Towards clinical translation of upscaled osteogenic grafts using human adipose tissue progenitors

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Sinan Güven

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auf Antrag von

Prof. Ueli Aebi, PhD
Prof. Ivan Martin, PhD
Prof. Mauro Alini, PhD
Dr. Arnaud Scherberich, PhD

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Prof. Dr. Martin Spiess (Dekan)
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Philosophisch – Naturwissenschaftlichen Fakultät Basel

PhD student Sinan Güven, MSc

PhD Committee:
Faculty responsible: Prof. Ueli Aebi, PhD
Thesis advisor: Prof. Ivan Martin, PhD and Dr. Arnaud Scherberich, PhD
Co-referee: Prof. Mauro Alini, PhD
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“It was the best of times, it was the worst of times
It was the age of wisdom, it was the age of foolishness
It was the epoch of belief, it was the epoch of incredulity
We had everything before us, we had nothing before us”

Charles Dickens

A Tale of Two Cities
Tissue engineering is an emerging strategy in medical field that focuses on regeneration or replacement of lost or damaged tissue and organs. Most of the tissues in human body have no or limited self renewal and regeneration potential which decrease with ageing. Today, bone is one of the most transplanted organs of human body every year. Bone defects or missing bone segments may occur due to trauma, injury, tumour removal and infections. Increased life span in modern society results in an increased demand for organ and tissue substitutes. So far, most of the tissue engineering approaches proposed solutions which only allow the generation of small scale of grafts with confined clinical relevance. Translation of tissue engineering and regenerative medicine approaches from bench to bedside faces with some vital issues that limit their immediate therapeutic applications. Providing autologous cells of clinical grade isolated from a relevant source to avoid potential clinical complications is the primary issue to be handled. Other than this, when enlarging the size of the engineered constructs vascularization of the graft upon in vivo implantation, the complexity and costs of the manufacturing protocols are among the other main problems. This thesis addresses possible solutions for limitations mentioned above by implementing stromal vascular fraction (SVF) cells from adipose tissue to provide pre-vascularization into up-scaled tissue engineered osteogenic constructs and by developing simplified approaches based on the coupling of reparative surgery to a streamlined cell isolation and intraoperative generation of osteogenic constructs from SVF cells.
A. INTRODUCTION

1. Bone

1.1 Bone repair

Bone is a complex organ in terms of structural organisation and functionality. Besides providing mechanical stability to the human body, bones play a role in the protection of internal organs like heart, lungs and brain, shape the body, play a major role in movement, control haematopoiesis by producing red blood cells and are responsible for many metabolic activities. Storing minerals like calcium and phosphorous, growth factors such as insulin growth factor, transforming growth factor and bone morphogenic protein, fatty acids, and heavy metals are also critical features of bone. Functioning as an endocrine organ is also among the extensive list of properties of bone. It secretes osteocalcin hormone which takes place in fat deposition and glucose regulation [1].

Bone has three major cell types, osteoblasts, osteocytes and osteoclasts [2]. Osteoblasts are derived from mesenchymal origin and are responsible for synthesising the organic extracellular matrix as well as regulating its mineralization. Osteocytes are post osteoblastic cells that form a network within ECM and function in the homeostasis of mineralization, signalling and mechanical sensing. Osteoclasts play role in resorbing the ECM of bone. They are of haematopoietic origin and together with osteoblasts, regulate the formation and remodelling of bone tissue.

Figure 1 Structure of bone.

http://academic.kellogg.edu/herbrandsonc/bio201_mckinley/skeletal.htm
Structurally, bone is comprised of two compartments [3]: compact (cortical) bone which is 80-90% mineralized tissue and provides most of the mechanical strength of bone and 15-25% mineralized trabecular bone that is primarily responsible for the metabolic activities and functions also as reservoir, as described above.

Bone defects or missing segments can result from trauma, injury, tumours and infections. Today, bone is one of the most transplanted organs of human body every year [4]. Increased life span of individuals in modern society results in an increased demand for bone repair [5]. Bone has a remarkable capacity to renew itself, which drastically decreases with age [3].

Healing of a bone fracture takes place in three steps. First, in the reactive phase, blood vessels adjacent to injury site constrict to stop bleeding and form a clot which allows only fibroblasts to survive and form loose granulation tissue. Secondly, the reparative phase is when fibroblasts in the granulose tissue differentiate into chondroblasts that produce hyaline cartilage. Accumulation of hyaline cartilage bridges the gap in the fracture and forms the callus. Hyaline cartilage is afterwards replaced with lamellar bone and this replacement is named endochondral ossification. Briefly, collagen matrix starts to mineralize, chondrocytes become hypertrophic and induce blood vessel ingrowth into the cartilagenous template. Chondrocytes are replaced by osteoblasts that start secreting bone extracellular matrix. As a final remodelling step, the newly formed trabecular bone is substituted with cortical bone. Osteoclasts start resorption of the trabecular bone, creating space for osteoblasts to deposit the newly compacted bone tissue [6].

![Figure 2. Bone repair mechanism (A) formation of hyaline cartilage, (B) lamellar bone and endochondral bone formation, (C) remodelling of newly formed bone, (D) compact bone [7].](image-url)
Another physiological pathway to regenerate bone tissue is through intramembranous ossification. In intramembranous ossification mesenchymal cells directly differentiate into osteoblasts. This process mainly occurs in flat bones like skull and mandible [8].

1.2 Gold standard in bone grafts
Despite the excellent self renewal capacity of bone in healing and regeneration, there often remains a need to support and guide the regeneration to reconstruct the defect. The bridging of the defect zone with callus and woven bone formation could be possible with natural mechanisms after restored alignment and stable fixation is provided. The gap can be bridged acutely by filling itself or continuously by distraction of the callus. These natural methods need a long time, and rigid fixation of the injury site however inadequate vascular supply might be the critical to obtain satisfactory results. Therefore, grafting will be definitely required after some clinical cases, such as resections of bone tumors, deformity corrections or large bone defects after comminuted fractures. Considering bone grafts, the clinically most relevant technique is microvascular transfer. Autologous cancellous bone is the best source for grafting and filling material. It can be harvested with its vascular pedicle, therefore, even the cells residing in the core of the graft may survive upon transplantation. Besides, they are osteoinductive, osteoconductive, osteoproliferative, angiogenic, and safe (regarding immunologic aspects) [9]. However, since these grafts are limited in their availability and the success of the procedure highly depends on the quality of the autologous tissue, not to mention the operator expertise, alternative approaches are desirable. Other potential limiting factors are morbidity at the harvesting site and/or inadequate material for extensive or multi-step reconstructions [8, 10, 11].

Alternatively, acellular bone allografts and synthetic biomaterials designed to substitute bone can also be used as defect-filling grafts. In large bone defects, since those materials lack vasculature and osteogenic cell, it takes a relatively longer time for them to be colonized and functionally engrafted. It has been shown that making those osteoconductive materials osteoinductive by incorporating signalling molecules that will recruit osteoprogenitors from the surroundings has a significant effect on reconstruction of the defect zone. However, clinical validation regarding efficacy and safety has not yet been achieved [12].
1.3 Tissue engineering

Drawbacks to the gold standard in bone healing have created a bridge between tissue engineering and the bone regeneration field. Advances in biomaterials and developments in cell biology could be a solution to satisfy the needs of custom prepared, ready to use, highly compatible treatments in bone regeneration. The tissue engineering approach aims to create in vitro designed systems which, upon transfer to the target organ, will enhance regeneration and healing [13]. In order to access the target organ successfully, tissue engineering tries to mimic its structural, mechanical, functional and biological properties. The tissue engineering paradigm can be summarized as thus: harvesting a biopsy from the healthy part of a target organ or site that contains stromal/progenitor cells, isolation and expansion of autologous cells or progenitor cells that are going to be directly used or differentiated, loading of the cells onto a carrier and then, either culture in vitro until certain maturation and/or transplanting directly to the defect site (Figure 3).

For engineering efficient and functionally successful osteogenic grafts, scaffolds for bone tissue engineering should provide sufficient mechanical stability, shape and porosity, considering the region of implantation. A scaffolding material can be of biological origin like collagen [14] and demineralized bone matrix [15] or synthetic in nature, such as porous metal [16], bioactive glass [17], synthetic polymers [18] or calcium phosphates like hydroxyapatite and tricalcium phosphate [19]. Scaffolds should also be osteoconductive, promoting and supporting osteoprogenitor cell attachment,
proliferation and differentiation and, therefore, bone formation [20]. But still, apart from composition, structure and bioresorbability, providing osteoinductive properties has remained one of the most challenging tasks for the development of bone graft substitutes [21].

Osteogenicity of the construct is provided by osteo progenitor cells and the mineralized matrix secreted by them. A large number of cells are demanded to generate tissue engineered grafts for clinically sized bone defects. Mesenchymal stromal cells have been shown to differentiate into osteogenic cells and produce bone [22]. Bone marrow is the most preferred source of mesenchymal stromal cells for bone tissue engineering. They can be easily be isolated from bone marrow aspirates, expanded, and differentiated in the presence of grow factors, hormones and other supplements like ascorbic acid [23] Differentiated cells should have high biosynthetic activity to support the development and integration into the host, express osteogenic markers to form real bone tissue and have a phenotypic stability to avoid nonspecific tissue development. A different and relatively newer source for mesenchymal stromal cells is adipose tissue [24]. Adipose tissue is much more easily accessed than bone marrow, more abundant and gives a higher yield of cells. Isolation of adipose tissue derived stromal (ATSC) cells causes a much reduced donor site morbidity as compared to bone marrow aspirates. Studies that compare ATSC and BMSCs indicate both similarities and differences in terms of surface markers and differentiation capacities in vitro and in vivo [25, 26].

2. Vascularization strategies in tissue engineering

In native tissue, cells are organized in a way that they can be supplied with oxygen, nutrients and signaling factors and be drained of their metabolic waste by endothelial-lined capillaries. The proximity of a capillary network therefore is vital for cell survival [27]. Vascularization is a key issue of tissue engineered grafts both in vitro and in vivo. In order to fabricate long lasting and functional tissues, one should first design the scaffold or carrier considering cell organization, select an ideal cell source for rapid vascular network formation and define an in vitro and in vivo graft maturation or implantation strategy. To overcome limitations related to vascularization and cell survival issues, different approaches were previously investigated:
2.1 Delivery of angiogenic signals

Angiogenesis is described as the formation of new blood vessels from already existing blood vessels by cell growth or sprouting. Angiogenic factors such as VEGF, FGF, PDGF and Ang1 are known to stimulate mobilization and recruitment of endothelial cells thereby inducing angiogenesis, vascular ingrowth and thus vascularization. Delivery of such angiogenic factors into tissue-engineered constructs can be done by direct addition, coating on the scaffold, controlled release by encapsulation into degradable micro- or nano-particles or the use of genetically modified cells, transduced to have sustained over expression of some of them. However, maintaining optimal concentrations of those specific proteins inside the graft or at the defect site of the host is challenging. With insufficient induction there might be a lack of improvement while excessive induction might lead to the formation of unstable vasculature. Such newly formed capillaries are often unstable, disorganized, leaky and hemorrhagic [28]. Newly formed capillaries are typically stabilized by pericytes and smooth muscle cells which are recruited through PDGF. More difficult can be to synchronize the dose and timing for more than one angiogenic factor. An indirect strategy is to stimulate cells that secrete such angiogenic factors by addition of sonic hedgehog homolog (SHH), hypoxia inducing factor 1 (HIF-1) [29] or bone morphogenic protein (BMP)-2, -4 or 6 [30]. In this case the dose of required angiogenic stimuli is regulated by the producing cell and often results in expression of the required physiological concentrations. Moreover, indirectly stimulated cells create a gradient of angiogenic factors which has been shown to be important for capillary morphogenesis and stimulation of the vascular ingrowth from the host. The procedure induces other proteins that also stabilize newly formed capillaries and play a role in the promotion of a functional vasculature. High costs and difficulties in clinical translations are the major disadvantages of both direct and indirect angiogenic factor delivery. Safety issues related to the use of transduced cells and to the control of the over expression remains a major limitation toward clinical applications.

2.2 Cellular approach

Using cells is another strategy to build the vasculature in vitro or in vivo in tissue-engineered constructs. Primary endothelial cells from different sources such as human umbilical vein, human dermal microvascular and peripheral blood have been co-cultured with fibroblasts, osteoblasts or mesenchymal stromal cells from bone marrow or adipose tissue in sequential culture conditions or simply by mixing cells together [31]. Endothelial cells alone are able to form capillary networks in vitro
and in vivo, which must be stabilized by pericytes (mural) shortly after formation. In vivo, pericytes interact with endothelial capillaries and enhance their function and stability. In tissue engineering, fibroblasts and mesenchymal stromal cells are easy to obtain and have been used as mural cells and in co-culture with endothelial cells to enhance vascularization of generated constructs. Co-culture of different cell types can be achieved in vitro to pre-vascularize the graft prior to implantation and is expected to create a vascular network which upon in vivo implantation is rapidly anastamosed with host vasculature [32]. The co-culture ratio of cell types, duration and culture conditions should be optimized for every cell types [27]. Besides cells that form vasculature, other cell types providing the functionality of the designed graft may often need different types of culture conditions for stimulation and differentiation [33]. Therefore, the implementation of these two aspects in one final product should be well optimized. On the other hand, sourcing of endothelial cells has ultimate importance in clinical perspectives. In tissue engineering research, HUVECs are the most frequently used endothelial cells which cannot be autologously sourced for clinical use. Endothelial progenitor cells originating from bone marrow are circulating in peripheral blood, having capacity to be used as an endothelial cell source for regenerative medicine [34]. However, those progenitor cells are very low in number and have to be expanded in vitro, which brings extra costs for the clinical translation. Freshly isolated adipose tissue-derived stromal cells, also known as the stromal vascular fraction (SVF), are a source for both endothelial cells and stromal cells. It has been shown that SVF cells can establish a pre-vascularization and differentiate into osteogenic cells and adipogenic cells, enabling large tissue engineered construct to be engrafted to the host tissue rapidly and functionally [35].

2.3 Biomaterial design

Achieving rapid and effective vasculature in tissue engineered construct also needs well designed scaffolds. Geometry, pore interconnectivity and scaffold material are the crucial points to be addressed considering the target organ [36]. Larger pores and higher interconnectivity significantly increase the vessel ingrowth in accordance with cell migration. The materials that enhance cell adhesion and migration and therefore also the vascularization should be considered [37, 38]. In some applications, the scaffolding material is coated with proteins such as collagen and fibronectin or chemically modified by immobilizing peptide motifs such as RGD, to promote cell seeding [39].
2.4 *In vivo* pre-vascularization

Another approach to vascularize tissue engineered constructs is to use living tissues. In *in vivo* conditions, host cells always tend to invade the implanted construct which brings the endothelial vasculature by branching and sprouting into the graft. Two possible strategies have been developed:

![Vascularization strategies in tissue engineering](image)

**Figure 4.** Vascularization strategies in tissue engineering a) delivering of angiogenic growth factors or cytokines, b) applying progenitor cells to the damaged area or to the construct, c) designing biomaterials that can favour vessel ingrowth or enhance vessel formation, d) implanting grafts that have been pre-vascularized *in vivo* with flap prefabrication or AV loop, e) generating novel strategies by combining two or more strategies. Adapted from [27].

1. **flap prefabrication:** Tissue engineered construct is implanted in a highly vascularized body part of the host, like muscle, that favors a rapid ingrowth of blood vessels and, once fully
vascularized, is transferred into the defect zone [40]. By using micro surgery, construct and blood vessels newly formed inside it during the pre-vascularization phase can be implanted at the defect site providing instantaneous perfusion of the whole construct. Major disadvantage of this approach is that it requires a two-step surgery and prior to the final implantation; the construct might need to be reseeded with cells of interest since a majority of cells inside the inner core of the construct most probably did not survive to the pre-vascularization step.

ii. AV loop formation: to shorten time of anastamosis of engineered construct, a pre-existing vascular network is integrated inside or around the graft by surgically creating an arterio-venous (AV) loop and by placing graft in close contact with this vascular bundle [32, 41]. The outgrowth of capillaries from the bundle forms a microvascular network in the construct and at the time of transferring to the required defect zone, the graft is harvested with the supplying artery making immediate perfusion of the entire construct. Complicated procedure and limitation of geometry of the construct make this strategy less appealing.

2.5 Combined strategies
It should be stated that there cannot be one ideal strategy to bring vascularization in tissue engineering applications. As different types of tissues and defects needs different treatments, vascularization approaches described above could be preferred one to another according to the feasibility. On the other hand, some of these techniques can be combined, increasing the efficiency and eliminating some limitations. For instance the angiogenic factors can be delivered in combination with endothelial progenitor cells that are co-cultured with mesenchymal progenitor cells. In this case, the dose of recombinant angiogenic proteins can be reduced which may reduce the effects of unstable vasculature formation like leaky vessels and also result in a significant decrease of the related costs. Specially-developed hydrogels mimicking the native tissue environment with defined stiffness, immobilized adhesive peptides and supplemented with growth factors can be seeded with different timing to provide optimal capillary formation and stability [38, 42].
3. Stem cells

Stem cells are defined by their self-renewal capacity, producing at least one identical stem cell and to produce one to undergo lineage differentiation [43]. Regarding their potency to produce one or more lineages, they can be identified as i) totipotent i.e. able to produce all cells and tissues of an organism, ii) pluripotent, having capacity to produce cells and tissues from all three germ layers – ectoderm, mesoderm and endoderm, iii) multipotent stem cells with the ability to produce more than one cell lineage or iv) unipotent which can differentiate only into a single cell phenotype [44].

Mesenchymal stem cells (MSC) are multipotent and can be found in most of the tissues in human body. However, regarding the isolation, availability and clinical applications the most relevant sources are bone marrow, skin, placenta and adipose tissue [45]. The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy have proposed three criteria to define MSC; (1) isolated cells should adhere on tissue culture plates, (2) more than 95% of adhering cells in the culture should express CD105, CD73 and CD90; and they should lack expression of CD34, CD45, CD14 or CD11b, CD79a or CD19 and HLA-DR markers, (3) MSC should differentiate into osteoblasts, adipocytes and chondroblasts in vitro [46].

3.1 Bone marrow stromal cells

Bone marrow mesenchymal stromal cells (BMSC) have been shown to have self-renewal capacity [47] and osteogenic potential [48] and been extensively used in bone tissue engineering since decades. However the frequency of putative stem cells is less than 0.01% [49] therefore requiring extensive in vitro expansion phases to be applied in tissue engineering applications. Various in vitro studies, preclinical and more recently human clinical trials have demonstrated the immunotolerance and immunomodulatory properties of allogeneic BMSC [50], [51]. Some clinical case studies showed that the seeding of autologous BMSCs into porous hydroxyapatite scaffolds and implantation in various limbs with 4-7 cm critical size defects can heal non-union fractures without any adverse effects [52]. However this strategy is not superior to traditional methods of bone grafting where this recovery is much faster. Bone tissue engineering using BMSC most of the time lacks enough vascularization in the case of large grafts [41]. To overcome this problem, many vascularization strategies have been developed. The most widely explored technique is where BMSCs are combined with endothelial cells from various different sources such as umbilical cord blood [53], buffy coat-
derived endothelial progenitors [54], human umbilical vein [55] or dermal microvasculature [56] to create vascular structures inside the osteogenic grafts that can anastamose with host vasculature upon implantation. Another approach aims to solve vascularization problems by enhancing the ingrowth of host vasculature into the graft. In this approach BMSCs are transfected with VEGF gene alone [57] or together with BMP-2 [58] thereby creating angiogenic zones through the graft by releasing angiogenic proteins which recruit endothelial progenitors from the surrounding tissue that can vascularize the construct. Although there are some promising studies, irrelevant cell sources, long and demanding in vitro cell manipulations and safety concerns put those approaches still far from regular clinical applications.

### 3.2 Adipose tissue derived stromal cells

Adipose tissue contains not only adipocytes but it is also an excellent source for progenitor cells [24]. Studies showed that subcutaneous adipose tissue homes hematopoietic, mesenchymal and vascular stromal cells that can be harvested with minimal invasive techniques isolated with enzymatic digestion and thereafter be used in stem cell research. The freshly isolated heterogeneous cell population from adipose tissue is named the stromal vascular fraction (SVF) which can be directly used or plated for expansion to amplify adherent population known as adipose-derived stromal/stem cells (ASC) [12]. The ASC present in the SVF are characterized as CD34⁻/CD105⁻ ASC. In addition to ASC, the SVF also contains blood-derived cells, such as erythrocytes and leukocytes characterized by expression of the pan-hematopoietic marker CD45, and other adipose-derived cells, such as vascular endothelial (known to be CD34⁻/CD31⁺) and mural cells [59]. ASC have multipotent stem cell capacities and can differentiate into osteogenic, chondrogenic, adipogenic and neurogenic cell types, attracting significantly high interest in regenerative medicine research and applications. Compared to bone marrow stromal cells, adipose tissue derived cells have higher clonogenic capacity that makes them more favorable in terms of stem cell yield [60].

Being more available and having various potential harvesting zones (abdominal, ties, breast etc.) lead many studies to try to characterize adipose tissue-derived stem cells and develop standardized harvesting methods for them. Despite high interest in ASC research, no true correlations and clear predictive indicator(s) could be defined between cell characteristic, cell yield, body mass index (BMI), age and harvesting site [61]. Adipose tissue-derived progenitors are widely used in reconstructive
surgery studies like for breast augmentation [62, 63]. Lipoaspirates are combined with SVF cells and implanted to augment breast volume and showed much more rapid and efficient engraftment. Also, clinical trials on wound healing of diabetic patients and radiation therapy tissue damage showed better healing by using SVF cells [64, 65]. The heterogeneous nature of SVF cells is hypothesized to enhance this engraftment via containing endothelial cells which contribute to vascular network formation. Clinical studies in orthopedics aim to involve ASCs and SVF cells in bone repair cases using their high capacity to differentiate into osteogenic lineage [12]. Clinical trial has shown that using autologous ASCs in combination with BMP-2 and β-tricalcium phosphate in low weight-bearing maxillofacial defect generate fully integrated and vascularized implants [66]. Some other clinical trials with adipose tissue-derived progenitors are initiated in the treatment of immune diseases like multiple sclerosis [67] and Crohn's disease [68, 69, 70].

4. Manufacturing challenges for bone tissue engineering

The major driving force behind stem cell research is focused to understand the biology behind them and place them as novel and efficient therapeutic agents in diverse fields of medicine and pharmaceutical applications. Some of the trials showed success in long term. However, following are many variables that have to be optimized to make stem cell therapies to become standardized and preferable to traditional treatments. Optimal cell source, in vitro culture conditions, scaffolds, dosage of differentiating factors, maturation time and implantation technique and costs are major items [1, 71, 72]. Sequential surgeries and long hospitalization time decrease donor comfort and increase the cost of the therapies. Most tissue engineering applications include implantation of autologous cells that are first isolated and expanded in good manufacturing practice (GMP) compliant laboratories. GMP facilities and running the protocols are costly and requiring big investments both in infrastructure and trained staff. Despite strict regulations, these manufacturing techniques still carry a risk of contamination and show limited reproducibility. An intraoperative approach in stem cell-based therapies may skip many of these in vitro handling phases and can complete all surgical manipulations in one step. Getting a biopsy, isolating cells and generating tissue engineered graft can be done in the same operation room, decreasing dramatically hospitalization time, costs and favoring patient comfort.
Automated systems are slowly being introduced for cell isolation and graft manufacturing in order to reduce costs and variables [73, 74]. The development of closed, aseptic and automated devices, allowing the isolation of adipose cells outside a GMP facility at reasonable costs, for instance inside an operation theater, and requiring minimal operator intervention, could overcome these limitations.

5. Aim of the thesis

Translation of tissue engineering and regenerative medicine approaches from bench to bedside is struggling with some key issues that limit their straightforward therapeutic applications. Providing autologous cells of clinical grade isolated from a relevant source to avoid possible clinical complications is the first issue to be considered. Besides that, so far, most of the tissue engineering approaches proposed solutions which only allow the generation of small grafts. The reasons for that are first vascularization problems upon in vivo implantation, when enlarging the size of the constructs and second the complexity and costs of the manufacturing protocols. This thesis addresses possible solutions for these two limitations by implementing stromal vascular fraction (SVF) cells from adipose tissue to provide pre-vascularization into up-scaled tissue engineered osteogenic constructs and by developing simplified approaches based on the coupling of reparative surgery to a streamlined cell isolation and intraoperative generation of osteogenic constructs from SVF cells.

Chapter I focuses on generating large osteogenic grafts using SVF cells and hypothesizes that the presence of endothelial progenitors could enhance the construct engraftment and uniformity of bone tissue formation in vivo. SVF cells have been shown to have intrinsic vascularization capacity making them able to form stable blood vessels within tissue engineered grafts in vivo [36]. Initially this chapter uses this feature of SVF cells as innovative strategy to avoid endothelial cell - mesenchymal cell co-culturing for formation of capillary network, thereby validating SVF cells as a more clinically relevant cell source for vascularization of large grafts in tissue engineering. Therefore, pre-vascularized large osteogenic grafts were generated aiming to anastamose with the host vasculature rapidly upon ectopic in vivo implantation in rats in order to provide better engraftment in terms of cell survival and integration. Finally as a result of rapid engraftment more uniform bone formation throughout the large grafts could be expected.
Thereafter, Chapter II looks for relevant and applicable ways to translate this outcome into more clinically relevant scenarios. The clonogenic cell yield after isolation of SVF cells is relatively high compared to other mesenchymal stem cell sources suggesting that they can be used directly without any in vitro manipulation. In this chapter the goal is to skip in vitro differentiation and expanding phases of SVF cells by applying an intraoperative approach. To achieve this, cell isolation and graft manufacturing is followed by direct ectopic implantation where major read out is success in engraftment and bone tissue formation in the engineered graft. This chapter focuses on the effects of key parameters such as cell seeding density and the dose of osteoinductive stimuli, namely bone morphogenetic protein-2 (BMP-2), on the success of the approach.

Finally Chapter III focuses on how to streamline this intraoperative approach by automating the manual cell isolation steps. Successful translation of laboratory developed methods into the clinics as an alternative or routine treatments needs to be validated to reach a large degree of standardization. An automated device can solve operator and procedure variability in SVF cell isolation; however phenotype and the function of isolated cells should not be altered. In this chapter, the protocol of the automated SVF cell isolation device (Sepax™) is validated and cells obtained in this way are compared to manually isolated SVF cells both phenotypically and also in terms of cell yield, viability, clonogenicity, and differentiation capacity.
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B. Experimental work

Chapter I

Engineering of large osteogenic grafts with intrinsic vasculogenic capacity

Engineering of large osteogenic grafts with rapid engraftment capacity using mesenchymal and endothelial progenitors from human adipose tissue

Enclosed is the pdf file of the paper published in Biomaterials, 2011;32:5801-5809
Engineering of large osteogenic grafts with rapid engraftment capacity using mesenchymal and endothelial progenitors from human adipose tissue

Sinan Güven a, Arne Mehrkens a, Franziska Saxer a, Dirk J. Schaefer a, Roberta Martinetti b, Ivan Martin a, s, Arnaud Scherberich a

a Departments of Surgery and of Biomedicine, University Hospital Basel, Hebelstrasse 20, CH-4031 Basel, Switzerland
b Fino-Ceramica Fanezza S.p.A., Via Rivugnana, 186, 48038 Fanezza, Italy

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A B S T R A C T

We investigated whether the maintenance in culture of endothelial and mesenchymal progenitors from the stromal vascular fraction (SVF) of human adipose tissue supports the formation of vascular structures in vitro and thereby improves the efficiency and uniformity of bone tissue formation in vivo within critically sized scaffolds. Freshly-isolated human SVF cells were seeded and cultured into hydronapagate scaffolds (1 cm-diameter, 1 cm-thickness) using a perfusion-based bioreactor system, which resulted in maintenance of CD34+/CD31+ endothelial lineage cells. Monolayer-expanded isogenic adipose stromal cells (ASC) and age-matched bone marrow stromal cells (BMSC), both lacking vasculogenic cells, were used as controls. After 5 days in vitro, SVF-derived endothelial and mesenchymal progenitors formed capillary networks, which anastomosed with the host vasculature already 1 week after ectopic nude rat implantation. As compared to BMSC and ASC, SVF-derived cells promoted faster tissue ingrowth, more abundant and uniform bone tissue formation, with ossicles reaching a 3.5 mm depth from the scaffold periphery after 8 weeks. Our findings demonstrate that maintenance of endothelial/mesenchymal SVF cell fractions is crucial to generate osteogenic constructs with enhanced engraftment capacity. The single, easily accessible cell source and streamlined, bioreactor-based process makes the approach attractive towards manufacturing of clinically relevant sized bone substitute grafts.

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1. Introduction

Bone loss following traumatic, neoplastic or degenerative events often requires the availability of large structural grafts, up to few cubic centimeters in size [1]. The gold standard of bone substitutes, namely autologous bone, is generally associated with relevant morbidity at the donor site [2], is available in limited amounts, and most often is not feasibly shaped into large implants of defined sizes and shapes [3]. The engineering of osteogenic grafts by the association of osteoprogenitor cells with porous scaffold materials may in principle overcome the limitations above, but has yet to face several challenges to demonstrate a reproducible clinical benefit [4]. Among the most critical limitations of engineered osteogenic constructs, especially those in a large size, is their reduced efficiency of engraftment. By 'engraftment' we refer here to the implant penetration by host tissue and connection with host blood vessels, ultimately regulating the efficiency and spatial uniformity of bone tissue formation. Indeed, when cylindrical scaffolds of up to 20 mm diameter were uniformly seeded with bone marrow stromal cells (BMSC) and implanted in an experimental rabbit model, bone tissue formation was restricted to the outer 1.8 mm region after 12 weeks, and the vast majority of the scaffold space was empty or necrotic [5]. In order to improve and accelerate engraftment of engineered tissues, the attractive strategy to co-culture vasculogenic cells along with the tissue-specific progenitors was successfully validated in the context of muscle regeneration [6]. Several studies have reported the feasibility of co-culturing osteoprogenitor and endothelial cells within 3D scaffolds and have characterized the functional crosstalk between the two cell types [7]. However, it is yet still to be demonstrated that this strategy can effectively enhance engraftment of large engineered, osteogenic constructs [8].

We have previously shown that progenitor cells freshly isolated from human adipose tissue, typically referred to as stromal
vascular fraction (SVF) cells, can be used to generate osteogenic grafts with intrinsic vasculogenic capacity if seeded and cultured for 5 days within hydroxyapatite porous scaffolds by using a perfusion-based bioreactor system [9]. The study critically established that human SVF-derived cells are effectively competent in generating frank bone tissue in vivo, even in the absence of exogenous osteoinductive signals. Moreover, the findings indicated that the endothelial lineage fraction within SVF cells retained the ability to form in vivo functional blood vessels, connected with the host vasculature [9]. In the present work, we first aimed at characterizing the phenotype, function and structural organization of the endotheial and mesenchymal progenitors from SVF during in vitro culture. We then used human SVF cells to engineer critically sized grafts and hypothesized that the presence of endothelial progenitors could enhance the construct engraftment and uniformity of bone tissue formation upon ectopic implantation into nude rats.

2. Materials and methods

2.1. Cell isolation and culture

Adipose tissue, in the form of liposuction or excision samples, was obtained from 15 healthy donors following informed consent and according to a protocol approved by the local ethical committee (EBRB Ref. 780/07). All donors were females between 20 and 65 years of age. Finely minced excision samples or liposuction samples were digested with 0.075% collagenase type II (355 U/mg, Worthington, Lakewood, NJ, USA) at 30–40 °C for 1–2 h. After centrifugation at 390 for 10 min, the lipid-rich layer was discarded and the cellular pellet was washed once with phosphate buffered saline (PBS, Gibco, Invitrogen, Grand Island, NY, US). Red blood cells were lysed by incubation for 2 min in a solution of 0.15% ammonium chloride. 1 mM potassium hydrogen carbonate (both Merck, Darmstadt, Germany) and 0.1 mM EDTA (Fuka Analytical, Sigma–Aldrich Chemie GmbH, Buchs, Switzerland). The resulting SVF cells were then resuspended in complete medium (CM), consisting of -MEM supplemented with 10% of fetal bovine serum (FBS), 10% FBS, 1% Sodium pyruvate and 1% Penicillin-Streptomycin-Glutamine (100-0) solution (all from Gibco), stained with crystal violet (Sigma) and counted in a Neubauer chamber. For monolayer expansion, SVF cells were seeded at a density of 2 × 10^3 cells/cm^2 onto tissue culture plates, cultured in CM supplemented with 5 mg/mL FGF-2 (R&D Systems) and serially replated in new dishes at a density of 3 × 10^3 cells/cm^2 when reaching confluence. The expanded adipose-derived cells will be hereafter referred to as adipose stromal cells (ASC), to make a distinction from the population of freshly derived SVF cells. Human bone marrow-derived mesenchymal stromal cells (BMS) were isolated and expanded from bone marrow aspirates of 3 donors (2 males of age 45 and 46 and one female of age 21) as previously described [40] and used as reference for a standard osteogenic cell population.

2.2. Cell characterization and sorting

The number of cloneogenic cells, generally referred to as colony-forming unit-fibroblasts (CFU-f), was determined by plating 5 × 10^5 SVF cells per 78 cm^2 petri dishes. Cells were cultured for 10–14 days in CM containing 5 ng/mL FGF-2. After fixation with 4% formalin for 10 min and staining with crystal violet, the colonies were counted and normalized to the number of plated cells. The phenotype of SVF cells and ASC was determined by cytofluorometric analysis with fluorescence-conjugated antibodies to human CD34 (from Abbi Scienze, Oxford, United Kingdom), CD90, CD31, CD34, CD146, CD45, CD14 and CD37. Isotype IgGs were used as control (all from BD Biosciences). Cells in suspension were incubated for 40 min with the different antibodies at 4 °C in CM, washed with PBS, resuspended in FACS buffer (PBS, 0.5% Human Serum Albumin, 0.5% Nu EDTA) and analyzed with a FACS calibur flow cytometer (BD Biosciences, USA). In some experiments, SVF cells were separated by using a FACs sorter (BD Influx cell sorter, BD Biosciences USA) based on staining intensity levels for human CD34 (APC-conjugated) and CD31 (FITC-conjugated). Osteoblastic differentiation capacity of SVF cells, ASC and BMS was assessed by 3 weeks' culture in CM supplemented with 10 ng β-glycerophosphate and 0.05% ascorbic acid (osteogenic medium, OM). Cells cultured with CM containing FGF-2 were used as a control. Deposition of mineralized matrix was detected by alizarin red staining, as previously described [9].

2.3. Assessment of cell vasculogenic capacity in vitro

The in vitro formation of vascular structures was tested by seeding unfractioned SVF cells, sorted CD34+/CD31- or CD34^{-}/CD31- populations from SVF cells, or ASC onto (i) Matrigel® (BD Biosciences)-coated culture plates, (ii) rapid-prototyped grid-shaped polylactic-co-glycolic acid (PLGA) scaffolds (2 mm × 2 mm) with a thickness of 1 mm, kindly provided by the Institute for Material Research and Macromolecular Chemistry, Freiburg, Germany, or (iii) hydroxyapatite–based Engineered® scaffolds (Fincaroma-Faenza, Faenza, Italy). Seed cells were cultured for 5–6 days in CM supplemented with 5 ng/mL FGF-2. Capillary network formation on Matrigel was observed with a phase contrast microscope. Constructs were analyzed by scanning electron microscopy (SEM) or by fluorescence microscopy. For SEM, cell-seeded constructs were fixed overnight at 4 °C with 4% formaldehyde and washed with PBS. Samples were gradually dehydrated with 30–50–70–90–100% ethanol, coated with gold and imaged with a Philips XL 30 ESEM microscope. For fluorescence microscopy, constructs were stained with a PE-conjugated anti-human CD31 antibody (from BD Biosciences) for 40 min at 4 °C, washed with PBS and observed using an iX50 fluorescence microscope (Olympus, Japan). Some constructs were assessed by confocal laser scanning microscopy (LSM710, Zeiss, Germany), following combined staining with anti-human CD31 (DakoCytomation, Denmark) and anti-human desmin (ABCum, UK) antibodies, followed by Alexa-conjugated series secondary antibodies (Invitrogen, Molecular Probes, USA) and with DAPI for nuclear structures.

2.4. Generation and analysis of large size ceramic-based scaffolds

Hydroxyapatite scaffolds (Engineered®, Fincaroma- Faenza, Faenza, Italy) in the form of large, porous cylinders (1 cm diameter × 1 cm thickness) were placed into custom produced chambers of a previously developed perfusion-based bioreactor system [11] and wetted with culture medium prior to cell seeding. 1.2 × 10^6 SVF cells or 3 × 10^6 either ASC or BMS were resuspended in CM supplemented with desamethasone, acetic acid and FGF-2 and perfused in alternating directions at a flow rate of 1 mL/min through the scaffold pores for 5 days, as previously described [9]. In order to assess the cell seeding homogeneity and viability, some constructs were stained with methyl-blue and observed under a microscope either at day 1 or at day 5, i.e. at the time of the in vivo implantation, according to the manufacturer's instructions. In order to extract cells, some constructs were perfused for 5 min with a 0.15% collagenase solution inside the perfusion bioreactor, rinsed with PBS and perfused with trypsin-EDTA for 5 min. Retrieved cells were assessed cytofluorometrically for the expression of CD31, CD45, CD43, CD90, CD105 and CD146 as described above. In order to assess osteogenic, vasculogenic and engraftment capacity, some constructs were implanted into nude rats as described below.

2.5. In vivo studies

Cell-seeded ceramic cylinders or fibrin hydrogels, generated as described above, were implanted subcutaneously in nude rats (CBA/Nu Charles-River, Germany) as previously described [9]. At the indicated time points, the animals were sacrificed by inhalation of CO₂. Maintenance of animals, surgical procedures and sacrificing methods were all performed in agreement with Swiss legislation and according to protocolapproved by the local veterinary office ("Kantonales Veterinäramt Basel-Stadt", permissio N°2167). Harvested ceramic-based constructs were dissected along the vertical axis, fixed overnight with 15% paraformaldehyde, subjected to a slow decalcification process by incubation in a solution of 7% w/v EDTA (Fuka Analytical, Sigma–Aldrich, Buchs, Switzerland), 10% w/v sucrose (Sigma–Aldrich) at 37 °C on an orbital shaker for 7–10 days and paraffin embedded.

2.6. Histological analysis

Histological sections (7 μm thick) of explanted constructs were first stained with haematoxylin/eosin to quantitatively assess the distribution of the newly formed tissue and of the bone nodules, thereby, microscopical pictures of the central sections of the full size constructs were taken. For tissue distribution, the distance from the surface of the construct to the deepest part of the tissue was measured. At least 6 independent constructs per experimental group were analyzed, performing 10 measurements per construct cross section. For the quantification of bone tissue formation, the number of pores exhibiting characteristic osteicle structures was counted and normalized to the total number of pores in the sections. In addition, the shortest distances from the deepest region of every pore containing bone tissue to the scaffold periphery were measured and their distribution plotted. In order to identify cells of human origin in the large constructs implanted in rats, sections were stained for human-specific Alu sequences by using the Zynofit CMV chromogenic in situ hybridization (CISH) Kit (Zytovision GmbH, Bremerhaven, Germany). Human and rat skin sections were used as positive and negative controls, respectively. Immunostaining for bone sialoprotein (BSP) was performed by using an anti-human BSP antibody (A22322, A22322, Immunodagnostik AG, Bensheim, Germany) followed by incubation with ABC-alkaline phosphatase complex kit (Dako, Glostrup, Denmark). The presence of blood vessels of human origin was assessed by using a biotin-conjugated anti-human CD31 specific antibody (Chemicon, Temecula, USA), as previously described [9].
3. Results

3.1. Characterization of freshly-isolated SVF cells and in vitro expanded ASC

Analysis of SVF cells freshly isolated from 4 donors indicated the presence of 23 ± 7% of mesenchymal lineage cells, as defined by the co-expression of CD90 and CD73 (Fig. 1A), and of 24 ± 8% endothelial lineage cells, positive for both CD31 and CD34 (Fig. 1B). SVF cells also comprised 35 ± 10% of hematopoietic cells, as assessed by the expression of the pan-hematopoietic marker CD45 (data not shown). These data are in general agreement with a previous report from our group [9] and other recent studies [12,13]. Upon monolayer expansion and repeated medium changes, mesenchymal lineage cells were selected over endothelial and hematopoietic ones. After 2 passages in culture, the great majority of ASCs were positive for CD73 (97.8 ± 2.5%) and CD90 (98.5 ± 1%) (Fig. 1C), whereas CD31 (Fig. 1D) or CD45-positive cells (data not shown) were negligible. Both SVF cells and ASC, when cultured in the presence of OM for 3 weeks, produced a mineralized extracellular matrix, indicative of an osteoblastic differentiation capacity, similar to BMSC (data not shown).

To investigate the clonogenic, osteogenic and vasculogenic potential of specific SVF subpopulations, CD34+/CD31−, CD34+/CD31+, and CD34+/CD31+ cells were sorted. As compared to unsorted SVF cells, CD34+/CD31+ cells displayed a similar capacity to deposit mineralized matrix when cultured in OM, as well as a similar CFU-f frequency (Fig. 2A and B). Instead, the other two subpopulations failed to produce mineralized matrix and contained a minimal percentage of CFU-f. SVF cells seeded onto Matrigel®, a highly standard assay of angiogenic capacity, formed a capillary network after 5 days of culture in the presence of FGF-2, whereas no capillary structures were observed with ASC (data not shown).

We next assessed the capacity of SVF cells, sorted populations from SVF and ASC to establish vascular structures in a more challenging model, namely within the void spaces of a porous scaffold. In order to simplify visual inspection and histological processing, a rapid-prototyped PLGA material was selected. After 5 days, SVF cells formed tubular structures positive for CD31, embedded within a high cell density tissue, as identified by DAPI staining, and in some instances in direct contact with cells positive for desmin, a pericytic marker (Fig. 2C). Capillaries were homogenously distributed throughout the scaffolds and had diameters in the range of 10 µm. None of the sorted populations was able to form similar tubular structures. Even the CD34+/CD31− population, including endothelial cells, resulted only in scattered clusters positive for CD31 (Fig. 2D). Ramified tubular structures were formed again throughout the construct by combining the CD34+/CD31− mesenchymal cells with the CD34+/CD31+ endothelial cells (Fig. 2E).

Scanning electron microscopy (SEM) documented the formation by unsorted SVF cells of long (mm-scale) vascular-like structures (Fig. 3A) with tubular shape and features suggestive of an open lumen with an average diameter of about 10 µm (Fig. 3B). Fenestrated motifs, highly similar to transmural pillar formation - a
3.2. Cell culture within porous ceramic scaffolds

In order to investigate the SVF capacity to form and vascularise large bone tissue grafts, we introduced a model based on scaffolds in a clinically relevant size (i.e., 1 cm diameter, 1 cm thick cylinders) and containing a mineral component (i.e., hydroxyapatite, HA), critically required to support ectopic bone formation by osteoprogenitor cells [8]. To achieve a homogenous seeding inside these materials, $1.2 \times 10^7$ SVF cells, or $3 \times 10^6$ expanded ASC from the same donors, were repeatedly perfused through the scaffold pores in alternate directions using a bioreactor system. The number of SVF cells and ASC was selected in order to seed a similar number of mesenchymal lineage cells, which was around four times higher in ASC than in SVF cells (according to data shown in Fig. 1). MTT staining of the cell-scaffold constructs after the first and the fifth day of perfusion culture was rather homogeneous (Fig. 4A and B). Cytofluorimetric analysis of extracted cells confirmed that the different SVF subpopulations negatively or positively stained for CD34/CD31 were still present inside the HA porous scaffolds after the perfusion seeding and 5-day culture (Fig. 4C). Therefore, this experimental group will be hereafter referred to as 'SVF', despite the in vitro cell processing and short-time culture. SEM analysis of the seeded HA constructs exhibited long tubular structures with features suggestive of open lumens, an average diameter of 10 µm and fenestrated motifs (Fig. 4D–F), similar to the ones observed on PLGA scaffolds.

3.3. In vivo analysis

Large HA cylinders seeded and cultured for 5 days with either SVF cells from 4 different donors, duplicate of those analysed above, or with expanded ASC from the same donors, were then subcutaneously implanted in nude rats. Scaffolds free of cells or seeded with BMSC were used as additional controls. After 1, 2 or 4 weeks in vivo, the penetration of tissue in SVF-seeded constructs was statistically significant deeper ($p < 0.05$, one-way ANOVA with Bonferroni's post-hoc tests, $n = 9$ measurements per condition from 3 independent experiments) than in all other experimental groups (Fig. 5A–D). The tissue formation in SVF cells-seeded constructs was concomitant with the presence of capillaries formed by hCD34^+ human endothelial cells, along with vessels of murine origin (Fig. 5E, respectively arrows and circles). ASC- and BMSC-seeded constructs did not show any positive staining for hCD34 (data not shown). The human capillaries were anastomosed to the host vasculature, as documented by the presence of erythrocytes in their lumens. Implants based on ASC or BMSC were fully colonized only after 8 weeks of implantation, and cell-free scaffolds maintained an empty core, void of any tissue, up to the last experimental time (Fig. 5I). After 8 weeks, both SVF cell- (Fig. 5F)

![Image](image_url)

Fig. 2. In vitro functionality of cell subpopulations. Representative alizarin red staining following culture with osteogenic medium (OM) or complete medium (CM) (A) and colony-formation efficiency tests (B) of stromal vascular fraction cells, unsorted (SVF) or sorted based on CD34 and CD31 expression. Data are from $n = 3$ donors. (C) Confocal microscopy following immunofluorescence staining for CD31 (red), desmin (green) and DAPI (blue) of unsorted SVF cells cultured for 5 days on PLGA scaffolds. (DE) Immunofluorescence for CD31 of SVF cells sorted as CD34^+CD31^- (D), alone (E) or combined with sorted CD34^+CD31^- (E) following 5 days culture on PLGA scaffolds. Pictures presented are representative of all 3 donors tested; higher magnifications are shown on the right of the pictures to highlight structural features. Scale bars – 30 µm (C) or 250 µm (DE). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
and ASC-seeded constructs (data not shown) displayed a homogeneous staining for Alu sequences, indicating that the implanted cells survived. Moreover, analysis of the SVF cell-based explants at the deepest front of tissue formation over time indicated the presence of Alu positive cells and of human endothelial cells, which reached the construct core after 4 weeks (Supplementary Figure 1, left and central columns).

After 8 weeks, constructs seeded with SVF cells from the 4 donors displayed formation of bone tissue within 18.5 ± 7.5% of the pores (Fig. 6A–C), with the bone matrix progressively filling the pore space starting from the area in contact with the ceramic material. Bone formation had a typical structure/cell morphology previously reported for small-scale constructs [9] and was accompanied by a temporally increasing amount of areas positively stained for BSP (Supplementary Figure 1, right column). Constructs seeded with ASC were void of any bone tissue, with the exception of one osteicle found in a peripheral pore of one construct (Fig. 6D, E).

Cell-free scaffolds, as expected, did not exhibit evidences of bone tissue (Fig. 6E). BMSC-seeded constructs showed relevant bone formation but in a significantly lower percentage of pores (p = 0.038, T-test, n = 6 measurements from at least 3 independent experiments) than SVF-seeded ones (Fig. 6C) and strictly limited to the most peripheral part of the constructs (Fig. 6F,G). The distribution of bone formation inside SVF- and BMSC-seeded constructs as a function of the distance from the outer surface quantitatively confirmed the morphological observations. In fact, bone tissue formed by BMSC was confined within 1.5 mm from the outer periphery, whereas SVF-seeded constructs exhibited bone formation up to a 3.5 mm depth from the surface (Fig. 6H).

4. Discussion

In this study, we have demonstrated that in vitro generation of organized vascular structures by SVF cells from human adipose tissue critically requires the cooperation of both endothelial and mesenchymal progenitor fractions. We further identified that a 3D model based on a porous scaffold and perfusion bioreactor system allowed maintaining in culture both SVF subpopulations, as opposed to the standard 2D culture in Petri dishes. The system resulted in the engineering of large 3D osteogenic and vasculogenic grafts with increased efficiency of engraftment and ultimately more homogenous bone tissue formation after ectopic implantation in nude rats.

The presence of vasculogenic cells within the adipose tissue-derived SVF fraction was first proposed by Miranville et al. [15] and thereafter confirmed by different groups [16–18], describing the presence of a CD34+/CD31- cell population with characteristics of endothelial progenitors and the capacity to rescue hindlimb ischemia. We have also recently identified that human SVF cells include endothelial lineage populations which can form functional blood vessels when ectopically implanted in nude mice [9,19,20]. In the present study, sorting of SVF cells based on both CD31 and CD34 allowed to determine that (i) the clonogenic mesenchymal progenitors predominantly reside in the CD34+/CD31- fraction, and (ii) the formation of tubular vascular structures requires the availability of both CD34+CD31- and CD34+CD31+ cells. Together with the perivascular detection of mural cells positive for desmin and negative for CD31 (Fig. 2C), these results suggest that the CD34+CD31- fraction includes not only osteoprogenitor cells, capable to produce mineralized matrix, but also cells with a pericytic function, necessary for stabilization of newly formed endothelial capillaries [21]. These data are also in accordance with a recent study showing that ASC co-cultured with primary endothelial cells from umbilical cord can establish vascular-like structures in vitro [22] and with the general recognition that putative mesenchymal stem cells from various tissues derive and share the function of pericytic/mural cells [23]. Alternatively, organized vascular structures in our model could have been generated by the endothelial differentiation of CD34-CD31+ cells, triggered by factors (e.g., CXCL12) produced by capillary endothelial cells [24].
While it was beyond the scope of the present study to dissect such molecular mechanism aspects, our results highlight that one single cell source, namely human adipose tissue, includes the different cell types required to generate tubular structures with features typical of blood vessels. The critical maintenance of such cell fractions in the proposed 3D culture model may be attributed to the physical entrapment of otherwise loosely adherent cells and/or to the establishment of a 3D stromal niche, required for the maintenance of CD34+CD31- cells (Fig. 4C) [25].

Large size scaffolds loaded with SVF cells resulted in faster engraftment upon in vivo implantation than materials seeded with ASC or bone marrow-derived mesenchymal stromal cells (BMSC). The rapid engraftment was associated with the formation of human origin blood vessels, which anastomosed with the host vasculature as early as 7 days after implantation and which remained functional up to the latest investigated time (i.e., 8 weeks), as assessed by the presence of erythrocytes in their lumens and the absence of haemorrhages. These results, as previously proposed for prevascularized tissue engineered skin grafts [26], suggest that the early engraftment of SVF-based constructs was the result of insoulation of the in vitro preformed vascular structures with the host capillaries, rather than merely neovascularisation, which is a slower process. Other groups previously reported vessel anastomosis by addition of endothelial cells into biodegradable, fibrin-based hydrogels [27] or by the co-culture of endothelial cells with mesenchymal progenitors [28], fibroblasts [29,30] or osteoblasts [31].

Moreover, inclusion of vascular and stromal elements was shown to enhance the in vitro performance of engineered human myocardium to improve its viability after transplantation [32]. However, our study is the first to associate the in vitro formation of vascular structures with a faster engraftment of engineered bone constructs in vivo. Additional investigations, such as intravital imaging, would be needed to more precisely characterize the functionality of these structures and their rheological properties as compared to native blood vessels.

The implant penetration by host tissue was accompanied by the presence of human origin cells at the edge of the tissue front, reaching the most central part of the construct after 8 weeks in vivo. Due to the technical challenge to histologically process ceramic-containing constructs until the pores are filled with tissue, it remains to be assessed whether human cells remained alive and functional in the centre of the construct until they were reached by host tissue (i.e., after 4 weeks). In fact, they could also have initially died and later been replaced by other human cells, surviving at the construct periphery and then migrating to the centre.

After 8 weeks of ectopic implantation, negligible bone tissue was formed in ASC-based grafts, consistent with the general understanding that an in vitro commitment is necessary to prime ectopic osteogenicity by human ASC (see [8] for a review). Instead, SVF-seeded constructs reproducibly formed bone ossicles, starting as typical for this model from the areas of contact with the ceramic scaffold. Due to the phenotypic differences observed between SVF cells and ASC subpopulations (Fig. 1), the result may be explained by the presence and maintenance in SVF of a diverse set of mesenchymal progenitors with intrinsic osteogenic capacity, but also by the possible crosstalk between endodermal and osteoprogenitor cells, which are known to be essential for bone formation and regeneration [31,33]. In fact, endothelial lineage cells are supported by the production by mesenchymal cells of proangiogenic factors, e.g. VEGF [34], and in turn are known to
Fig. 5. Characterization of critically sized constructs in vivo. Hematoxylin-Eosin stained sections of constructs retrieved after 1 week implantation in nude rats and based on hydroxyapatite scaffolds without cells (A), seeded with expanded adipose stromal cells (ASC) (B) or seeded and cultured with freshly-isolated stromal vascular fraction (SVF) cells (C). (D) Graph summarizing the kinetics of tissue penetration in implanted constructs seeded with the indicated cell types, using bone marrow stromal cells (BMSC) as controls. Asterisks (*) indicate statistically significant differences between SVF cells and all other experimental groups at the indicated time point. (E) Immunostaining for human-specific CD34 of a representative SVF cell-seeded construct 1 week after in vivo implantation. Arrows show blood vessels of human origin with erythrocytes in the lumens, whereas dashed lines show host (rat) blood vessels. (F) In situ hybridization for ALU sequences specific for human cells of a representative SVF cell-seeded construct 8 weeks after in vivo implantation. Dashed lines delineate the contour of the cross section. The inset shows ALU staining in individual cells at higher magnification. All data are representative of 3 donors for BMSC and ASC, and 4 donors for SVF. Scale bars = 2 mm (A, B, C, F) or 25 μm (E).
secrete key osteogenic factors like BMP-2 and BMP-4 [35]. The maintenance of an endothelial cell population from SVF cells in the 3D perfusion cultures and the formation of a prevascular network could thus have contributed to the osteogenic commitment of the mesenchymal CD34<sup>+</sup>/CD31<sup>-</sup> cell fraction. Importantly, as compared to SVF-seeded constructs, those based on typical and well-accredited osteogenic cells, namely BMSC, displayed lower amounts of bone formation, due to the spatial restriction to the outer regions. The deeper production of bone tissue by SVF cells was consistent with the more efficient penetration of host tissue and associated vessels, and allowed for the production of bone tissue up to 3.5 mm from the periphery of the construct. Over the 206 bones found in the human body, the one with largest continuous dimension is the human femur shaft, with an integral bone thickness of 7–10 mm [36]. Therefore, the proposed approach could overcome the challenge of osteogenic tissue engraftment for virtually any bone defect. Obviously, this will require further validation at an orthotopic site of an immunocompetent model, where biomechanical and inflammation/immune-related aspects can better mirror a clinical case scenario, although the species specificity of SVF biology may introduce a relevant bias. The process implementation using a streamlined bioreactor system, bypassing the labor-intensive phase of monolayer expansion and reducing the culture time, provides the basis for standardized, automated and possibly cost-effective graft manufacturing [37].

5. Conclusion

Our findings support the concept that vascular progenitors derived from human SVF cells accelerate the engraftment of critically sized osteogenic constructs, ultimately improving the efficiency and uniformity of bone tissue formation. The use of a single, easily available and abundant cell source for both osteogenic and vasculogetic progenitors offers a practical and clinically attractive approach.

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Appendix. Supplementary material


Fig. 6. Bone formation within critically sized constructs. Hematoxylin-Eosin stained sections of constructs retrieved after 8 weeks implantation in nude rats and based on hydroxyapatite scaffolds seeded with stromal vascular fraction (SVF) cells (A,B); expanded adipose stromal cells (ASC) (C), no cells (D), or bone marrow stromal cells (BMSC) (E,G). (C) Quantification of the percentage of pores containing bone tissue in the constructs seeded with the indicated cell source. (H) Distribution of pores containing bone tissue plotted as a function of the distance from the scaffold surface for constructs generated by SVF cells or BMSC. All data are representative of 3 donors for BMSC and ASC, and of 4 donors for SVF. Scale bars = 2 mm (A, D, E, F) or 200 μm (B, G).
References


Supplementary Figure 1. In situ hybridization for human-specific ALU sequences (left column), immunostaining for human CD34 (central column) and for bone sialoprotein (BSP) (right column) on histological sections of stromal vascular fraction (SVF) cell-based constructs implanted in nude rats for the indicated time (1, 2 or 4 weeks). Images were acquired at the deepest front of tissue penetration and are representative of those observed for 4 donors. Scale bars = 200 µm.
Chapter II

Intraoperative approach in bone tissue engineering

Intraoperative engineering of osteogenic grafts combining freshly harvested, human adipose-derived cells and physiological doses of bone morphogenetic protein-2

Paper submitted
Intraoperative engineering of osteogenic grafts combining freshly harvested, human adipose-derived cells and physiological doses of bone morphogenetic protein-2

Arne Mehrkens 1*, Franziska Saxer 1*, Sinan Güven 1, Waldemar Hoffmann 1, Andreas M. Müller 1, Marcel Jakob 1, Franz E. Weber 2, Ivan Martin 1**, Arnaud Scherberich 1

1. Departments of Surgery and of Biomedicine, University Hospital Basel, Hebelstrasse 20, 4031 Basel, Switzerland.
2. Oral Biotechnology & Bioengineering, Division of Cranio-Maxillo-facial and Oral Surgery, University Hospital Zürich, Frauenklinikstrasse 24 8091 Zürich, Switzerland

* These authors contributed equally to the work acting as co-first authors

** Corresponding author:
Prof. Ivan Martin
Institute for Surgical Research & Hospital Management University Hospital Basel
Hebelstrasse 20, ZLF, Room 405
4031 Basel, Switzerland
Telephone: +41 61 625 23 84, Fax: +41 61 265 39 90

Running head: Ectopic bone by non-expanded adipose cells
Abstract

Engineered osteogenic constructs could replace the use of autologous transplantation for bone surgery but typically involve long and costly fabrication processes, due to a limited availability of cells from the donor. Freshly isolated human adipose-derived cells provide precursors in large amounts, allowing production of the grafts and their immediate, intraoperative implantation, despite the reported requirement to include a molecular trigger into the construct to induce full osteogenicity of the cells in vivo. The present study evaluated recombinant human BMP-2 (rhBMP-2) as a potential inductive supplement in this context. The stromal vascular fraction (SVF) was isolated from human adipose tissue from 7 healthy donors by enzymatic digestion. Immediately after, porous silicated calcium-phosphate granules (Actifuse®, Apatech) were mixed with 1x10^6 or 4x10^6 fibrin-embedded SVF-cells, supplemented or not with rhBMP-2 (250 ng / 0.06 cm^3 construct). These constructs were thereafter implanted ectopically for eight weeks in nude mice. Upon explantation, constructs were analyzed histologically while the effect of rhBMP-2 on osteoblastic differentiation of SVF cells was assessed in vitro. Bone tissue was formed only in the presence of rhBMP-2, at a dose which could not induce ectopic ossification by itself. The reproducibility of bone tissue formation was improved by increasing the density of SVF cells, the latter not only supporting but directly contributing to bone tissue formation. In vitro, rhBMP-2 did not involve an increase in the percentage of SVF cells recruited to the osteogenic lineage, but rather induced a stimulation of the osteoblastic differentiation of the committed progenitors. This study confirms the feasibility of the generation of fully osteogenic grafts intra-operatively, and the mechanisms involved. An extension to an orthotopic, immuno-competent animal model is the next required step towards its validation for clinical use.
Introduction

The standard of care in the treatment of bone defects in orthopaedic, trauma or reconstructive surgery is the transplantation of autologous bone grafts. Alternative options are the implantation of allografts or osteoconductive materials, the local treatment with osteoinductive growth factors such as BMP-2 or BMP-7, or combinations thereof (Berner et al., 2011; De, Jr. et al., 2007; Saxer F et al., 2010). The engineering of osteogenic bone graft substitutes based on osteoconductive scaffolds combined with autologous osteoprogenitors (mesenchymal stromal cells, MSC) as a biologically active component could provide an attractive alternative, but its translation into clinical practice has proven to be highly challenging (Berner et al., 2011; Cuomo et al., 2009; Evans et al., 2007). Low MSC numbers found in the bone marrow generally require a step of cell expansion for graft manufacturing. This not only is known to be associated with a progressive loss of osteogenic differentiation capacity (Banfi et al., 2000), but also requires processing under costly and tightly regulated Good Manufacturing Practice (GMP) conditions. Thus, cost-effectiveness of the classical bone tissue engineering paradigm still needs to be verified (Meijer et al., 2007).

One possible solution proposed to overcome the limitations above is based on the 3D expansion of MSC directly within porous scaffolds (Braccini et al., 2005). This was shown to reduce intra-individual differences, increase quality of grafts and streamline manufacturing in perfusion bioreactors, with the potential to introduce automation and thus reduce costs (Martin et al., 2009).

Another approach has more radically addressed the problem, by trying to eliminate the expansion phase, i.e. reducing the manufacturing process to a one-step surgical procedure. Such an intra-operative approach poses the essential requirements to identify an autologous source of cells that have (i) intrinsic osteogenic capacities in vivo without prior culture or osteoinduction and (ii) are available in sufficient numbers directly upon isolation. Freshly isolated bone marrow-derived cells, possibly harvested using a reamer-irrigator-aspirator (Cox et al., 2011; Stafford and Norris, 2010), concentrated by immunoselection (Asian et al., 2006) or modified genetically (Evans et al., 2007), have been proposed to be directly used for bone repair. Despite the promising data collected so far, the reproducible collection of a sufficient number of MSC across different patients remains to be demonstrated. The freshly-isolated stromal vascular fraction (SVF) of human adipose tissue represents a possibly better cell source for a one-step surgical procedure, given its up to 500-fold larger number of clonogenic progenitors per volume of tissue sample compared to human bone marrow (Fraser et al., 2006; Scherberich et al., 2007). Two studies (Helder et al., 2007; Vergroesen...
et al., 2011) tested bone formation by autologous SVF cells, intraoperatively processed to generate grafts implanted in a goat spinal fusion model. Those studies demonstrated a superior bone healing when implants were loaded with SVF cells, but the model was not designed to assess the direct osteogenic properties of the SVF-based grafts. Our group recently demonstrated that ectopic implantation in nude mice of human SVF cells seeded on porous hydroxyapatite scaffolds results in the formation of human origin blood vessels and dense osteoid matrix, but no ‘frank’ bone formation (Muller et al., 2010). These findings suggested that, in the absence of in vitro commitment, additional cues (e.g. osteoinductive factors) might be needed to support ectopic bone tissue generation in vivo.

In the present study, recombinant human bone morphogenetic protein-2 (rhBMP-2) was therefore used as an osteoinductive stimulus (Chen et al., 2004; Jeon et al., 2008) for the implanted SVF cells, at doses known to be not sufficient to induce by themselves bone tissue formation (Fujimura et al., 1995). RhBMP-2 was introduced in fibrin-ceramic-based constructs simultaneously with the freshly-isolated/SVF cells and immediately implanted ectopically in nude mice. Bone formation and the contribution of SVF cells to this process were studied 8 weeks after implantation. In vitro experiments were also performed to address whether rhBMP-2 enhances SVF cell osteogenic differentiation and/or the osteogenic recruitment of clonogenic SVF populations.

Material and Methods

Cell isolation

Adipose tissue, in the form of liposuction or excised fat samples, was obtained from 7 healthy female donors following informed consent and according to a protocol approved by the local ethical committee (EKBB, Ref. 78/07). Minced tissue from excised fat samples or lipoaspirates were processed as previously described (Guven et al., 2011; Muller et al., 2010) and the cell pellets resuspended in complete medium (CM), consisting of □-MEM supplemented with 10% of foetal bovine serum (FBS), 1% HEPES, 1% Sodium pyruvate and 1% of Penicillin-Streptomycin Glutamate (100x) solution (all from Gibco, www.invitrogen.com).
Cell Characterisation

**Fluorescence activated cell sorting (FACS)**

SVF cells were analyzed by cytofluorimetry with antibodies to CD105, CD90 and CD73 (mesenchymal markers), CD31 and CD34 (endothelial markers), the monocytic marker CD14 and the pan-haematopoietic marker CD45 (anti-CD105 antibody from AbD Serotec, www.abdsrrotec.com, all others from Becton Dickinson Bioscience, wwwbdbiosciences.com), as previously described (Gronthos et al., 2001; Guven et al., 2011).

**Frequency of clonogenic cells**

The ratio of colony forming unit-osteoblasts (CFU-o) to the total number of formed colonies (colony forming unit-fibroblasts, CFU-f) (Friedenstein et al., 1970) (Baksh et al., 2003) was determined by plating 100 SVF cells/well into six well plates. Cells were cultured with CM or osteogenic medium (OM), consisting of CM supplemented with 100 nM dexamethasone, 10 mM beta-glycerophosphate, and 0.05 mM ascorbic-acid-2-phosphate (Sigma-Aldrich, www.sigm Aldrich.com) for 14 days, in the presence or absence of the indicated concentration of rhBMP-2 (R&D Systems, www.rndsystems.com). CFU-o were defined as colonies stained positive for alkaline phosphatase (ALP) activity, using a commercially available kit (104-LL kit, Sigma-Aldrich). The CFU-o/CFU-f ratio was determined following counter staining with buffered neutral red solution (N6264, Sigma-Aldrich), which allowed to count the total number of CFU-f.

**In vitro stimulation with rhBMP-2**

SVF cells were plated on tissue culture plastic and grown to confluence in the presence of CM. Cells were then cultured for 14 days with either CM or OM, alone or further supplemented with 50 or 500 ng/mL BMP-2 (produced as previously described (Weber et al., 2002)) were analysed by reverse transcriptase real time polymerase chain reaction (RT-rt-PCR). Cells were then treated with lysis buffer (Qiagen, http://www.qiagen.com) enriched with 1/100 (V/V) β-mercaptoethanol (Sigma-Aldrich). RNA was extracted by using a NucleoSpin® RNA II kit (Macherey-Nagel, http://www.mn-net.com). The RNA was eluted in RNase-free water and transcription into cDNA was performed as previously described (Barbero et al., 2003). The samples were analysed by using a GeneAmp® PCR System 9600 (Perkin Elmer, www.perkinelmer.com) and the transcription levels of osteopontin (OP) and osteocalcin (OC) quantified, with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as reference housekeeping gene (Frank et al., 2002). SVF cells were similarly plated on
tissue culture plastic, grown to confluence and cultured for 7 days with CM, alone or further supplemented with 500 ng/mL BMP-2 (R&D Systems, www.rndsystems.com). Cells were then detached with trypsin (Invitrogen) and analysed by cytofluorimetry with fluorochrome-conjugated antibodies to alkaline phosphatase and osteocalcin (ALP and OC, both from R&D systems, www.rndsystems.com).

Generation and assessment of SVF cells-fibrin-ceramic constructs

One or four millions SVF cells were suspended in the fibrinogen phase (30 µL) of a polymerizing fibrin gel (Tisseel®, Baxter, www.baxter.com), as described previously (Bensaid et al., 2003; Muller et al., 2010), with or without addition of 250 ng of recombinant human BMP-2 (R&D Systems). Briefly, following mix with the thrombin phase (30 µL), the solution was poured onto a volume of approx. 0.06 cm$^3$ of hydroxyapatite granulates of 1-2mm size (Actifuse® ABX, ApaTech, www.apatech.com) pre-stacked in the wells of a 96-well plate. After 1-2 min, when the gels polymerized, constructs were covered with CM and transferred into a humidified incubator (37°C, 5% CO$_2$) for 10 min. Directly after fabrication, some constructs were incubated for 2 hours at 37°C in a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) solution at a final concentration of 0.05 mg/mL and the distribution of the blue/purple metabolized substrate of MTT was inspected macroscopically to assess cell viability. Other constructs were fixed overnight in 4% formalin, paraffin-embedded, sectioned and stained with haematoxylin/eosin (H&E) for qualitative assessment of the spatial distribution of the seeded cells. The remaining constructs were implanted in nude mice as described below.

In vivo implantation in nude mice and explant analysis

The maintenance, surgical treatment and sacrifice of animals were performed in accordance with the guidelines of the local veterinary agency ("Kantonales Veterinäramt Basel-Stadt", permission #1797). Constructs were implanted in the subcutaneous tissue of nude athymic mice (CD1 nu/nu, Charles River, www.criver.com) and harvested after eight weeks following mice sacrifice by inhalation of CO$_2$. Tissues were fixed in 4% formalin overnight, subjected to slow decalcification in 7 % w/v EDTA and 10% w/v sucrose (both from Sigma-Aldrich) at 37°C on an orbital shaker for 7-10 days and paraffin-embedded. Samples were then cross-sectioned (12 µm thickness) and processed for histological, histochemical and immunohistochemical stainings as follows. Standard
H&E staining and Masson trichrome staining (Kit Trichrome de Masson-Vert lumière, Réactifs RAL, www.ral-diagnostics.fr) were performed to identify bone tissue formation and maturation stage. Safranin-O staining allowed investigating the presence of sulphated proteoglycans inside the construct, characteristic of cartilaginous tissue. Tartrate resistant alkaline phosphatase (TRAP) staining (leukocyte acid phosphatase kit, Sigma-Aldrich) was used to identify osteoclasts, while the presence of osteoblastic cells and osteoid structures was assessed by immunostaining for human bone sialoprotein (BSP, Immundiagnostik AG, www.immundiagnostik.com) (Minkin, 1982; Papadimitriopoulos et al., 2011). The presence of donor-derived, human blood vessels was demonstrated by immunostaining with a biotin-conjugated antibody for human CD34 (Abcam, www.abcam.com), as previously described (Scherberich et al., 2007). All human cells in the explants were identified by chromogenic in situ hybridization for the human-specific sequence ALU, using a biotin-conjugated DNA probe (ZytoVision, http://zytovision.com), as previously described (Muller et al., 2010; Roy-Engel et al., 2001).

Results

The percentage of CFU-f in the SVF preparations from different human adipose tissue samples averaged 14.7 ± 6.8 % (n = 4). The fractions of different SVF subpopulations were highly variable across different donors, as assessed by the large standard deviations in the percentage of positive cells for different typical surface markers (Figure 1A) and in accordance with previous reports (Muller et al., 2009; Muller et al., 2010). Once embedded in a fibrin gel around ceramic granules, SVF cells were viable and homogenously distributed throughout the construct, as evidenced by MTT metabolic staining (Figure 1B). The structure of the construct was investigated by H&E staining of sections of decalcified samples (Figure 1C and D). It allowed visualizing the structural components of the constructs prior to implantation, including the fibrin gel (pink stain in Figure 1C and D), the embedded cells (blue stain in Figure 1D) and the porous ceramic granules (void spaces in decalcified samples, Figure 1C).
Figure 1: Characterization of cells and constructs in vitro
(A) Cytofluorimetric analysis of freshly-isolated SVF cells derived from 7 donors. For every CD marker, the average percentage of cells positive for the marker is plotted. Error bars represent standard deviations. (B) Representative picture of a tetrazolium-based metabolic staining (MTT assay) performed on SVF cells-fibrin gel-ceramic granules sample constructs to demonstrate the distribution of viable cells. (C and D) Macroscopic (C) and microscopic (D) pictures of hematoxylin/eosin staining performed on histological sections of decalcified, paraffin-embedded samples.

In the absence of incorporated BMP-2 and independently of the initial cell density (1 or 4 million SVF cells per 100 mm³ construct), constructs explanted after subcutaneous implantation for 8 weeks in nude mice did not display any evidence of frank bone tissue formation, as assessed by H&E staining (data not shown) and by fluorescence microscopy (Figure 2, left column). These findings are in accordance with our previous results (Muller et al., 2010). The addition of 250 ng of rhBMP-2 inside the fibrin gel resulted in a significant increase in the formation of bone tissue with 71% of cases showing bone inside the construct (5/7 donors). A trend indicating a lower reproducibility in bone formation with a lower number of cells (71 % vs 33% of the donors with respectively 4 million vs. 1 million cells) was shown (Figure 2, right column). Because this comparison was not essential for the study, a limited number of replicates (with cells from 3 independent donors) were performed with 1 million SVF cells, and a relevant statistical analysis between the 2 cell seeding densities was therefore impossible.
Bone tissue formation was confirmed by H&E staining both around the entire construct, in between the ceramic granules and within their pores (Figure 3A, black arrows). Bone tissue displayed the typical features of an ‘ossicle’ structure, including a dense collagenous matrix with embedded osteocytes and a rim of osteoblasts depositing osteoid tissue starting from the ceramic material (Figure 3B). Masson trichrome staining further qualified that the bone tissues was at various stages of maturation, with local spots of red stained regions, indicating the presence of elastic proteins and characteristic of a more mature bone tissue (Figure 3C, black arrow). Neighbouring already developed bone ossicles, areas of pre-osteoid tissue were also identified by positive immunostaining for bone sialoprotein (Figure 3D, black arrow). As a control group, implantation of ceramic-fibrin-rhBMP-2 constructs without cells resulted in the formation of a merely fibrous tissue with no bone (data not shown).
Figure 3: Characterization of newly-formed bone tissue
Histological analysis of sections of explanted, fixed and decalcified constructs seeded with $4 \times 10^6$ SVF cells and rhBMP-2. (A) Hematoxylin/eosin. Arrows show newly-formed bone tissue both in the pores of granules and in a shell around the construct. (B) Higher magnification of the same sections showing compact bone matrix (b) and osteocytes embedded therein. (C) Masson trichrome staining. The green dye stains dense collagenous matrix identifying bone tissue (b) in contact with the ceramic granules (gr.), with various stages of maturation, in particular zones with red staining characterizing elastic proteins (arrows). (D) Immunostaining for BSP. The arrow indicates a zone with BSP-positive, osteoblastic cells where initial bone formation is ongoing. Areas with mature bone tissue (b) do not contain osteoblastic cells.

In order to study the contribution of implanted human SVF cells to the formation of tissue inside the construct, in situ hybridization for human-specific ALU sequences was performed. Cells of human origin were identified both embedded within the bone matrix (putative osteocytes, black arrow in Figure 4A) as well as at the bone matrix deposition front (putative osteoblasts, open arrow in Figure 4A). ALU staining was also positive in the lumen of capillaries (putative endothelial cells, red circle in inset, Figure 4A). The contribution of human vascular cells from the SVF to blood vessel formation was further confirmed by immunostaining for human CD34 (Figure 4B, open arrows). The presence of erythrocytes in the lumen of the human capillary structures demonstrated functional connection with the host vasculature. Negative safranin-O staining indicated the absence of structures containing cartilage-specific glycosaminoglycans (Figure 4C). TRAP staining identified the presence of multinucleated cells, likely of host origin, in contact with the newly formed bone (putative osteoclasts, arrow in Figure 4D), suggesting an active remodelling process.
Figure 4: Characterization of the contribution of SVF cells to tissue formation
Histological analysis of sections of explanted, fixed and decalcified constructs seeded with $4 \times 10^6$ SVF cells and rhBMP-2. (A) In situ hybridization for human-specific ALU sequences. osteocytes (black arrows) and lining osteoblasts (open arrows) of human origin are identified by their nuclear ALU staining. Inset shows ALU nuclear staining of human endothelial cells at the level of human capillaries (red dashed line). (B) Immunostaining for human CD34 shows human blood vessels (bv, open arrows), filled with erythrocytes. (C) Safranin-O staining with no specific red staining, indicative of sulphated proteoglycans and therefore of the generation of cartilaginous tissue inside the constructs. (D) TRAP staining showing the presence of multinucleated, osteoclastic cells in contact with newly formed bone (arrow). (b) indicates bone and (gr.) ceramic granules.

We next investigated the effect of rhBMP-2 on the percentage of osteoprogenitors recruited in vitro within the SVF cell population and the level of osteogenic induction of those osteoprogenitors in vitro. The CFU-o/CFU-f ratios, representing the fraction of clonogenic SVF cells displaying osteogenic properties, were higher in OM than in CM, but were not affected by rhBMP-2 at both tested concentrations (Figure 5A). The level of osteogenic induction of SVF cells in vitro was assessed by the mRNA expression of osteoblastic markers, namely BSP and OP. The expression of these genes was not affected by medium supplementation with 50 ng/ml rhBMP-2, whereas it was consistently enhanced by the use of 500 ng/ml rhBMP-2, independently of the use of CM or OM (Figure 5B). To confirm this trend in the effect of rhBMP-2 on the differentiation of the osteoprogenitors, the effect of 500 ng/ml rhBMP-2 on cells cultured with CM was tested by cytofluorimetry and compared to untreated cells (negative control) and cells cultured with OM (positive control). RhBMP-2 significantly increased the differentiation of osteoprogenitors, at levels
similar to OM, based on the expression of ALP (Figure 5C) and based on cells co-expressing ALP and ALP (Figure 5D).

**Figure 5: In vitro effect of rhBMP-2 on human SVF cells**
(A) Effect of 2 different doses of rhBMP-2 (50 and 500 ng/ml) on the CFU-o/CFU-f ratio of human SVF cells cultured with either complete medium (CM) or osteoblastic induction medium (OM). Experiments were performed in triplicates with cells from n=6 independent donors. Average ± s.d. is represented. (B) Effect of 2 different doses of rhBMP-2 (50 and 500 ng/ml) on the expression levels of bone sialoprotein (BSP) or osteopontin (OP) mRNA. Results are represented as average ± s.d. of the ratio between marker’s expression levels and expression levels of GAPDH. Experiments were performed in duplicate with cells from n=3 independent donors. (C and D) Effect of 500 ng/ml rhBMP-2 and OM on the percentage of cells expressing the osteoblastic markers alkaline phosphatase (ALP, C) and ALP+osteocalcin (OC, D). Experiments were performed with cells from n=5 independent donors. Average ± s.d. is represented.

* and ** indicate significant differences (p<0.05 and p<0.01, respectively) as tested by one-way ANOVA test followed by Newman-Keuls comparison of the different groups.
Discussion

This study validates an intraoperative manufacturing for the generation of grafts with osteogenic/vasculogenic potential derived from human adipose tissue. The formation of bone tissue was shown to require the delivery of a low dose of rhBMP-2, which could not induce ectopic ossification by itself. The reproducibility of bone tissue formation might well be improved by increasing the density of implanted SVF cells, which not only supported but directly contributed to bone tissue formation. The in vitro results suggest that the mechanism of action of rhBMP-2 was not involving an increase in the percentage of SVF cells recruited to the osteogenic lineage, but rather a stimulation of the osteoblastic differentiation of the committed progenitors.

Previous reports demonstrated that SVF cells, freshly isolated from adipose tissue and immediately implanted, can enhance bone healing in orthotopic experimental animal models (reviewed in (Scherberich et al., 2010)). Autologous SVF cells have also been used in a on-the-spot intraoperative approach in a few clinical cases, demonstrating safety and a favourable clinical outcome (Lendeckel et al., 2004; Pak, 2011). However, in all these studies, the direct contribution of the implanted cells to bone formation was not addressed and therefore the intrinsic osteogenic capacity of freshly harvested SVF cells had not yet been demonstrated. The experimental setup used in the present work, instead, namely an ectopic implantation site in a nude mouse model, allowed to investigate the fate and mode of action of the implanted human SVF cells and therefore to conclude that SVF cells can directly form bone tissue, but only when stimulated in situ by rhBMP-2.

The induction of bone formation by BMPs in vivo has been described for the first time in 1965 (Urist, 1965). Numerous in vitro and in vivo studies have later demonstrated enhanced bone repair by rhBMP-2, which is now FDA-approved in spinal, trauma and maxillo-facial surgery (Govender et al., 2002; Hsu and Wang, 2008; Jones et al., 2006; Smith et al., 2008). The clinical use of rhBMP-2 is based on the principle of induction of osteogenesis by resident precursor cells and requires very high and non-physiological doses, which have been reported to be associated with aberrant bone formation (Deutsch, 2010), neurotoxicity (Smith et al., 2008) or cancer development (Carragee et al., 2011). As compared to commercially available products, which contain 1.5 mg/ml of rhBMP-2, the concentration used in the present study (2.5 g/ml of construct) was about three orders of magnitude lower. The dose, which to the best of our knowledge is lower than the minimal one ever reported for stimulation of adipose derived cells in vivo (Jeon et al., 2008), was not intrinsically
associated with osteoinductivity and supported bone formation only by acting in concert with the implanted cells. Moreover, one can no exclude that the combination of implanted human cells and rhBMP-2 could have recruited circulating cells from the host which may have directly contributed to bone formation inside the constructs after their stimulation by rhBMP-2, in parallel with implanted bone-forming cells.

The actual osteogenic responsiveness of mesenchymal stromal cells to BMPs, in particular for osteoprogenitors derived from adipose and bone marrow tissues, is still controversial. Indeed, while some groups reported no significant difference of bone formation after addition of rhBMP-2 (Chou et al., 2011; Diefenderfer et al., 2003; Osyczka et al., 2004; Zuk et al., 2011) or transfection with hBMP-2 gene (Peterson et al., 2005), some others reported induction of bone repair by BMP-2 stimulation of adipose-derived osteoprogenitors (Jeon et al., 2008; Lee et al., 2010). This discrepancy may result from factors such as the high inter-donor variability, the variety of animal models and experimental settings as well as the doses of rhBMP-2 used (Zara et al., 2011). Also age and sex of the donor seem to influence the osteogenic potential of osteoprogenitors (van, V et al., 2003; Zhu et al., 2009), as well as their responsiveness to rhBMP-2 (Kim et al., 2008). Our in vitro results indicate that rhBMP-2 specifically stimulated the osteoblastic differentiation of SVF cells. No effect of rhBMP-2 on adipose-derived cell differentiation was seen with 50 ng/mL, confirming a previous report (Zuk et al., 2011) showing no effect at doses ranging 10-100 ng/mL. We however demonstrated in this study, both at the gene expression and protein expression level, that a 500 ng/mL concentration stimulated osteoblastic differentiation of adipose-derived cells.

Based on those in vitro data, it was challenging to establish which dose of rhBMP-2 had to be added to the constructs to mimic this effect during in vivo implantation. Indeed, the release profile - 3 repetitive doses for monolayer culture in vitro vs. burst release from the gel in vivo- and the different cell mixes - pure ASC in vitro and SVF cells (containing ASC, endothelial and hematopoietic cells) in vivo- could not easily be compared. Therefore, the cumulative dose of rhBMP-2 in vitro (1.5 µg) was used to normalize the dose in the constructs, which was then further adjusted from 1.5 µg/ml of construct to 2.5 µg/ml of construct in order to account for a very high density of SVF cells seeded in the constructs. This dose was then tested in acellular constructs in vivo and rhBMP-2 demonstrated no intrinsic osteoinductive capacity by itself in this setting. Interestingly, there have been promising results in enhancing bone formation by adipose tissue cells also by addition of vitamin D3 (Song et al., 2011), alendronate (Wang et al., 2010) or platelet-
rich plasma (Liu et al., 2008). Whether or not addition of these substrates, alone or in combination with rhBMP-2, could lead to a more reliable bone formation in the proposed setup will also have to be investigated. Finally, although rhBMP-2 was previously reported to have the potential to stimulate angiogenesis (Deckers et al., 2002; Peng et al., 2005), in our study the presence of human endothelial cells (positively stained for ALU sequences and for human CD34) in graft vascularisation did not appear to be increased in the presence of rhBMP-2 (data not shown).

**Conclusion**

This study reinforces the feasibility of an intra-operative use of autologous SVF cells for bone regeneration. The approach requires only one surgical procedure, similar to autologous bone grafting but clearly with reduced morbidity at the donor site. Moreover, it does not require extensive processing and culture of the isolated cells, thereby also reducing the costs and regulatory burdens otherwise associated with advanced cellular therapies. The clinical translation of the proposed strategy still requires an extended validation to an orthotopic model, in order to investigate the bone regenerative capacity of SVF cells in the context of a bony and injured/inflamed environment. Recent work on the established interaction between the immune system and osteoprogenitor cell function (Liu et al., 2011) also prompts for further studies in immunocompetent models. However, the introduction of alternative in vivo models requires the use of animal as opposed to human adipose-derived cells, which are known to have markedly different biological properties and osteogenic potential (Levi et al., 2011) and thus would limit the potential clinical relevance of the generated findings. In this regard, one of the most compelling challenges in the routine clinical implementation of this approach is related to the large variability in phenotype and bone forming capacity of human adipose-derived cells derived from different donors (Scherberich et al., 2007). Therefore, one additional effort will have to involve the identification of reliable quality control/potency markers of the implanted cells, in order to ultimately define the number of cells with a specific phenotype which should be introduced per unit of construct volume to ensure reproducible bone formation.

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References:


Chapter III

Automated cell isolation system for streamlining intraoperative approach

Validation of an Automated Procedure to Isolate Human Adipose Tissue-Derived Cells by Using the SEPAX® Technology

Validation of an Automated Procedure to Isolate Human Adipose Tissue–Derived Cells by Using the Sepax® Technology

Sinan Güven, Ph.D.,1,2 Marianna Karagianni, M.D.,3 Mandy Schwaibe,3 Simone Schreiner, M.D.,1,2 JIan Fathadi, M.D.,4 Sylvain Buia, M.Sc.,5 Karenblech, Ph.D.,3 Ivan Martin, Ph.D.,1,2 and Arnaud Scherberich, Ph.D.1,2

The stromal vascular fraction of adipose tissue has gained popularity as a source of autologous progenitor cells for tissue engineering and regenerative medicine applications. The aim of this study was to validate a newly developed, automated procedure to isolate adipose-derived mesenchymal stem/stromal cells (ASCs) from adult human liposaptries in a closed and clinical-grade device, based on the Sepax® technology. Using a total of 11 donors, this procedure was compared with the standard operator-based manual separation in terms of isolation yield, clonogenic fraction, phenotype, and differentiation potential of ASCs. As compared with the manual process, automation resulted in a 62% higher isolation yield, with 2.6±1.2×10^5 nucleated cells per mL of liposuction, and a 24% higher frequency of clonogenic progenitors. The variability in the isolation yield and clonogenicity across different preparations was reduced by 18% and 50%, respectively. The cytometric profile and in vitro differentiation capacity into mesenchymal lineages were comparable in the cells isolated using the two procedures. The new Sepax-based process thus allows an efficient isolation of ASCs with higher and more reproducible yields than the standard manual procedure, along with minimal operator intervention. These results are expected to facilitate the use of ASCs for clinical purposes, either within an intraoperative setting or in combination with further in vitro cell expansion/cultivation.

Introduction

Mesenchymal stem/stromal cells (MSCs), a rare population of nonhemopoietic stromal cells, were initially defined within the rodent bone marrow,1 as the adherent population on tissue culture plastic and by their expression of various molecules, including CD90, CD105, and CD73, and the absence of markers like CD34, CD45, and CD14.2,5 Upon adherence, these MSCs have the capacity to form clones, defined as colony-forming unit–fibroblasts (CFU-f), and to extensively proliferate. MSCs are able to differentiate into mesenchymal lineages and thus generate bone, cartilage, adipose, and muscle tissues. These properties have made them a promising tool for cell-based tissue repair and tissue engineering approaches.3

Cells with properties similar to bone-marrow-derived MSCs were later derived from other tissues and organs, including brain and muscle,2 skin,3 or adipose.4,7 Indeed, adipose tissue, when digested with collagenase and centrifuged to remove differentiated adipocytes floating in the aqueous phase, forms a cellular pellet made up of a highly heterogeneous population of cells, typically referred to as the stromal vascular fraction (SVF) and includes fibroblastic colony-forming cells, vascular/endothelial cells, erythrocytes, and other hematopoietic cells. These SVF cells are either freshly used for therapeutic applications6,9 or seeded onto tissue culture plastic in order to select the adherent population and then expanded to generate what is generally referred to as adipose derived mesenchymal stem/stromal cells (ASCs). ASCs share several characteristics of bone marrow MSCs and recently became, due to their ease of harvest and availability, a cell source raising great scientific and clinical interest. Numerous preclinical studies, defining various potential applications for ASCs in human therapy and clinical applications, have indeed documented the ability of ASCs to repair not only musculoskeletal tissues, but also cutaneous and endodermal tissues or organs, in the field of gastroenterology, neurology, orthopedics, reconstructive surgery, and related

Departments of 5Surgery and 6Biomedicine, University Hospital Basel, Basel, Switzerland.
6German Red Cross Blood Service Baden-Württemberg-Hessen, Medical Faculty Mannheim, Institute of Transfusion Medicine and Immunology, Heidelberg University, Mannheim, Germany.
7Department of Plastic Surgery, Westminster Bridge Road, St. Thomas's Hospital London, United Kingdom.
8Biosile Group SA, Eysins, Switzerland.
clinical disciplines (reviewed in Refs. 10,11). The first clinical trials with SVF cells and ASCs are ongoing, in the form of phases I (e.g., myocardial infarction, skin ulcer, or graft versus host disease), phase II (e.g., rectovaginal fistula), phase III (e.g., enterocutaneous fistula), and phase IV (e.g., breast reconstruction) studies.10,11) Such clinical trials in humans require the supply of clinical grade, generally autologous, SVF cells. The preferable solution to provide such cells is to process adipose tissue in a Good Manufacturing Practice (GMP) facility. For that reason, clinical centers aiming to apply adipose-cell-based therapies require access to such a GMP facility, supported by a highly specialized staff of technicians and qualified persons. This greatly limits the potential applications of adipose-cell-based therapies to larger clinical centers capable of housing such facilities and thus results in a cost-ineffective therapeutic approach. The development of closed, aseptic, and automated devices would allow for the isolation of SVF cells outside of a GMP facility, for instance, directly inside an operating theater, thus reducing such current limitations as cost effectiveness and operator intervention and error.

Automated cell isolation systems are currently being developed by several groups to facilitate clinical implementation of cell-based therapies. Among these, the CE-marked device Sepax® (Biosafe SA) has been previously developed to isolate and to concentrate nucleated cells from umbilical cord, peripheral, or bone marrow blood.12,13 In this study, we aimed to validate a newly developed automated procedure based on the Sepax technology to isolate SVF cells from human adipose tissue in a closed, clinical-grade setting. To validate this new procedure, we compared it with the standard operator-based manual separation of the SVF cells in terms of isolation yield, cytometric profile, and differentiation capacity into mesenchymal lineages. This study was performed independently in two research centers to confirm the reproducibility of the process.

Materials and Methods

Tissue source

Adipose tissue, in the form of tumescent liposuction samples from subcutaneous abdominal fat, was obtained from 11 healthy female donors (age 20-69) following informed consent and according to a protocol approved by the local ethical committee (EKBB, Ref. 78/07 and 2006-192N-MA [extended in 2009]). Liposuction samples were centrifuged and concentrated to remove tumescent solution immediately in the operating room. The same liposuction samples were used as starting material in parallel for the two isolation procedures as described below.

Manual isolation of the cells

Adipose samples were digested for 60 min at 37°C in 0.15% (W/V) collagenase NB 6 GMP Grade from Clostridium histolyticum (0.12 U/mg collagenase; SERVA Electrophoresis GmbH) diluted in phosphate-buffered saline (PBS, Gibco). After centrifugation at 190 g for 10 min, the lipid-rich layer was discarded and the cellular pellet was washed once with PBS. For analysis, red blood cells were lysed by incubation for 2 min in a solution of 0.15 M ammonium chloride. 1 mM potassium hydrogen carbonate (both from Merck; www.merck-chemicals.com) and 0.1 mM EDTA (Fluka Analytical, Sigma-Aldrich Chemie GmbH). The resulting SVF cells were then resuspended in complete medium (CM), consisting of alpha-minimum essential medium (α-MEM) supplemented with 10% of fetal bovine serum (FBS), 1% HEPES, 1% sodium pyruvate, and 1% of penicillin-streptomycin-glutamine (100 x) solution (all from Gibco). For the experiments testing proliferation rate, cells were expanded in Dulbecco's minimal essential medium (DMEM) containing 10% FBS or 10% human AB serum (FBS).14

Sepax-based automated isolation of the cells

Liposuction samples were introduced in 400 mL transfer bags (Terumo) and NB 6 GMP Grade collagenase diluted in PBS was added at a final concentration of 0.15% (W/V). The bag was placed into an incubator at 37°C for 60 min. After digestion (Fig. 1), the bag was connected to a CS-400.1 kit (Biosafe SA) specifically modified for this purpose and the kit was installed on a Sepax device (Biosafe SA; www.biosafe.ch) for automated digestion. After priming and staining of the digested adipose tissue into the separation chamber, a centrifugation step was performed to remove the supernatant. It was followed by a washing procedure of the pellets with saline solution. When more than 200 mL of adipose was processed, a two-phase digestion by the machine was necessary, which slightly increased the time for procedure. For cell collection, the system diluted the residual pellets (± 10 mL) and extracted the content into the final bag, and then automatically rinsed the

FIG. 1. Schematic description of the isolation process. The adipose tissue sample is digested with collagenase in a transfer bag. The bag is connected to the Sepax kit and the kit is installed on the device. The output bag contains the stromal vascular fraction (SVF) that can be used for in vitro expansion or infusion into a patient. Color images available online at www.liebertonline.com/tec
chamber twice for an optimal cell recovery. The minimum final volume used was 40 mL. The re-suspension medium was as described for the manual isolation process.

Cell count and characterization by flow cytometry

Nucleated cells were counted using a Neubauer chamber after staining with Crystal Violet (Sigma), whereas cell viability was determined by counting the blue (dead) and transparent (alive) cells after trypan blue (Sigma) staining. The phenotype of SVF cells was determined by cytometric analysis with fluorocromes conjugated antibodies to human CD105 (from AbD Serotec), CD90, CD31, CD34, CD45, or CD73. Isotype IgGs were used as control (all from BD Biosciences). About 10^6 cells in suspension per condition were incubated for 30 min with the different antibodies at 4°C in CM, washed with PBS, resuspended in FACS buffer (PBS, 0.5% human serum albumin, and 0.5 mM EDTA), and analyzed with a FACSCalibur flow cytometer (BD Biosciences). 7-Aminoactinomycin D (7-AAD) was used to determine the number of dead cells inside the isolated SVF cell population. Expanded ASCs were analyzed after the first passage, investigating 7-AAD-negative cells for expression of CD105 (eBioscience), CD144 (Reckman Coulter), CD44, CD29 (Biolegend), CD90, and CD73 (BD Biosciences).

Cell culture and colony-forming assays

For preparation, SVF cells were seeded at a density of 2×10^3 cells/cm² onto tissue culture plates, cultured in CM supplemented with 5 mg/ml fibroblast growth factor-2 (FGF-2; R&D Systems), and serially replated in new dishes at a density of 3×10^5 cells/cm² upon subconfluence. To compare the effects of PBS and FS supplementation, cells were serially replated at a density of 2000 cells/cm². Cumulative population doublings (PD) were calculated using the formula: PD=[log2(A)-log2(A_0)])/log2(2), where N1 is the number of seeded cells and N2 is the cell number counted after expansion. The number of CFU-fs was determined by plating 5×10^3 SVF cells per 75 cm² Petri dishes. Cells were cultured for 10-14 days in CM containing 5 mg/ml FGF-2. After fixation with 4% formalin for 10 min and staining with crystal violet, the colonies consisting of at least 40 cells were counted and the number of colonies was normalized to the number of plated cells.

Osteoblastic differentiation assays

The osteoblastic differentiation capacity of SVF cells was tested after 3 weeks of culture in z-MEM supplemented with 10 mM β-glycerophosphate, 10 mM ascorbate, and 0.05 M dexamethasone. Cells cultured with CM containing FGF-2 were used as a control. Deposition of mineralized matrix was detected by a 2% alizarin red S staining, as previously described, or by quantification of hydroxyapatite deposition using the Osteoimage Mineralization Assay (Lonza), following manufacturer's instructions.

Chondrocytic differentiation assay

The chondrocytic differentiation capacity of SVF cells was investigated in pellet culture by using a chemically defined, serum-free medium consisting of DMEM containing 4.5 mg/mL D-glucose, 0.1 mM monosodium L-glutamate, 1 mM sodium pyruvate, 10 mM HEPES buffer, 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.29 mg/mL l-glutamine (all from Gibco) further supplemented with ITS-1 (10 ng/mL insulin, 5.5 mg/mL transferrin, 5 ng/mL selenium, 0.05 mg/mL bovine serum albumin, and 4.7 mg/mL linoleic acid), 1.25 mg/mL human serum albumin, 0.1 mM ascorbic acid 2-phosphate, and 1×10^7 M dexamethasone (all from Sigma), and 10 ng/mL TGF-β1 (R&D). All aliquots of 5×10^5 SVF cells were centrifuged at 250 g for 5 min in 15 mL polypropylene conical tubes (Sarstedt) to form spherical pellets, which were placed onto a 3D orbital shaker (Block Scientific at 30 rpm). Pellets were cultured for 2 weeks at 37°C/5% CO₂. Some pellets were fixed and paraffin embedded, and then 5 µm sections were serially stained with hematoxylin, Fast Green, and safranin-O as previously described. To quantify the sulfated glycosaminoglycan (GAG) content, other pellets were digested with a protease K solution, incubated with a dimethylmethylene blue dye, and read spectrophotometrically, with chondroitin sulfate as a standard, as previously described. The GAG content was normalized to the deoxyribonucleic acid (DNA) amount that was measured using a CyQUANT cell proliferation assay kit (Molecular Probes), with calf thymus DNA as a standard.

Adipocytic differentiation assay

The adipocytic differentiation was induced by culture of SVF cells on tissue culture plastic, which upon confluence were treated with 10 µg/mL insulin, 1 µM dexamethasone, 100 µM indomethacin, and 0.5 mM 3-isobutyl-1-methylxanthine for 72 h and then with 10 µg/mL insulin for 24 h, as previously described. The 96-h treatment cycle was repeated three times. These adipogenic-inductive supplements were added to z-MEM containing 10% FBS. At the end of induction, cells were fixed with 4% formalin, stained with oil red O dye (Sigma), and observed with a phase-contrast microscope.

Results

Liposuction samples, in volumes ranging from 40 to 400 mL, were processed in paral el by following either the standard manual procedure described previously, or the newly developed automated procedure based on the Sepax technology (Fig. 1). Both processes were typically completed within 1.5-2 h, depending on the initial volume of input and including 1 h for the digestion of the sample by collagenase.

Isolation yields and phenotype

The number of nucleated cells from SVF in the final suspension was evaluated after both procedure types and normalized to the volume of liposuction input processed. The manual procedure yielded 1.6±1.0×10^7 nucleated cells/mL of liposuction, whereas the Sepax-based procedure isolated 2.6±1.2×10^7 cells/mL of liposuction (n=6, Fig. 2A). This represents a 62% increase in isolated nucleated cells, and the difference was highly significant as tested by t-test. The yield in terms of isolation of CFU-fs was 24% higher with Sepax (Fig. 2B), which represented a limited but significant difference (n=18, triplicate determinations performed for six donors). The variability in the isolation yield and clonogenicity, that is, the standard deviation as a percentage of the mean, was always higher with Sepax, with a reduction of 18% and 50%, respectively, as compared with the manual procedure.
chamber twice for an optimal cell recovery. The minimum final volume used was 40 mL. The re-suspension medium was as described for the manual isolation process.

Cell count and characterization by flow cytometry

Nucleated cells were counted using a Neubauer chamber after staining with Crystal Violet (Sigma), whereas cell viability was determined by counting the blue (dead) and transparent (alive) cells after trypan blue (Sigma) staining. The phenotype of SVF cells was determined by cytofluorimetric analysis with fluorochrome-conjugated antibodies to human CD105 (from AbD Serotec), CD30, CD31, CD34, CD45, or CD73. Isootype IgGs were used as control (all from BD Biosciences). About 10^6 cells in suspension per condition were incubated for 30 min with the different antibodies at 4°C in CM, washed with PBS, resuspended in FACs buffer (FBS, 0.5% human serum albumin, and 0.5 mM EDTA), and analyzed with an FACSCalibur flow cytometer (BD Biosciences). 7-Aminoactinomycin D (7-AAD) was used to determine the number of dead cells inside the isolated SVF cell population. Expanded ASCs were analyzed after the first passage, investigating 7-AAD negative cells for expression of CD105 (Bioscience), CD144 (Becton Coulter), CD44, CD29 (Biorad), CD50, and CD73 (BD Biosciences).

Cell culture and colony-forming assays

For monolayer expansion, SVF cells were seeded at a density of 2 × 10^5 cells/cm² onto tissue culture plates, cultured in CM supplemented with 5% FBS, 1% pen/strep, 10 μM FGF-2 (R&D Systems), and 1% ITS +1 (10 μg/mL insulin, 5.5 μg/mL transferrin, 5 μg/mL selenium, 0.5 μg/mL bovine serum albumin, and 4.7 μg/mL linoleic acid). 1.25 μg/mL human serum albumin, 0.1 μM ascorbic acid 2-phosphate, and 10^-7 M dexamethasone (all from Sigma), and 10 μg/mL TGF-β1 (R&D). Aliquots of 5 × 10^5 SVF cells were centrifuged at 250 g for 5 min in 1.5 mL polypropylene conical tubes (Sarstedt) to form spherical pellets, which were placed onto a 3D orbital shaker (Bibby Scientific) at 30 rpm. Pellets were cultured for 2 weeks at 37°C/5% CO₂. Some pellets were fixed and paraffin embedded, and then 5 μm sections were serially stained with hematoxylin, fast green, and safarin-O as previously described. To quantify the sulfated glycosaminoglycan (GAG) content, other pellets were digested with a pronase K solution, incubated with a dimethylmethylen blue dye, and read spectrophotometrically, with chondroitin sulfate as a standard, as previously described. The GAG content was normalized to the decyribonucleic acid (DNA) amount that was measured using a CyQUANT cell proliferation assay kit (Molecular Probes), with calf thymus DNA as a standard.

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Results

Liposuction samples, in volumes ranging from 40 to 400 mL, were processed in parallel by following either the standard manual procedure described previously or the newly developed automated procedure based on the Sepax technology (Fig. 1). Both processes were typically completed within 1.5-2 h, depending on the initial volume of input and including 1 h for the digestion of the sample by collagenase.

Isolation yields and phenotype

The number of nucleated cells from SVF in the final suspension was evaluated after both procedure types and normalized to the volume of liposuction input processed. The manual procedure yielded 1.6 ± 0.9 × 10^6 nucleated cells/mL of liposuction, whereas the Sepax-based procedure isolated 2.6 ± 1.2 × 10^6 cells/mL of liposuction (n = 6, Fig. 2A). This represents a 62% increase in isolated nucleated cells, and the difference was highly significant as tested by t-test. The yield in terms of isolation of CFU-Cs was 24% higher with Sepax (Fig. 2B), which represented a limited but significant difference (n = 18, triplicate determinations performed for six donors). The variability in the isolation yield and clonogenicity, that is, the standard deviation as a percentage of the mean, was always lower with Sepax, with a reduction of 18% and 50%, respectively, as compared with the manual procedure.
FIG. 3. Cytometric analysis of SVF cells obtained from liposuction samples processed by manual or Sepax procedure. SVF cells obtained from three different donors were analyzed by cytomtery for the expression of CD45 (top graph) and CD34/CD31 (middle and bottom graphs). Results are expressed as mean ± SD of the positivity for the indicated markers. NS, in percentage of cells was found between the two procedures as tested by paired t-tests.

the Sepax technology and compared it with the current standard, namely, the manual isolation of SVF. The automated procedure resulted in higher and more reproducible isolation yields of SVF cells, with phenotypic and functional characteristics similar to those isolated by the manual procedure. The isolation yield of SVF cells not only depends on how the tissue is processed, but also on various factors, including interdonor variability, anatomical harvest site, aspiration procedure, storage time, sample processing prior to digestion (e.g., washing or concentration by centrifugation), and concentration/type of enzyme used. Those factors explain the high variability of yields described in the literature, from as low as 4 × 10⁶ cells/ml²⁵ to around 3 × 10⁷ cells/ml of adipose tissue.²⁶ Another factor affecting yield variability is whether the adipose tissue is in the form of a solid resection or a liposuction sample, whether or not tumescent solution was used for liposuction, and/or whether or not centrifugation is performed after collection. All of which greatly modify the characteristics of the source material used for isolation. Typically, our standard manual isolation procedure results in an average yield of 1.5–2 × 10⁷ cells/ml of adipose tissue. To avoid these interdonor (liposuction vs. resection) and technical sources of variability, we divided a given tissue sample into two equal fractions that were then processed via the automated or manual procedure. The average isolation yield achieved with Sepax was in the range of 2.6 × 10⁷ nucleated cells per ml of liposuction sample. This average yield is comparable with the previously described Celution™ system from Cytori Therapeutics,²⁵ for which an average yield of 2.6 × 10⁷ cells per ml of adipose was reported. The reported frequency of CFU-fs in SVF also varies in the literature, ranging from 0.3% to 15%, likely due to different donor densities of seeding, different media used, or a discrepancy in the definition of a CFU-f colony.²⁴,²⁵,²⁶ Frequencies between 10% and 15% of CFU-fs are reported here, with only a slight difference between manual and automated processing for a given donor. The variability seen in the manual processing was reduced with the automated procedure, likely due to a better standardized performance of the critical steps (e.g., removal of the supernatant after centrifugation).

The investigation of the phenotypical and functional characteristics of the isolated cells showed no difference between ASCs isolated manually or by Sepax. We recently identified CD34⁺/CD31⁺ as the population containing CFU-fs and pericytes within SVF, with CD34⁺/CD31⁻ and CD45⁺ cells as the endothelial and hematopoietic
FIG. 5. Differentiation into mesodermal lineages of SVF cells obtained from liposuction samples processed by manual or Sepax procedure. (A) Alizarin red staining after manual (left picture) and Sepax (right picture) procedures; SVF cells were grown to subconfluence and induced with either standard medium (left well) or osteoblastic induction medium (right well) for 3 weeks. (B) Apatite deposition by ASCs obtained either by manual isolation or by Sepax and cultured for 3 weeks with osteoblastic induction medium quantified by using the Osteoimage kit. Results are expressed as mean±SD of 10 fluorescence intensity values obtained from three independent donors. No significant difference in fluorescence intensity was found between the two procedures as tested by unpaired t-test. (C) GAG/DNA quantification and (D) safranin-O staining on sections of samples from ASCs obtained either by manual isolation (left) or by Sepax (right) and cultured for 2 weeks with chondrogenic induction medium. (E) Fluorescence microscopy pictures after oil red O staining of ASCs obtained either by manual isolation (left) or by Sepax (right) and cultured for 2 weeks with adipogenic induction medium. GAG, glycosaminoglycan.

Color images available online at www.liebertonline.com/toc

fractions, respectively. No difference between manual and Sepax processing was observed in the percentage of the different cell populations or in the expression of other mesenchymal markers (CD73⁺ or CD90⁺, data not shown). From a functional standpoint, ASCs from SVF proliferated and differentiated similarly toward the three mesenchymal lineages (adipogenic, osteogenic, and chondrogenic), independently of the isolation procedure. The results of the present study therefore validate Sepax as an automated alternative to manual processing of adipose tissue, leading to higher yields of SVF cells of comparable quality.

The potential benefit of the automated device/process is manifold. (1) For research purposes, it could help to standardize and simplify the processing of adipose material. This could allow for a better comparison of the results generated in different experimental runs or research laboratories. (2) For clinical purposes, the presented approach would enable the direct coupling of the technology with another bioreactor system for cell expansion/culture, resulting in a streamlined and perhaps fully automated approach to generate adipose-cell-based grafts. (3) Alternatively, the Sepax system may be employed for intraoperative transplantation of SVF
cells. This approach is already in clinical use for cell-assisted lipotransfer in breast augmentation and breast reconstruction,28 but so far relies on a manual isolation of the SFV cells.29 In this latter context, our group is currently planning to clinically test the efficacy of osteogenic/vasculogenic grafts intraperitoneally generated by cells automatically isolated from autologous adipose tissue for bone defect repair in osteoporotic patients.

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Address correspondence to:
Ivon Martin, Ph.D.
Departments of Surgery and Biomedicine
Institute for Surgical Research and Hospital Management
University Hospital Basel
Hebelstrasse 20
CH-4031 Basel
Switzerland

E-mail: imartin@uhbs.ch

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C. CONCLUSIONS AND FUTURE PERSPECTIVES

Since two decades, tissue engineering is an emerging research field in regenerative medicine. The idea to regenerate a tissue or even a whole organ stimulated science and medicine and raised expectations in society. Indeed, in today’s world need for tissue and organ replacement is growing tremendously, every day the waiting list for donors is extending. Initial experiments in laboratories and clinical trials promised that tissue engineering approach would soon satisfy those hopes. Unfortunately, few applications and products have found a place in clinics and on the medical market. Knowledge gained in the stem cell field and their translation into the tissue engineering field further increased the potential of those approaches. However, despite efforts and investments made on tissue engineering, researchers and biotechnology industry so far failed to actually provide relevant and applicable therapies and novel products, mostly because of the following reasons: i) most of the developed strategies failed to demonstrate higher clinical potential over the conventional techniques, ii) the reproducibility of successful approaches is often not satisfactory, iii) the manufacturing and treatment techniques are too costly and therefore not taken in charge by health insurances. This thesis focuses on bone tissue engineering and aims to solve some of the limitations related to the upscaling from laboratory scale osteogenic grafts to a size adapted to applicable and standardized clinical applications in bone tissue repair. If successful, this study and its outcomes could likely be adapted to other applications in the regenerative medicine field.

Considering vascularization as one of the major obstacles in upscaling of tissue engineered construct, in Chapter I, in vitro pre-vascularized large osteogenic grafts were generated and shown to be able to engraft more rapidly than non-prevascularized ones and to form highly homogenous bone tissue in vivo. The study shows that large osteogenic grafts can be pre-vascularized by the use of stromal vascular fraction (SVF) cells from human adipose tissue which results into more uniform and abundant formation of bone tissue inside the graft upon ectopic implantation in rat. Primary endothelial progenitors from SVF can substitute other primary endothelial cells (i.e HUVEC, cord or peripheral blood-derived EPCs, etc.) commonly used to study pre-vascularization of tissue-engineered constructs. Moreover, those primary cells lack clinical relevance and require further costly
in vitro manipulations. It should be stressed that the in vitro performance of SVF cells to build capillary networks and their potency to become functional blood vessels through rapid anastomosis upon implantation is likely the key mechanism enabling the successful engraftment of the tissue-engineered, osteogenic constructs. The 3D perfusion bioreactor system used in Chapter I maintains endothelial progenitors inside the scaffold for 5 days of in vitro culture. Moreover, in such large scale grafts, a homogenous cell seeding provided by the perfusion system appears essential not only for a uniform distribution of the pre-vascularization but also for a uniform distribution of osteogenic cells and their secreted extracellular matrix. This study also suggests that SVF cells, though typically described with a lower intrinsic bone-forming capacity than BMSC, managed to compete with them and even showed a higher performance both in terms of reproducibility and amount of bone tissue formation, in the context of a large osteogenic graft.

Many approaches to overcome the size limitations of engineered osteogenic grafts involve irrelevant and impractical endothelial cell sources as well as transduction of mesenchymal cells with angiogenesis-stimulating proteins. The strategy used in the present study identified novel and efficient tools allowing demonstrating that endothelial progenitors from human SVF can support deeper bone formation not only in the periphery but also throughout a large construct and that, due to their availability and abundance, SVF cells constitute a unique source of both vasculogenic and osteogenic cells, likely showing a superior potential as compared to BMSCs. As a continuation of this study, scaffolds made of different materials and obtained by various production techniques, able to fulfill any specific shape or mechanical features, will have to be validated. Also, animal models better reflecting targeted clinical scenario, such as large orthotopic models, should be designed to further validate the described approach.

Chapter II introduces in vivo manufacturing of osteogenic constructs producing bone tissue ectopically. SVF cells were directly implemented into the construct during manufacturing of the graft. The time consuming and costly in vitro manipulations typically related to engineered constructs was skipped by stimulating SVF cells with low doses of rhBMP-2, known to induce osteogenesis and vascularization. It should be stressed that by eliminating the in vitro culturing phases, this strategy shifts the cell-seeded construct from being truly a tissue engineered product into becoming rather a transplant. As a clinical relevance, this method introduces an intraoperative approach by making the
whole procedure applicable in the timeframe and environment of one surgery procedure. One should also note that the approach decreases related costs by reducing hospitalization time and need of GMP facilities. Follow-up of this project, by applying a similar manufacturing process but by using an orthotopic model with a critical size defect is ongoing. Based on forthcoming results, clinical trials using volunteer patients are currently planned.

Finally, in Chapter III, the standardization of the cell isolation procedure to manufacture tissue engineered osteogenic grafts or implants was assessed. Newly developed therapeutic approaches using tissue engineering suffer from a lack of standardization and reproducibility and therefore hardly find clinical applications and mostly stay at the clinical trial phase. In this part of the thesis, an automated adipose tissue derived cell isolation device was developed and validated. The automated cell isolation device gives higher yield of viable cells with preserved clonogenic and differentiation potential as compared to manual isolation techniques used in the laboratory. Adipose tissue derived stromal cells isolated with this automated method are not exposed to risk of contamination and are functionally similar. Making cell isolation techniques easy, reproducible, applicable, operator and environment independent and might widespread tissue engineering approach in clinics. Minimizing labor intensive work and safety concerns will also result in decrease of costs changing the preferences towards newly developed strategies in regenerative medicine.

Taken together, these results suggest that bone tissue engineering using freshly isolated SFV cells is pretty close to be applied as a regular treatment in orthopedics. As indicated before, results presented in this thesis could easily be adapted to other medical fields where stem cell based tissue engineering therapies such as skin, muscle, vascular and periodontal tissue engineering.
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Sinan Güven

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E. CURRICULUM VITAE

First name, Surname: ŞİNAN GÜVEN

Date of Birth: May 7, 1979
Nationality: Turkish
Gender: Male

185 Elm Street
Cambridge 02139 MA, USA
sinansnp@yahoo.com

Education:
2007 - 2011 Ph.D. Department of Biomedicine, University of Basel, Switzerland
2003 - 2006 M.S. Department of Biotechnology, Middle East Technical University, Ankara - Turkey
1999 - 2003 B.S. Department of Chemistry, Middle East Technical University, Ankara - Turkey

Language:
Excellent command of both spoken and written English
Good command of both spoken and written Bulgarian
Turkish native language

Positions and employment:
2012 - present Post-doctoral fellow, Harvard-MIT Health Sciences and Technology, Massachusetts, USA
2011 - 2012 Post-doctoral fellow, University Hospital Basel, Switzerland
2007 - 2011 PhD Student, University Hospital Basel, Switzerland
2006 - 2007 Visitor scientist, Ludwig Boltzmann Institute for Experimental and Clinical Traumatology, Vienna - Austria
2004 - 2007 Teaching and Research Assistant, Faculty of Pharmacy, Hacettepe University, Ankara - Turkey

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Awards:

2011 University of Basel, Travel award

2011 Orthopaedic Research Society Annual Meeting,

New Investigator Recognition Awards Finalist, Long Beach - USA

2010 Tissue Engineering and Regenerative Medicine Int. Society EU,

50 Best Abstracts Awards, Galway - Ireland.

2006 European Union Leonardo da Vinci Scholarship, Ankara - Turkey

Certificates and Qualifications:

January 2010 Laboratory Animal Experiments course Zürich - Switzerland


June 27-28, 2006 Nanotechnology Workshop, Hacettepe University, Ankara - Turkey
June 1-5, 2006  1st Marie Curie Cutting Edge InVENTS Conference on New Developments on Polymers for Tissue Engineering, Replacement and Regeneration, Funchal, Madeira - Portugal

Nov.24-26, 2004  Basic Principles on Cell Culture Technology and Artificial Organs Workshop, Department of Bioengineering, Ege University, Izmir - Turkey

September, 2003  Preparation and Characterization of Multicomponent Polymer Systems, Summer School, Technical University Darmstadt, Darmstadt - Germany

Interests:  Angiogenesis, Tissue Engineering, stem cells, biomaterials, nanotechnology