented the results after 8 weeks from operation for the perfusion image (I), blood-pool image (II) and the uptake of the delayed image (III). ROI was set on the segment of porous titanium pylon (*blue*) and symmetric ROI was set on contralateral normal area as control (*yellow*). All the parameters (i.e., PI, BPR, and DR) are significantly increased in the animal with porous intramedullary pylon seeded with osteoblast-like fibroblasts. Thus the planar delayed image (III) of the animal with the pylon treated with fibroblasts induced into osteoblast differentiation (C) showed the gross area of the tracer uptake (red solid arrow) in the femur due to osteogenesis in comparison to the animals A and B.

Figure 9.

Longitudinal section of intramedullary part of the SBIP system implanted into the rabbit's femur (A), 0.3X, H&E. The histological analysis was performed 8 weeks after the operation. (B) Internal device pores filled with bone marrow tissue or fibrovascular connective tissue 10X, H&E. (C) Bone in direct contact with the pylon 10X, H&E.

Figure 10.

Longitudinal section of transcutaneous part (blue arrows) of the SBIP system attached to the intramedullary component (red arrows) (A), 0.3X, H&E. Histological assay was performed two months after the attachment of the transcutaneous component. (B) Dermal fibrovascular connective tissue along the side of exposed device. 4X, H&E. (C) Fibrovascular connective tissue inside the pores of the transcutaneous pylon. 10X, H&E.

Figure 11.

Schematic presentation of the fibroblasts function seeded into the pores of the titanium pylon in the collagen gel. Due to the production of various cytokines, including fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF) fibroblasts increase the processes of the angiogenesis and formation of the fibrovascular connective tissue inside the pores of the pylon. Fibroblasts can also form extracellular matrix that facilitates the migration of the host mesenchymal stem cells (MSCs) into the pores of the device.

Figure 12.

Representative scanning electron micrographs of the porous intramedullary pylon after 5 (**A**, **B**) and 30 days (**C**, **D**) of being inserted into the rabbit's femur. A higher magnification (**B**, **D**) shows fibrovascular connective tissue as well as host cells which migrated into the pores of the pylon (shown by red solid arrows). (**E**) Porous titanium implant without treatment with fibroblasts. (**F**) Porous pylon seeded *in vitro* with fibroblasts for 7 days.

Table 1

Displayed are the mean PI, BPR, and DR observations across the five groups and four time periods. Standard deviations are included in parentheses. The p-values are for the null hypothesis that there is no difference among the 5 groups (I-1 through I-5) for a given parameter-week combination. Significant p-values ($p < 0.05$) are indicated by an asterisk (*).

Group I-	PI 1	BPR 1	DR 1	PI 2	BPR 2	DR 2	PI 4	BPR 4	DR 4	PI 8	BPR 8	DR 8
1	1.1 (0.10)	1.19 (.10)	1.14 (0.09)	1.13 (0.13)	1.22 (0.13)	1.23 (0.12)	1.09 (0.08)	1.19 (0.10)	1.2 (0.11)	1.19 (0.02)	1.36 (0.07)	1.15 (0.09)
2	1.77 (0.08)	1.08 (0.11)	1.23 (0.07)	2.39 (0.53)	1.73 (0.13)	1.79 (0.23)	2.94 (0.24)	2.39 (0.25)	2.5 (0.41)	4.11 (0.18)	3.69 (0.35)	3.35 (0.26)
3	1.73 (0.12)	1.09 (0.02)	1.15 (0.06)	2.95 (0.35)	1.86 (0.08)	1.77 (0.23)	3.72 (0.14)	3.04 (0.06)	2.92 (0.16)	4.72 (0.31)	4.25 (0.16)	3.91 (0.20)
4	1.96 (0.08)	1.27 (0.18)	1.14 (0.08)	2.91 (0.63)	2.3 (0.60)	1.78 (0.54)	4.81 (0.41)	4.71 (0.43)	3.79 (0.24)	6.04 (0.25)	5.81 (0.29)	4.64 (0.72)
5	1.27 (0.09)	1.44 (0.18)	1.25 (0.18)	1.22 (0.18)	1.43 (0.11)	1.34 (0.05)	1.4 (0.12)	1.42 (0.12)	1.47 (0.13)	1.56 (0.06)	1.63 (0.08)	1.84 (0.14)
p-value	0.014*	0.081	0.742	0.025*	0.014*	0.127	0.009*	0.009*	0.010*	0.009*	0.009*	0.011*

Table 2

p-values from the Mann-Whitney test comparing group I-4 to whatever happens to be the next highest group in its parameter-week combination.

Scintigraphy parameters	Week 1	Week 2	Week 4	Week 8
PI	0.05*	0.5	0.05*	0.05*
BPR	N/A	0.13	0.05*	0.05*
DR	N/A	N/A	0.05*	0.2