PROJECT FINAL REPORT

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Project co-ordinator name:	Prof. Dr. Heike Walles
Project co-ordinator organisation:	Fraunhofer-Gesellschaft zur Foerderung der angewandten Forschung e.V.
Phone:	+49 931 31 88828
Fax:	+49 931 31 81068
E-mail:	Heike.walles@igb.fraunhofer.de
Project website address:	http://www.vascubone.fraunhofer.eu/
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Contact:
Prof. Dr. Heike Walles
Fraunhofer-Institute Interfacial Engineering and Biotechnology, IGB
Translational Center Würzburg
´Regenerative therapies in oncology and musculoskelettal diseases` (Würzburg branch)
Röntgenring 11
97070 Würzburg
Germany

VascuBone Consortium – Month-60-Meeting

Table of Contents

1. Executive Summary

The goal of VascuBone was to develop a "**tool box**" composed of combinable parts to optimize bone regeneration taking into account the respective patient's situation. At the end of VascuBone the "tool box" includes **modified CeraSorb®M (β-TCP) materials and new developed composite materials** for the reconstruction of not critical and critical size defects. The major improvement of this new bone implant materials is the newly developed and **standardized modification** with different **diamond particles (DP)** ranging from nano- to microcrystalline sizes improving the hydrophilicity of the materials due to high surface wettability of the diamond itself and promoting cellular proliferation, differentiation, and bone formation in small (mice, rats) as well as in large (sheep) animal models. **New methods to surface-modify** the nDPs by polylactide and benzoquinone have been established: composite scaffold of poly(LLA-co-CL)/polylactide modified nanodiamond (n-DP-PLA/poly(LLA-co-CL)), and poly(LLA-co-CL)/benzoquinone modified nanodiamond (n-DP-BQ/poly(LLA-co-CL)).

The **company DiaCoating GmbH**, Innsbruck Austria **was founded** by VascuBone members and the production processes were established and tested for reproducibility. Process documentation due to ISO-standards was developed and is available. DiaCoating will bring the new material as well as the new modification technology on the market and **ensures the sustainability** of materials developed in VascuBone. The new materials were tested according to DIN ISO 10993-12: 2009 and found to be non cytotoxic and biocompatible. Furthermore, an intravenous dose toxicity study of the nano diamond particles prove that the nDP appeared to be safe. Beside the material development one major focus of VascuBone was to develop **new tools to control and ensure safety**, immunological acceptance and efficacy of new implants including nano-materials and ATMPS. A highlight to this respect is the development of **organotypic tissue models** to proof tumorigenicity of new materials. Additionally, a successful design has been established to investigate in vivo environmentally-induced carcinogenesis and to monitor implanted materials/scaffolds by **bioluminescence** in an attempt to surpass the limitations of the long term rodent assays. Last but not least all these new models were used for in vitro – in vivo correlation studies to validate the new models.

Other important components of the VascuBone "tool box" are based on in vivo imaging of the bone implant to visualize existing vessels and the **formation of neovessels** in the healing bone. Multiple optimized **angiography contrast agents** for in vivo MRI investigation of blood flow were developed in the consortium and are included in the VascuBone "tool box". The imaging agents were various and included gadolinium-based agents and iron oxide-based agents, for positive contrast and negative contrast in ^{1}H MRI, respectively.

At the end of the project components of this new tool box were combined with cells and the prevascularised Scaffold BioVaSc-TERM® to develop translational approaches for regenerative therapies of critical size defects. To ensure safety of these **ATMPs** a novel quality assessment technology for **non-invasive online monitoring** of cellular aging during cell culture was developed. For this, an electric cell-substrate impedance sensing was devised. The **sensors** were able to trace significant impedance changes after bmMSC seeding upon cell spreading and adhesion. The system was further proven suitable for continuous monitoring of cellular behavior up to four weeks and allowed to validate the initiation of osteogenic and adipogenic induction.

To reach the overall aim of VascuBone, a pre-vascularized bone implant that allows connection to a patient's circulatory system, methods to seed the vascular parts of the BioVaSc-TERM® with endothelial cells and to fill the lumen with bone replacement material (diamond coated β-TCP) and bmMSC (**BoneVaSc-TERM®**) were standardized in GMP laboratories. In vitro critical size defect of the sheep mandible as well as an in vivo critical size defect of the sheep mandible was standardized. The prevasularized BoneVaSc-TERM® was implanted successful up to six month in a **critical size defect of the tibia and the mandible** and could document improved bone healing of both critical bone defects. During VascuBone the **first prevasularized bone implants** were sussefully implanted and based on these preclinical data e an "Advanced Therapeutical Medicinal Product" will be developed in further projects, in order to start the clinical evaluation in phase I / II trials.

2. Summary description of the project context and the main objectives

VascuBone was focusing on the following scientific objectives and major goals:

- Optimization of osteoconductive, and osteo- and angioinductive properties of clinical applied biocompatible materials
- Enhancement of mechanical, surface and morphology properties of scaffolds
- Process development for efficient binding of active bio-molecules in low dose on a modified/coated implant surfaces by an innovative coating technology
- New strategy for the development of vascularized ATMPs using a BioVaSc
- Evaluation and comparison of stem cell sources for regenerative therapies
- Design and development of regeneration inducing materials and therapies adapted on the needs of older people considering the demographic trend in Europe
- Development of a tool box for the production of bone implant materials including vascularized materials for critical size defects (medical need); the translation of this tool box to other regeneration applications
- Carry regenerative medicine towards clinic

To reflect the value chain of advanced bone implants the objects can be classified into four groups – **materials – cells – non invasive monitoring technologies – animal models**.

Materials

One major objective of VascuBone addressed the production and modification of two types of materials with the commercially available β-TCP materials CeraSorb®M and the newly developed composite materials. Both materials were modified with different diamond particles (DP) ranging from nano- to microcrystalline sizes, one of the new products of the VascuBone consortium is DiaSorb (CeraSorbM + nDPs). The modified surfaces promote cellular proliferation, differentiation, and bone formation in vitro and in animal models. Additionally the consortium could demonstrate that nano diamond particles can be efficiently bio functionalized by physisorption with growth factors like Ang-1, VEGF to stimulate angiogenesis and BMP-2 to enhance bone formation after implantation. The nDP-PHY scaffolds (scaffolds modified by nDP and physisorbed growth factors) used in critical-sized bone defects for the first time appear to have promise compared to growth factors adsorbed onto a scaffold alone and the short distance effect prevents adverse systemic side effects. Interestingly by using nDP-PHY it is possible to use very low BMP-2 dose of 1 µg / critical size bone defect to stimulate bone formation.

Emanating from state-of-the art biomaterials like Cerasorb® M, chronOS® or the novel polymer scaffolds, as L-lactide and ε-caprolactone and the corresponding copolymers the modification of the biomaterials based on hydrophilic diamond particles has been carried out. A reproducible fabrication chain of modified, tailored

biomaterials could be realized with respect to scaling-up and regulation aspects. Both complex geometries (e.g. cylinders, blocks, etc.) as well as granula for further application as BoneVaSc® could be produced in large scale with high quality and reproducibility.

In addition PPSorb™, a tin-free blend of two homo-polymers, was developed with reproducible quality with minimum environmental impact and suitable for sterilization by gamma-irradiation.

All methods allow the control of morphology, porosity and degradation time. The developed composites have been tested in terms of biocompatibility (DIN ISO 10993) and cytotoxicity test according to DIN ISO 10993-5. The degradation process and influence on cell growth and interaction was studied *in vitro* and *in vivo*.

The interfaces between different materials play a crucial role in many applications. In VascuBone various theoretical methods and calculations were applied to tailor and design reactive nDP surfaces, and to bio-functionalize these surfaces.

The osteoconductive, and osteo- and angioinductive properties of the biomaterial have been improved by coating with growth factors (GF) or components of the ECM. For an efficient and stable grafting of these molecules on the nDP-coated implant material first theoretical modelling using example substances like chitosan and fibronectin were performed.

The company DiaCoating GmbH, from Innsbruck (Austria), was founded by VascuBone members and the production processes were established and tested for reproducibility. Process documentation due to ISO-standards was developed. DiaCoating will bring the new material as well as the new modification technology on the market and ensures the sustainability of materials developed in VascuBone.

Cells

The biggest hurdle in translation of cell based implants for critical side defects are the missing pre-vascularised scaffolds an technologies to seed human endothelial cells (EC) on the preformed vascular structures and establishing co-cultures with tissue specific cell types, in bone engineering this could be bone marrow derived mesenchymal (MSC) stem cells.

To solve these problems technologies to load cells into synthetic materials have to be devloped. To facilitate loading the VascuBone consortium investigated and established bioreactor systems which provide sufficiently stable conditioning of cell nurture for both cell types EC and MSC and the co-culture of them, to engineering the first pre-vascularised bone implants for critical size defects in the mandibular as well as the tibia. The prime feature of the bioreactor technology was not only testing cell attachment onto substrates but also to optimize for long-term cellular growth in a controlled and reproducible manner in a three-dimensional macroscopic environment. This technical advancement firstly enabled to assess biocompatibility between cells and artificial materials in a reliably fashion. Besides proprietary developments also commercially available solutions for single use were implemented.

During the process development of each cell based implant the ideal cell source is one major objective. For every pre-vascularised implant EC are necessary, this cell type could be isolated from skin biopsies an alternative are endothelial precursor cells (EPCs). In VascuBone a Endothelial Cell Growth Medium based on vericyte® technology was developed. This Medium allows an increased number of population doublings of EC during cultivation in comparison to current available standard media, and does not contains serum and other non-defined compounds to match the current GMP requirements.

Additionally and EPC subtype was thoroughly characterized by assessing different specific markers and various cell based assays aiming at the biological functionality of the cells. The EPCs possess the cell culture specific characteristic of clonal proliferation and can be re-plated and passaged. Through the optimization of the isolation technique, by using erythrocyte depletion, changing the seeding density and addition of a new attachment factor, the yield of starting points for the clonal outgrowth could be significantly increased. These improvements made it possible to standardize the isolation procedure for future use in patients.

To reach the overall aim of VascuBone, a pre-vascularized bone implant Bone VaSc-TERM® that allows connection to a patient's circulatory system was developed. Therefore methods to seed the vascular parts of the BioVaSc-TERM® with endothelial cells and to fill the lumen with bone replacement material (diamond coated β-TCP) and MSC (BoneVaSc-TERM®) were standardized. Thereafter intense in vitro characterization, in vivo studies and the transfer into a GMP production process was realized during the VascuBone project.

Non invasive monitoring technologies

The success of cell-based therapies depends on several critical issues, including the route and accuracy of cell transplantation, the fate of cells after transplantation, and the interaction of engrafted cells or scaffolds with the host microenvironment and the vascularization of the implant. To assess these issues, it is necessary to monitor transplanted cells as well as blood flow non-invasively in real-time.

Magnetic resonance imaging (MRI) is a tool uniquely suited to this task, given its ability to image deep inside tissue with high temporal resolution and sensitivity. Within the project framework, various optimized angiography contrast agents for in vivo MRI investigation of blood flow were developed. The imaging agents were various and included gadolinium-based agents and iron oxide-based agents, for positive contrast and negative contrast in 1H MRI, respectively. These imaging agents were tested in vivo in healthy rats in comparison to the standard, marketed clinical contrast agent, Magnevist (Gd-DTPA). The in vivo MR results obtained with the various agents showed that the optimized iron oxide-based contrast agent (FeraSpin XS-Type) induced the most significant signal changes in the bone marrow within the rat femur. Indeed, this particular agent clearly aided in the characterization of the existence of active blood flow and is, thus, potentially suited for the visualization of angiogenesis in healing bone and remodeled biomaterials.

The second important objective in this field is to control the cellular fate during in vitro expansion ideal non invasive and marker free. Experimental setups for the study of the crosstalk of MSCs and EPCs were successfully established in VascuBone. We focused on the changes of global gene expression patterns of human primary EPCs after having been subjected to conditioned medium of human primary MSCs or after direct cell-cell contact, and vice versa, respectively. The bioinformatical analysis of the microarray data revealed that several genes related to osteogenesis and angiogenesis are differentially regulated in MSCs and EPCs due to treatment with conditioned medium as well as after direct cell-cell contact. Assays derived from these results might prove useful in terms of quality control for tissue engineering procedures. This study will help to better understand the crosstalk of MSCs and EPCs, which will finally aid to the improvement of vascularized tissue engineering constructs.

To ensure safety of these ATMPs a novel quality assessment technology for non-invasive online monitoring of cellular aging during cell culture was developed. For this, an electric cell-substrate impedance sensing was devised. The sensors were able to trace significant impedance changes after MSC seeding upon cell spreading and adhesion. The system was further proven suitable for continuous monitoring of cellular behavior up to four weeks and allowed to validate the initiation of osteogenic and adipogenic induction.

Animal models

A qualified animal model has to be developed independently of any specific drug as a basis for the safety and efficacy study. The animal model should provide a defined framework for the submission, review, and regulatory acceptance of a new drug, scaffold or cell based product. The use of a qualified model in multiple drug/product development programs targeting a particular disease or condition eliminates the need to develop specific models for each investigational new drug. The objective in VascuBone-TERM® was to develop such defined bone defect and critical size animal models and to test the first materials, cell based products in these models. At the end of the project an intense characterisation of the models and of the first tested materials and cell based bone implants should be available as standard values to compare further new materials regarding their capacity to induce bone formation and vascularisation and to heal critical size defects. Beside a variety of small animal models, a large animal model of the avascular necrosis of the femoral head and large bone defects of the mandibular could be standardized. First implantation of the pre-vascularized BoneVaSc® underline the functionality of the critical size defect model in the mandible as well as the vascularized bone regeneration capacity of the BioVaSc-TERM® technology.

3. Description of the main S & T results/ foregrounds

In the following, the main results are described for every scientific work package (WP).

WP2: Monitoring and quality control

Biocompatibility, safety and efficacy of refined resorbable synthetic scaffolds

The selection of the most appropriate material to produce a scaffold for bone/cartilage tissue engineering applications is an important step towards the construction of a tissue engineered product, since its properties determine, to a great extent, the properties of the scaffold. Scaffolds based on polylactic acid, and their co-polymers are believed to be suitable materials for bone tissue engineering. In addition, ceramics, as beta-tricalcium phosphate (β-TCP) have been widely used in the biomedical engineering and bone substitution/regeneration field due to the fact of being osteoconductive and osteoinductive.

One of the most important objectives of the VascuBone project was addressing the production and modification of two types of materials which were included in the project: the commercially available β-TCP materials CeraSorb®M and the newly developed composite materials. These scaffolds were tested by indirect and direct contact cytotoxicity assays according to DIN ISO 10993-12: 2009 and found to be noncytotoxic and biocompatible. Furthermore, in vivo animal experiments were performed by the consortium to evaluate the host responses and tissue reactions. The results of biocompatibility tests, degradation, and inflammation in vitro and in vivo have been presented and reported in several articles published by the consortium.

Furthermore, the materials were modified for i) improvement of hydrophilicity, ii) increasing of active surface area and iii) controlling and localizing offer of growth factors within the scaffolds. Both of TCP and polymer scaffolds were modified with different diamond particles (DP) ranging from nano- to microcrystalline sizes improving the hydrophilicity of the materials due to high surface wettability of the diamond itself. The surfaces of the scaffolds modified by nDP were found to biocompatible and shown to promote cellular proliferation, differentiation, and bone formation in small (mice, rats) as well as in large (sheep) animal models. Furthermore, it has been found that nano diamond particles can be efficiently biofunctionalized by Ang-1, VEGF and BMP-2 which remain active upon physisorption. The capacity of binding growth factors (BMP-2, Ang-1, VEGF) as well as the bioactivity of such bond GFs on nDPs was determined. Such functionalized nDP will provide increased active area (area in contact with bio-entities) in modified scaffolds and simulate a cell niche via nano-structure mimicking extra cellular matrix (ECM).

The nDP-PHY scaffolds (scaffolds modified by nDP and physisorbed growth factors) used in critical-sized bone defects for the first time appear to have promise compared to growth factors adsorbed onto a scaffold alone and the short distance effect prevents adverse systemic side effects. Interestingly by using nDP-PHY it is possible to use very low BMP-2 dose of 1 µg / critical size bone defect to stimulate bone formation.

The biocompatibility and efficacy of the surfaces modified by the diamond particles were demonstrated by

the consortium and reported in the 36, 48, 60 month technical reports. Furthermore, an intravenous dose toxicity study of nano diamond particles was performed. The data from the studies showed that the nDP in 3 concentrations (0.7, 3.5, and 7 mg/animal) appeared to be safe to use in future preclinical studies.

Evaluation of tumor promoting potential of nDP

One of the main Tasks of the VascuBone project was to investigate the safety of the developed scaffolds and the putative tumor promoting effects of scaffolds and their degradation products. This effect was investigated by evaluating the ability of the functionalized scaffolds to initiate and/or enhance an invasive phenotype in premalignant cells. As reported in the 36 and 48 month technical reports, protocols for in vitro tumorigenicity assays were established. Experiments have been carried out investigating the tumorigenic potential of luciferase expressing dysplastic oral keratinocytes (DOK^{Luc}) after being cultured on the different scaffolds for 1 week using in vitro functional tumorigenicity assays.

Furthermore, a successful design has been established for in vivo tumorigenicity assessment model in NOG mice using the combination of the premalignant cells and successfully transfecting cells for in vivo imaging (presented in 48 month report). A recently developed in vivo environmentally-induced oral carcinogenesis model to screen the tumorigenic potential of bone tissue engineered scaffolds has been successfully developed to monitor the scaffolds by bioluminescence in an attempt to surpass the limitations of the long term rodent assays. The developed model has been applied on testing nano diamond modified poly(LLA-co-CL) scaffolds with or without BMP-2. This model was used to investigate the tumorigenic potential oft he copolymer scaffolds modified with nDP [nDP] or with nDP + physisorbed BMP-2 [nDP -PHY], keeping only copolymer as the control scaffold which was used for developing the model.

In vitro tumorigenicity assays

Extracted DOK^{Luc} were cultured in non-adherent surface to account for sphere formation, which show the ability of a cell to grow independent of anchorage, hence giving an understanding to its tumorigenic potential (Fig. 1).

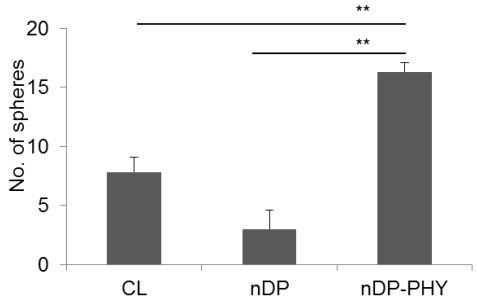

Fig. 1: Average number of spheres formed after 21 days in culture. The spheres counted are those larger than 40 µm in diameter.

In our studies we attempted the replication of an oral mucosa that mimics the primary tissue by co-culturing epithelial and mesenchymal (stromal) cell in a 3 dimensional organotypic model (OT). To assess the invasive

potential of the DOK^{Luc} extracted from the scaffolds, they were seeded on a matrix incorporating different types of fibroblasts (gingival fibroblasts or carcinoma associated fibroblasts (CAF)) (Fig. 2).

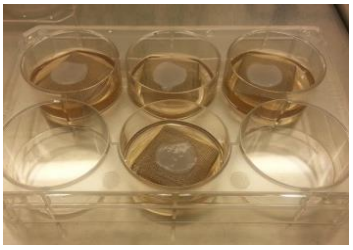

Fig. 2: Organotypics showing DOK^{Luc} cells extracted from scaffolds grown on collagen gels containing GF or CAF in an air-liquid interface.

Invasion of the epithelium formed was assessed and differences evaluated between cells grown on different scaffolds (Fig. 3).

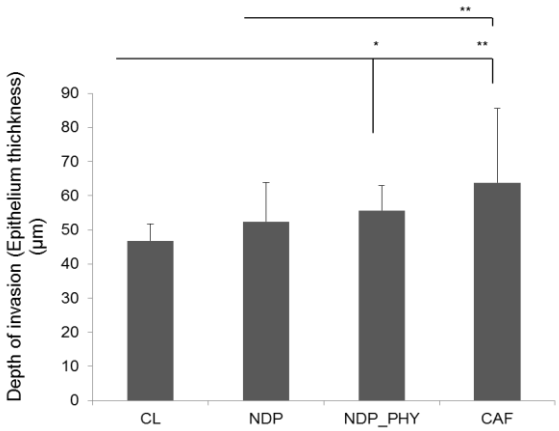

Fig. 3: Depth of invasion measured from organotypics. Highest and most significant invasion was seen from cells after being cultured in nDP -PHY scaffolds and from OT that were formed using CAF (which is used as a positive control).

Anti-Ki67 staining was carried out to identify the proliferating cells in the basal/ parabasal region and the suprabasal region of the epithelium in the organotypics evaluating the tumorigenic potential of these cells after being cultured on the different scaffolds (Fig. 4).

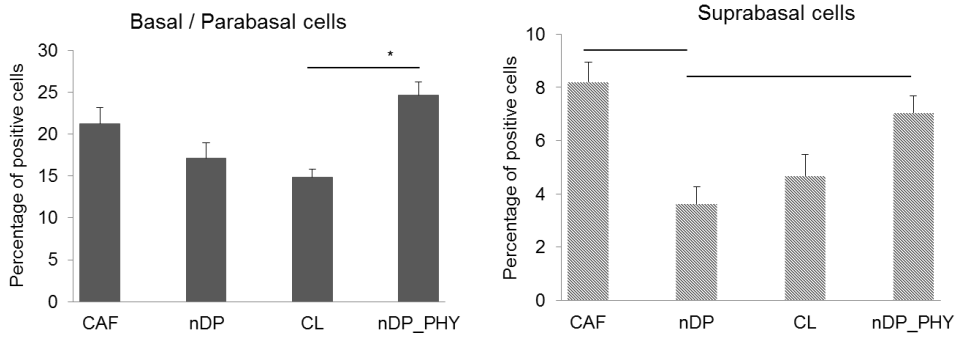

Fig. 4: Percentage of proliferating cells in the basal/ parabasal and suprabasal layers of the epithelial compartment of the organotypics. Cells extracted after being cultured in nDP scaffolds showed significantly reduced proliferation in suprabasal layers compared to other groups, hence demonstrating the least tumorigenic potential.

The different scaffolds cultured with either DOK^{Luc} or DOK^{Luc} + CAF were implanted subcutaneously in NSG mice; BLI was used to non-invasively monitor tumor formation and total photon count of negative and positive controls and plotted (Fig. 5).

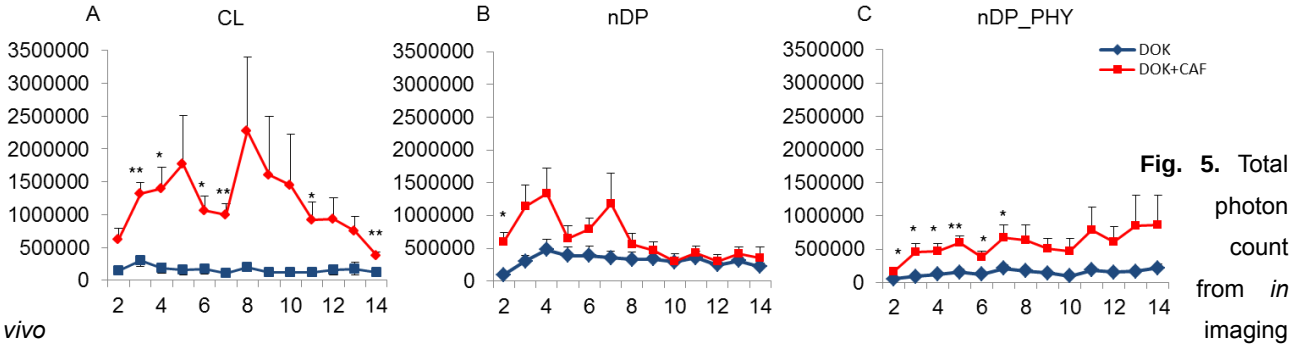

Fig. 5. Total photon count from *in vivo* imaging of different scaffolds xenotransplanted with DOK^{Luc} alone and DOK^{Luc}+CAFs throughout 14 weeks of imaging (n=6).

Within the VascuBone project, we have developed and validated a novel, sensitive, non-invasive and reliable model of microenvironmentally induced carcinogenesis by using high potency BLI enabling the early longitudinal real-time *in vivo* and consistent surveillance of tumors post implantation of scaffolds.
In vitro functional tumorigenicity demonstrated that the cells when grown in nDP modified scaffolds they form significantly less number of spheres. However, modified scaffolds with BMP-2 had the highest number of spheres due to the action of BMP-2 in increasing aggressiveness of tumors. This suppressing effect of the tumorigenic potential was also seen when comparing the invasiveness and proliferation of the cells in the OT, compared to the scaffolds modified with BMP-2 and to the OT grown with CAF as a positive control.
In vivo BLI results demonstrated that the tumors formed in nDP scaffolds had lower total photon count compared to CL and after 10 weeks, the positive tumors decreased and showed similar total photon count as negative tumors until the end of the experiment. When adding BMP-2 the total photon count of positive tumors was still higher than negative but still less than CL. In conclusion, the nDP does not show an increase in the tumorigenic potential of poly (LLA-co-CL) scaffolds and results show signs of an anti-tumorigenic effect that need further support.

In addition, the ability of tumor cells to proliferate in response to nano diamond particles used to modify the scaffold surfaces was investigated. The experiments were performed aiming to determine if the treatment of tumor cells with nano diamonds (ND) would affect the ability of the cells to proliferate. Accordingly, three cells lines were chosen for this part of the work: a prostate cancer cell line (LNCaP), and two breast cancer cell lines namely MDA MB 231 and MCF-7. Furthermore, a system based on a decellularized porcine jejunum scaffold was established to study tumor cells in 3D environment. Data from experiments done seem to suggest that the presence of nano diamonds does not induce Epithelial Mesenchymal Transition (EMT), which is implicated in cancer progression and metastasis. On the contrary, they might promote the reverse process called Mesenchymal Epithelial Transition (MET), which could possibly reduce the risk of tumor metastasis.

Monitoring and imaging of implant ingrowth

WP 2 concern in vivo imaging of the bone implant for the visualization of existing vessels and the formation of neovessels in the healing bone and remodeled biomaterials. Within the project framework, various optimized angiography contrast agents for in vivo MRI investigation of blood flow were developed. The imaging agents were various and included gadolinium-based agents and iron oxide-based agents, for positive contrast and negative contrast in ^{1}H MRI, respectively. These imaging agents were tested in vivo in healthy rats in comparison to the standard, marketed clinical contrast agent, Magnevist (Gd-DTPA). The in vivo MR results obtained with the various agents showed that the optimized iron oxide-based contrast agent (FeraSpin XS-Type) induced the most significant signal changes in the bone marrow within the rat femur (Fig. 6).

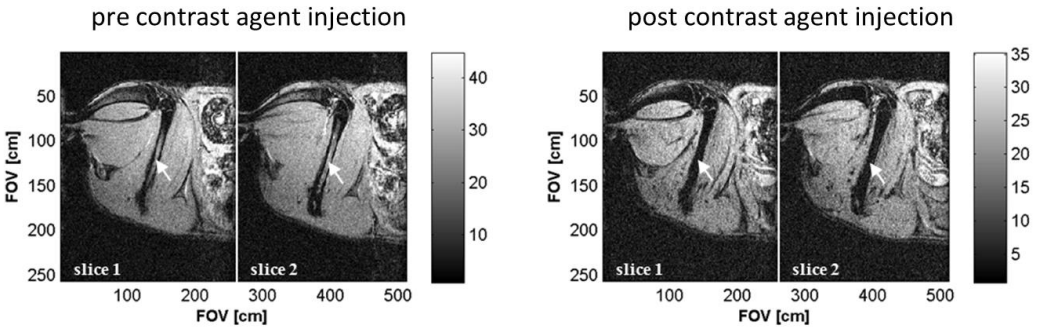

Fig. 6: Successful application of an iron oxide-based contrast agent (FeraSpin XS) for detection of blood flow in the rat femur (arrows).

Indeed, this particular agent clearly aided in the characterization of the existence of active blood flow and is, thus, potentially suited for the visualization of angiogenesis in healing bone and remodeled biomaterials.

Subsequently, after obtaining results in the rat, a strong focus was placed on the translation of the MRI method using the selected SPIO contrast agent from the rat to the sheep. So as to allow for a translation from the rat study to the large animal sheep study, the synthesis of the relevant SPIO contrast agent was optimized in the lab, not only to produce larger volumes of the agent to cover the dosing for the sheep study but also to increase the concentration of the signal-enhancing moiety, to enable a reduction of the required injection volume per animal. Of course, the synthesis modifications with the aim of increasing the particle concentration as well as overall volume had to be undertaken accordingly so as to maintain nanoparticle colloidal stability. Furthermore, the imaging agent was formulated by pharmaceutical methods to obtain a sterile, isoosmolar dispersion with a biocompatible pH for application into sheep.

The synthesis of the SPIO imaging agent for the sheep study was performed successfully. Furthermore, In preparation of the planned clinical studies in projects following the VascuBone project and based on the preclinical data of the VascuBone project, 3D UTE (Ultra Short Echo) MR imaging sequences have been implemented and tested on the 3T MRI scanner. Even though the spatial resolution is still limited, due to their short echo times, the UTE sequences allow measuring tissues which otherwise provide only little MR signal and appear dark in the MR images because of their short relaxation time constants.

WP 3: Design and Fabrication of Scaffolds

The activities of work package (WP) 3 providing a construction kit for tailor-made vascularized bone implants are focussed on the following aspects:

- development, modification and production of implantable and biocompatible material for regeneration of large bone defects in the jaw
- engineering of mechanical, chemical and surface properties, morphology of scaffolds suitable for cell adhesion and tissue generation
- theoretical calculation to predict surface chemistry for efficient binding of bio-molecules on nano-diamond particles as carrier
- process development for efficient binding of active bio-molecules in low dose on a modified/coated implant surfaces
- implementation of new quality standards in bone tissue engineering

Development, modification and production of implantable and biocompatible material for regeneration of large bone defects in the jaw

As explained in WP 2 the selection of the most appropriate material to produce a scaffold for bone tissue engineering applications is an important step towards the construction of a tissue engineered product. Therefore, the following materials have been applied and developed in VascuBone:

1. a commercially available beta-tricalcium phosphate (β-TCP)
2. biocompatible hybrid polymer scaffolds, which are developed within the consortium
3. modification of these biomaterials with tailored nano diamond particles (nDPs) improving the hydrophilicity of the materials and increasing the active area for a better interaction with cells and a better binding of active bio-molecules or proteins.

Emanating from state-of-the art biomaterials like Cerasorb® M, chronOS® or the novel polymer scaffolds, as L-lactide and ε-caprolactone and the corresponding copolymers the modification of the biomaterials based on hydrophilic diamond particles has been carried out. A reproducible fabrication chain of modified, tailored biomaterials could be realized with respect to scaling-up and regulation aspects. Both complex geometries (e.g. cylinders, blocks, etc.) as well as granula for further application in the BioVaSc could be produced in large scale with high quality and reproducibility.

With respect to the polymer-based scaffold materials the polymer synthesis process was up-scaled, well controlled and optimized. The scaffold can be prepared by both salt-leaching and 3D plotting methods and both types of polymer scaffolds can be modified by nDPs. The scaffold thickness could be expanded to more than 3 cm for the applications of critical size defects in jaw.

In addition PPSorb™, a tin-free blend of two homo-polymers, was developed with reproducible quality with minimum environmental impact (production of these blends using non-chlorinated solvents) and suitable for sterilization by gamma-irradiation.

All methods allow the control of morphology, porosity and degradation time. The developed composites have

been tested in terms of biocompatibility (DIN ISO 10993) and cytotoxicity test according to DIN ISO 10993-5. The degradation process and influence on cell growth and interaction was studied *in vitro* and *in vivo*.

Further modification of the scaffold materials with help of nano-materials has three aims: i) improvement of hydrophilicity, ii) increase of active surface area and iii) controlled and localized offer of growth factors/bio-molecules within the scaffolds. Within the project focus was laid on nano diamond particles. These particles have been tailored with well defined properties. Analysis confirmed these properties, all requirements have been realized and could be provided for all bone graft substitutes. The processes were established and tested for reproducibility and can be summarized as follows:

- development of a robust and highly reproducible method for the production of hydrophilic nanodiamond colloids with single digit nanometre size
- development of stable suspension of functionalized nDPs in organic solvents, e.g. THF for the incorporation into the polymer matrix
- development of strategies for the stabilization of nanodiamond colloids under physiological conditions using glucose solution
- synthesis and characterization of various surface functionalized nDP materials for biocompatibility testing and other cell tests
- synthesis and characterization of fluorescently labelled nanodiamonds.

Finally, it was possible to modify different synthetic bone graft substitutes homogenously in the entire volume of large scaffolds with tailored nDPs without abrasive wear due to diamond (reduction of pore size in perfusion method).

Engineering of mechanical, chemical and surface properties, morphology of scaffolds suitable for cell adhesion and tissue generation

As one of the hardest and multifunctional material, nDPs have the potential to bring specific properties to the biomaterials, especially to i) poly(LLA-co-CL) and ii) CeraSorbM scaffolds. Customized and functionalized nDP will provide increased active area (area in contact with bio-entities) in modified scaffolds and will simulate a cell niche via nano-structure mimicking extra cellular matrix (ECM).

i) poly(LLA-co-CL):

Severe phase separation when the two materials were mixed by direct blending occur. This issue was addressed due to the divergent surface chemistry of the nanodiamond particles. New methods to surface-modify the nDPs by polylactide and benzoquinone have been established: composite scaffold of poly(LLA-co-CL)/polylactide modified nanodiamond (n-DP-PLA/poly(LLA-co-CL)), and poly(LLA-co-CL)/benzoquinone modified nanodiamond (n-DP-BQ/poly(LLA-co-CL)). The modification improved the surface interaction with poly(LLA-co-CL).

The scaffolds produced by two methods were well characterized chemically and biologically. n-DP-PLA/Poly(LLA-co-CL) and n-DP-BQ/Poly(LLA-co-CL) scaffolds were characterized for the mechanical properties. n-DP-PLA/Poly(LLA-co-CL) showed significant improved modulus, whereas n-DP-BQ/Poly(LLA-co-CL) composite could not be mechanically improved by particle addition. As a scaffold designed for bone

regeneration, the samples should be biocompatible. Therefore, the cell viability of n-DP-PLA/Poly(LLA-co-CL) was characterized confirming its good biocompatibility which is independent to the ratio of modified nanodiamond.

ii) DiaSorb (CeraSorbM + nDPs):

To quantify the increase of the surface due to nDP retained in the scaffold, the Brunauer-Emmet-Teller (BET) isotherm was determined using N2 at 77 K using a ASAP 2420 surface area and porosimetry system (Micromeritics). The established BET-method was used to calculate the surface area of the scaffold material before and after nDP modification. The surface measurement of unmodified scaffold material resulted in 0.12 m^2/g whereas the surface of nDP (6% wt.) modified scaffold was identified to 11.2 m^2/g, an increase by factor 100. The overall amount of carbon (nDP) for the applied solution (6% wt.) could be determined with 39 mg in 1 g scaffold material by oxidation of the carbon at increasing temperature and quantification of the resulting amount of CO_2 using the carbon content analyzer LECO CS 600. The surface area measurement of CeraSorbM+nDP (DiaSorb) scaffold was conducted by Mercury intrusion porosimetry according DIN 66133 additionally. It becomes apparent that the pore size of the nDP modified scaffold decreased slightly with increasing amount of nDP.

The active area (area in contact with bio-entities) was increased by nano-structured particle films and a cell niche via nanostructure is simulated mimicking extra cellular matrix (ECM). Due to different termination of the dangling bonds of the diamond particle surface properties like surface energies, band structure, polarity, electrostatical properties, H-bonds etc. have been varied. The possibility to generate closed or open porosities/morphology and higher mechanical stiffness/thickness of the layer leads to preconditions to bear loads.

Theoretical calculation to predict surface chemistry for efficient binding of bio-molecules on nano-diamond particles as carrier

The interfaces between different materials play a crucial role in many applications. This is especially the situation for bio-diamond interfaces. In silico calculations with the purpose to i) tailor and design reactive nDP surfaces, and ii) bio-functionalize these surfaces, have been performed using various theoretical methods in a multiscale approach (i.e. ranging from quantum mechanical calculations for the surface reactivity studies, to force field based calculations for the diamond-bio interfaces).

The role of surface termination (e.g. chemisorption of small and reactive species by saturation of the carbon dangling bonds) is to control the phase-purity of diamond and its surface reactivity, and to influence its specific surface properties e.g. wettability and polarity.

By chemical functionalization of diamond surfaces, tailor-made anchoring of growth factors (e.g. Angiopoietin-1 and BMP-2) can be triggered. Chemical surface patterns can be obtained which allow the guided assembly of GFs on predefined areas on the surface via different chemical or physical interactions. DFT-based calculations have been used in studying the effect of surface termination on the diamond surface reactivity. Ranging from surface functionalization to bio-diamond interfaces effects like entropy, temperature, conformation, etc. have been considered.

These calculations accompanied and supported the whole process of material development since these techniques are necessary to gain a thorough understanding during design of interface materials. Significant results can be summarized as follows:

- for non-solvent diamond-biomolecule systems, the results show that adhesion affinities are strongly dependent on biomolecule molecular weights
- when including a water based solvent in the systems, the results show good physisorption affinities between proteins and diamond
- by comparing the biomolecular structural changes during the adhesion processes, it can be concluded that both the general structures, as well as the binding pocket structures, were kept intact after the adhesion to the diamond surfaces (regardless of the adhesion affinities).

All results lead to predictions of experimental parameters and supported the interpretation of experimental results (see next section).

Process development for efficient binding of active bio-molecules in low dose on a modified/coated implant surfaces

The osteoconductive, and osteo- and angioinductive properties of the biomaterial have been improved by coating with growth factors (GF) or components of the ECM. For an efficient and stable grafting of these molecules on the nDP-coated implant material first theoretical modelling using example substances like chitosan and fibronectin were performed. Both of these molecules were found to bind strongly to the diamond surfaces, with a clear preference for chitosan. Based on these model calculations all applied proteins have been considered and clear recommendations for surface termination of nDP acting as carrier for GFs were taken into account.

Two binding-strategies are followed in the VascuBone project: adsorption (physisorption) and covalent binding. The different grafting methods have been implemented and compared with calculated results. The experiments confirmed the calculated predictions and the optimized determination of conditions for GF binding was achieved. To improve the osteoconductive, and osteo- and angioinductive properties of the materials, bioactive molecules like bone morphogenetic protein 2 (BMP-2) and Angiopoietin (Ang-1) have been bound to the materials applying these grafting methods. The development of a reagent free coupling of proteins to the nDP surface lead to reproducible production and characterization of BMP-2 and Ang-1 functionalized nanodiamond. The capacity of binding growth factors (BMP-2, Ang-1, vascular endothelial growth factor (VEGF)) as well as the bioactivity of such bound GFs on nDPs was determined.

The following experiments have been performed to learn about the behaviour of binding techniques and to define the most suitable strategy for production of efficient scaffold systems for preclinical and clinical application:

- detachment of VEGF-165 and angiopoietin from the surfaces of nanodiamond particles (nDP) by means of radiolabeling was successfully realized

- physisorption of BMP-2 to nDP-modified CeraSorbM® resulted in a higher alkaline phosphatase (ALP) induction in comparison to non-modified materials treated with BMP-2 The biological activity of the physisorbed BMP-2 could be verified
- method to functionalize porous scaffolds and subsequently covalently bound growth factors was developed. New technique to combine nDP and the polyester based scaffolds during the scaffold fabrication was successfully applied
- synthesis of orthogonally functionalized nanodiamond materials with different orthogonal groups have been developed and can be used for the simultaneous grafting onto a polymer scaffold and the grafting of functional molecules, e.g. growth factors
- first preliminary results showed that Quartz Crystal Microbalance (QCM) analysis can be a suitable technique for analyzing the interactions between PLA and poly(LLA-co-CL) and nDP, fibronectin and BMP-2

These findings and methods have been transferred with respect to the preparation, data evaluation and approval for future clinical trials. Biofunctionalization with growth factors, release measurements, evaluation and optimization for active, stable immobilization have been performed successfully. *In vivo* and *in vitro* testing have been performed to test and confirm the biocompatibility and bioactivity of the bound molecules and to evaluate proper dosage in the range of □g. The results are described in corresponding work packages.

Additionally it was shown that surface-tailored nDPs tend to aggregate in plasma and form a protein corona. The interaction of proteins differ between diamond-free scaffold materials and the diamond coated variants.

Implementation of new quality standards in bone tissue engineering

The optimized scaffold materials were used for experimental applications at the partners and for analysis to achieve all required data for the planned clinical trials according to EN DIN ISO 10993 and 14155 as well as FDA-guidelines with respect to nano-particles and for nanoparticles on medical devices. The production processes were established and tested for reproducibility. Process documentation due to ISO-standards was developed and is available.

The regulation aspects for the first planned clinical trial (8% DiaSorb granula in "split mouth" based study for lateral augmentation of to narrow mandibular crests before insertion of dental implants) have been determined and the required tests and documentation were carried out.

8% DiaSorb granula were produced in line with the defined protocols in high reproducibility and large batches. Sterile nDPs (filter-sterilized) have been prepared according protocols. Characterization of the novel implant material (DiaSorb as medical device class III) for pre-clinical studies have been realized within the consortium and at external accredited and authorized laboratories. New methods have been developed for approval of the medical device class III since there are no standardized tests for nano-based biomaterials. These new methods are defined as follows:

1. Quantification of nDPs and release of nDPs in/from DiaSorb:
    - release and leaching analysis – techniques developed, no release could be observed

- structural analysis of DiaSorb – BET surface analysis, increase of surface area up to 100 fold, pore size not influenced
- method development for label free detection and quantification of nDPs
- determination of size by NTA: no alternative to Malvern

2. Biocompatibility studies of nDPs for pre-clinical trials confirmed the safety of the novel implant materials

- AMES test: novel method for nano particles established
- single dose escalation toxicity study in rats: nDP in glucose in higher concentration applied

Based on these results Standard Operation Procedure (SOPs) to ensure i) safety, ii) quality and iii) verification of efficacy have been generated. For the safety aspects all developed and modified scaffolds and their break-down products have been tested for biocompatibility regarding EN DIN ISO 10993 guideline. Quality criteria of the production of DiaSorb material for pre-clinical and clinical applications have been defined with respect to safety/risk and approval issues. Production processes and analytical methods of DiaSorb and its components (nDP, CeraSorbM) are developed, optimized and defined as standards for quality monitoring.

Therefore, analytical investigation of three large-scaled batches have been performed for the components of DiaSorb: nanodiamond particles, CeraSorbM (clinical grade released by curasan AG) and the final product DiaSorb, i.e.:

Nanodiamond particles:

- Fourier transform infrared spectroscopy (FTIR)
- dynamic light scattering (DLS) – size distribution
- zeta potential – stability of collodial solution
- carbon amount/concentration
- extended single dose toxicity study in rats
- genotoxicity (Bacterial Reverse Mutation Assay (AMES), in vitro micronucleus assay)

DiaSorb:

- C-content
- BET – surface area
- SEM – morphology, crystallinity
- Hg intrusion - porosity
- heavy metal (according ASTM F1088)
- pilot stability study – degradation of CeraSorbM and DiaSorb in PBS
- biodistribution of possible nDP release of implanted DiaSorb in jaw bone/sheep

All data confirmed that the processes consistently produce material meeting the predetermined specifications and corresponding quality attribute. A final dose and formulation has been selected for completion of the pre-clinical test package. Minipig study and cell interaction studies were finalized and the results are presented in WP 2 and WP 8.

The scientific findings of WP 3 are published in joint publications peer-reviewed journals and on conferences and workshops (see dissemination table).

WP 4: Clinical observational study

An important aim of the VascuBone consortium was the development of new therapeutic approaches for the regeneration of bone defects based on the application of bone marrow derived progenitor cells and biocompatible scaffolds. The translational aspect of this project is the design and executions of preclinical and clinical phase I trials addressing vascularized bone and soft tissue regeneration in:

1. avascular necrosis of the femoral head
2. small maxillary defects
3. large bone defects of the facial skeleton
4. bronchotracheal defects

The common feature of all four clinical trials is that the medicinal product is classified as an ATMP (Advanced Therapy Medicinal Product). For the AVN trial, human MSCs belong to the group of somatic cell therapy medicinal products where else for the other trials the therapeutic factor belong to the tissue-engineered products. Local and national authorities tightly regulate the use of ATMPs in clinical trial. By addressing patient safety of these novel therapeutic options, the application of these products have to fulfil safety standards on biodistribution, non-tumor formation, and non-immunogenic potential. Furthermore, the production of these ATMPs for clinical trials has to be performed under GMP (Good Manufacturing Practice).

In the VascuBone consortium, most of these requirements have been fulfilled in the WPs 7 and 8. The pre-clinical data have been achieved by in vitro studies as well as in small and large animal trials. Visualization and monitoring of implant ingrowth and remodeling in mandibular defects was successfully established by MRI. The manufacturing process for vascularized tracheal construct was adapted to GMP. An authorization for manufacturing of the TraVaSc was requested by local authorities.
Although clinical trials in human have not been started within the funding period, the prosperous establishment of a “clinical trial environment” is one of the major outcome of WP4. These includes the writing of essential documents for clinical trial application (clinical trial protocol, investigational medicinal product dossier). Furthermore, quality management systems were established in the variant trial sites to gain pre-clinical and clinical data under standard operation protocols (SOPs). Of same importance, participants in WP 4 have gained a unique expertise in the complex field of regulatory affairs. Specific knowledge on ATMP in clinical trials and the collected pre-clinical data on safety issues provides a powerful tool for the proposed clinical trials and for further clinical trials in the field of vascularized implants.

WP 5: Cell/Tissue scaffold interactions

The center piece of tissue engineering is integration of bioactive factors, cells, and scaffolds made of biosimilar materials that are to be bionically structured. VascuBone was also aiming at this high target, in

particular however to firstly provide selected tools and measures which are complimentary matching the proprietary tool kit of the consortium. In line with this concerted work towards this common goal, research and development was stratified in several layers: it included a variation of biocompatible biomaterials and cell types, a combination of FDA approved growth factors in order to trigger osteoinductive and/or angiogenic biologic responses, material modification technologies in order to build bionic niches for ex vivo amplified cells, together with computer-assisted simulation and biomedical analytical tools to allow molecular imaging based on established in vivo diagnostics such as MRI and PET/CT.

Production of advanced polymer scaffolds was concerned with improving biocompatibility, bioactivity and degradation effects in vivo and in vitro. For the standardized production of the biomaterials as well as the cellular components quality criteria were defined and monitoring techniques according to official standards had to be established. In parallel two different cell types that are believed to work in concert to bring forth enhanced healing and regeneration; these are endothelial progenitors that form new blood vessels and mesenchymal stromal cells that firstly build major structural parts of most tissues and organs and secondly greatly support vessel stability. The individual cell types can be isolated from biopsies. Mesenchymal cells also readily proliferate in culture and can be rapidly amplified. This cell type is most often used for clinical therapies and is currently investigated in plethora of clinical trials.

Developing technology to load cells into synthetic materials was a considered key. The artificial environment needs to suit the cells. More than solely offering the biological spacing and physical properties the material eventually has to promote macroscopic bone development. Hence a major task was the optimization of loading techniques. This went along with definition of markers for quality control that allowed defining cell numbers, types and ratios for seeding with optimal outcomes. In this context differentiation of mesenchymal cells was an important issue as it became apparent in the literature that material stiffness and surface textures besides inductive biofactors can force mesenchymal progenitors to proliferate and form bone precursor cells. Eventually only a compound of cells and scaffold materials may develop into living bony tissue.

To facilitate loading yet also to provide sufficiently stable conditioning of cell nurture bioreactor systems were established. The prime feature of the bioreactor technology was not only testing cell attachment onto substrates but also to optimize for long-term cellular growth in a controlled and reproducible manner in a three-dimensional macroscopic environment. This technical advancement firstly enabled to assess biocompatibility between cells and artificial materials in a reliably fashion. Besides proprietary developments also commercially available solutions for single use were implemented. Working along this line, two new aliphatic polyester co-polymer scaffolds were examined in parallel to ceramic materials that are already in clinical use for several years. The latter exhibits insufficient hydrophilicity and thus implantation suffers from a lesser healing by insufficient cell ingrowth and slow regeneration over time. This in mind, the scaffold materials were modified by hydrophilic diamond powder. This way of physical functionalization of materials proofed very interesting for two reasons. Firstly scaffolds bearing diamond nanoparticles enhanced osteogenesis, yet diamond also provided further functionalization for stably binding bioactive factors. Hydrophilic diamond provides firm interactions with biopolymers. Playing this trick, factors can be

immobilized onto scaffolds thus perfecting the material in a bionically-tailored way.

Based on the combination of these technical refinements osteogenic differentiation of MSC on scaffold materials structurally comparable to the natural osseous environment could be induced. Cells were still viable after many weeks of differentiation. Based on this development it can be anticipated that macroscopic bone grafts can now be produced in vitro.

WP 6: Evaluation of cell sources for implant loading

Age related quantification of osteogenic and angiogenic subpopulations in MSC

The question to be resolved in this task was whether adult stem cells, in particular MSC are at risk to fail in clinical applications, because they may be prone to cellular aging. IAW established a novel quality assessment technology which allows non-invasive online monitoring of cell cultures. For this an electric cell-substrate impedance sensing was devised. The sensors were able to trace significant impedance changes after bmMSC seeding upon cell spreading and adhesion. The system was further proven suitable for continuous monitoring of cellular behavior over many days up to four weeks and allowed to validate the initiation of osteogenic and adipogenic induction in bmMSC already within a few days, which when processed according to state-of-the-art standard protocols can only be determined after weeks of culture time. In the context of medical cell production in a GMP-compliant process, the here presented interdigitated electric microsensors technology allows the documentation of MSC quality in a fast, efficient and reliable fashion. This monitoring system was designed to enable continuous monitoring of cell behavior and was used to characterize cultivation and differentiation of MSC over a period of many days and weeks. These sensors are sensitive to adherent cell layers. Compared to the culture medium, biological cells exhibit a considerably higher ohmic resistance. Covering the sensor surface with an excess of rounded spheroidal cells alters the electric field. When cells are evenly spread and concomitantly flattening on top of the substrate, this is again reflected by the electrical impedance. Conclusively, the sensors serve as a quantitative measure for morphological changes as a consequence of cell adhesion.

UWue cloned a reporter vector for cellular senescence, which can be easily transduced and used in primary cells of interest to monitor the development of cellular senescence in cells grown on different scaffolds or under different conditions. The readout used was the activity of the p16 promoter cloned downstream of a GFP or a luciferase reporter. P16 is known to be activated during cellular senescence. These experiments show that the p16 driven luciferase construct is active and can be stimulated by Doxorubicin, a known agent to induce cellular senescence. Therefore UWue established a reliable and sensitive test system for the development of cellular senescence that contributes to the VascuBone toolkit.

IAW was working on the distinction of optimal MSC populations and conditions to efficiently induce osteogenesis. This work is based on previously established genomic and functional analyses which were designed to determine as well as to instruct the naïve cell to develop into an appropriate precursor cell type thus enabling in situ osteogenic differentiation. Applying 4-methyl umbelliferron (4MU) is propagating a fate

decision in osteogenesis greatly mimicking the terminal stage of the osteogenic process. Notably cells cease proliferation, which is certainly of pertinent importance when employing cultivated MSC in vivo, as it is essential to warrant no further cell growth thus minimizing the risk of cancer formation. Furthermore, 4MU is capable of inducing osteocytogenesis. It is assumed that osteocytes are a robust long-living cell type, which builds bone matrix through the production of efficiently calcifying matrix. Mesenchymal cells when isolated from an environment of advanced age or otherwise when replicatively aged through excessive rounds of cell proliferation in vitro often exhibit a decreased propensity to differentiate. Treatment with a small molecule, which may also be infused at later time points during tissue engineering or presumably, also in vivo post operation is a potent tool to stimulate and propel osteogenesis in a skewed situation of an elderly organism.

Up-Scaling of EPC *ex vivo* expansion

Endothelial precursor cells (EPCs) play an important role in postnatal vasculogenesis. An intact vascular system is crucial for the survival of all tissues including bone.

Medicyte has developed within the VascuBone project a vericyte® Endothelial Cell Growth Medium, which allowed an increased number of population doublings of EC during cultivation in comparison to current available standard media. The vericyte® Endothelial Cell Growth Medium contains serum and other non-defined compounds. The medium doesn´t match with the current GMP standards necessary for autologous cell expansion and re-implantation. Therefore, Medicyte has developed a chemically defined Endothelial Cell Growth Medium, based on vericyte® technology, that matches the current GMP requirements. The addition of defined serum components such as alpha1-anti-trypsin or alpha2-macroglobulin led even to a further improvement of this chemically defined medium. The proliferation doubling time of the cells grown in vericyte® defined Endothelial Cell Growth Medium without serum equals the doubling time of cells cultured in vericyte® Endothelial Cell Growth Medium with serum. The endothelial cells cultured in chemically defined Endothelial Cell Growth Medium retain their primary cell characteristics such as expression of cell-specific markers, CD31 and van Willebrand factor, over the entire course of the expansion.

Novel bone tissue engineering approaches using scaffolds seeded with mesenchymal stem cells (MSCs) raised the idea of combining MSCs with EPCs to improve bone regeneration. For this purpose EPCs were isolated by UWue by Ficoll-Paque density gradient centrifugation from human Buffy Coat and characterized using fluorescence-labeled antibodies against specific surface markers including FITC-UEA as well as by Dil-acLDL uptake.

An alternative cell source for the vascularization could be Blood Endothelial Outgrowth Cells (BOEC). The BOECs represent proliferating EPC. They are also described under the synonym Endothelial Colony Forming Cells. The successfully from Medicyte isolated BOECs were thoroughly characterized by assessing different specific markers and various cell based assays aiming at the biological functionality of the cells. BOECs have the cell-type specific characteristics of endothelial cells regarding the key molecule expression like vWF, CD31, VEc, UEAI, etc. as well as their biological functionality like capability to uptake LDL protein and cell migration. They possess the cell culture specific characteristic of clonal proliferation and can be re-plated and passaged. Through the optimization of the isolation technique, by using erythrocyte depletion,

changing the seeding density and addition of a new attachment factor, the yield of starting points for the clonal outgrowth could be significantly increased. These improvements made it possible to standardize the isolation procedure for future use in patients and can be used as part of VascuBone's toolkit.

Molecular elucidation of MSC-EPC cell communication to better understand niche interactions

This task focused on the changes of global gene expression patterns of human primary EPCs after having been subjected to conditioned medium of human primary MSCs or after direct cell-cell contact, and vice versa, respectively. Experimental setups for the study of the crosstalk of MSCs and EPCs were successfully established at UWue including Affymetrix microarray analysis. The bioinformatical analysis of the microarray data revealed that several genes related to osteogenesis and angiogenesis are differentially regulated in MSCs and EPCs due to treatment with conditioned medium as well as after direct cell-cell contact.

At UWue MSCs were isolated from human bone marrow. EPCs were isolated from buffy coat. Two experimental setups were applied. A: EPC or MSCs received conditioned medium from the other cell type. B: MSCs and EPCs were cultured in direct co culture (after labeling green or orange), subsequently separated by FACS sorting and compared to single cultured cells.

Microarrays (n=4) were performed for both experimental setups after RNA isolation using the Affymetrix GeneChip® HG-U133 Plus 2.0 array. Results have been re-evaluated by RT-PCR. Bioinformatical analyses revealed a number of overrepresented pathways in MSCs and EPCs subsequent to a co-culture period compared so single-cultured cells. Regulated genes belong to several clusters e.g. osteogenesis and angiogenesis. Assays derived from these results might prove useful in terms of quality control for tissue engineering procedures. This study will help to better understand the crosstalk of MSCs and EPCs, which will finally aid to the improvement of vascularized tissue engineering constructs.

At UIB primary human ECs and MSCs were seeded onto poly(L-lactide-co-1,5-dioxepan-2-one) (poly(LLA-co-DXO)) scaffolds produced by KTH and grown in dynamic culture before subcutaneous implantation in immunocompromised mice for 1 and 3 weeks. Cellular activity, angiogenic stimulation and vascular assembly in cell/scaffold constructs seeded with ECs or ECs/MSCs in a 5:1 ratio was monitored at UIB with real-time RT-PCR, ELISA and immunohistochemical microscopy analysis. A quiescent phenotype of ECs was generated, by adding MSCs to the culture system. Decreased proliferation of ECs, in addition to up-regulation of selected markers for vascular maturation was demonstrated. Baseline expression of VEGFa was higher for MSCs compared with EC with subsequent up-regulated VEGFa-expression for EC/MSC constructs before and after implantation. Furthermore, an inflammatory response with CD11b+ cells was generated from implantation of human cells. A higher vascular density was shown for both cellular constructs compared with empty control scaffolds.

Application of Manufacturing Authorization and GMP production of EPC

Although the resources for GMP production were available, the adoption of a production process for EPCs to GMP requirements has not been achieved, despite the successful propagation of the EPCs. The experimental setting for the BioVaSc requires a huge number of cells that has not been obtained. Therefore

activities were focused on establishing methods to characterize the growth properties of cells like EPCs on the BioVaSc and to establish bioreactor systems to cultivate cells under controlled conditions to high density.
The BioVaSc production process was successfully optimized to fulfil the safety limits set for the residual amounts of endotoxines in the final product without using antibiotics during the isolation procedures. Depletion of the acellularization agent was further improved allowing an efficient reseeding of the matrix with human cells. A special focus was to setup methods to isolate and quantify porcine DNA from the matrix material during and after the isolation procedure and human DNA after reseeding with cells.

WP 7: Development of a pre-vascularized bone implant

The overall aim of work package 7 was the construction of a pre-vascularized bone implant that allows connection to a patient's circulatory system in order to approach the lack of sufficient vascularization in bone implants as one of the most challenging hurdles for cell-based bone implants. This task required the combination of a range of different techniques and insights from other WPs. The different aspects required are:

- Suitable biomaterial for the bone aspect as well as the vascular aspect of the implant
- Cell sources
- Bioreactor development
- Influence of dynamic culture conditions in comparison to static culture conditions
- Optimization of 3D dynamical co-culture in a bioreactor system
- Transfer of pre-vascularized bone implants to GMP conform manufacturing

The pre-vascularized bone implant was composed of β-TCP granules for the bone aspect of the implant and the BioVaSc-TERM for the vascular structures. The β-TCP granules were validated (CeraSorb®M), of a size of 1000 – 2000 µm with a pore size of 150 – 500 µm and a porosity of 65%.
The BioVaSc-TERM is a naturally derived collagen scaffold. For its production, porcine jejunal segments get decellularized and cleansed from residual DNA and endotoxins.

Bioreactor development

A bioreactor system for the re-seeding of the BioVaSc-TERM with endothelial cells was developed. The system allows the controlled perfusion of the BioVaSc-TERM's vessels at physiological pressure conditions. This setup, shown in Fig. 7, enables the possibility of seeding cells into the vessels.

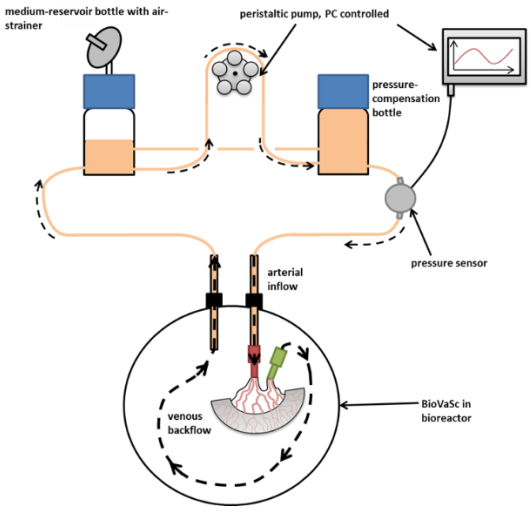

Fig. 7: Schematic of the bioreactor system for 3D dynamical culture of the BioVaSc-TERM. Illustration of the essential components necessary to seed and culture a BioVaSc-TERM under dynamic conditions.

Combination of BioVaSc and modified bone material

Endothelial progenitor cells were chosen for the re-functionalization of the BioVaSc-TERM due to their accessibility. Their isolation is based on density gradient centrifugation of peripheral blood and subsequent seeding on fibronectin coated surfaces. A protocol was established that allows endothelial progenitor cell isolation from human and ovine peripheral blood. The cells were shown to grow inside the BioVaSc-TERM's structures and repopulate all the vessels (Fig. 8 and 9).

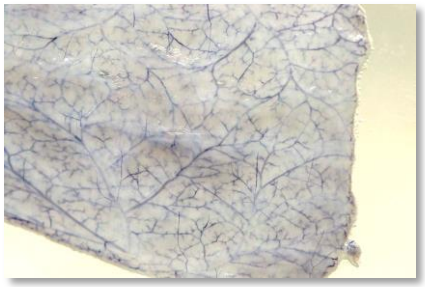

Fig. 8: Endothelial progenitor cells in the vessel structures of the BioVaSc-TERM after one week of dynamic culture. Live cells stained by MTT.

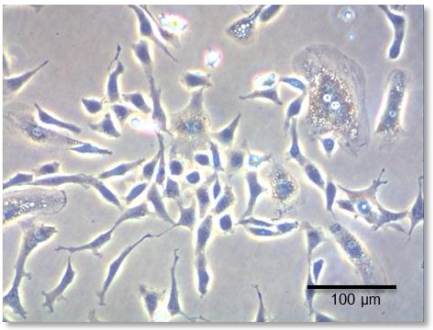

Fig. 9: Endothelial progenitor cells isolated from ovine peripheral blood by density gradient centrifugation.

The combination of the two components of the implant is visualized in Fig. 10.

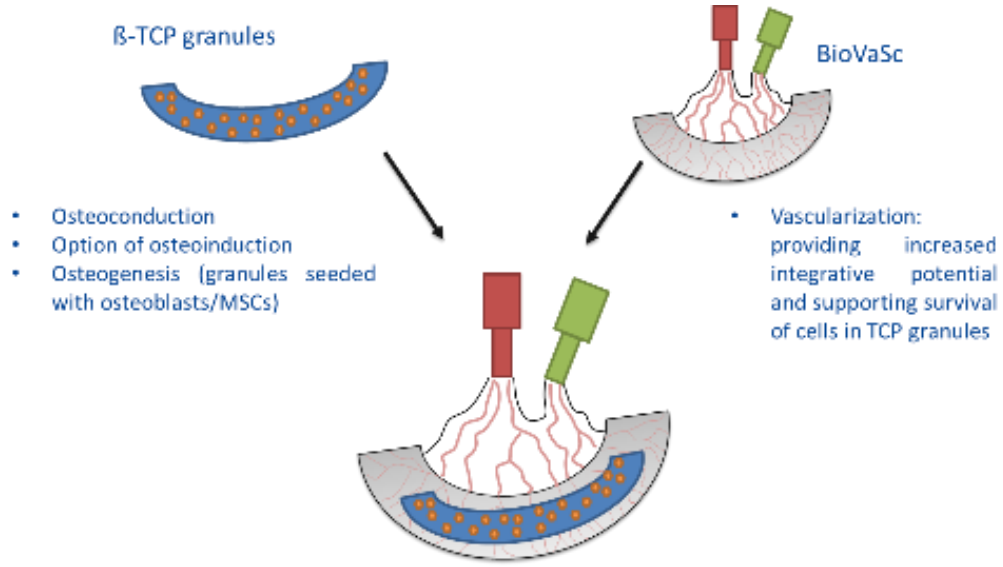

Fig. 10: Combination of BioVaSc-TERM and β-TCP granules.

Additionally to the aforementioned bioreactor, WP 7 included the development of a bioreactor system specifically for the investigation of the influence of a dynamic medium perfusion and mechanical stimulation of mesenchymal progenitor cells seeded on bone substitute scaffolds. Calcification assay analyses suggested increased calcification in samples during dynamic conditions as compared to static conditions (Fig. 11).

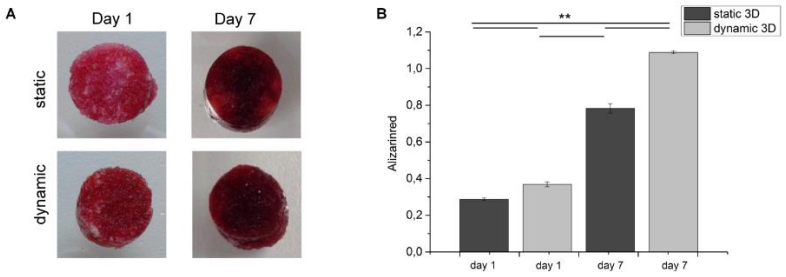

Fig. 11: Calcification assay. Dynamic culture compared to static culture.

A further increase in calcification could be achieved by the application of mechanical stress onto the samples (Fig. 12).

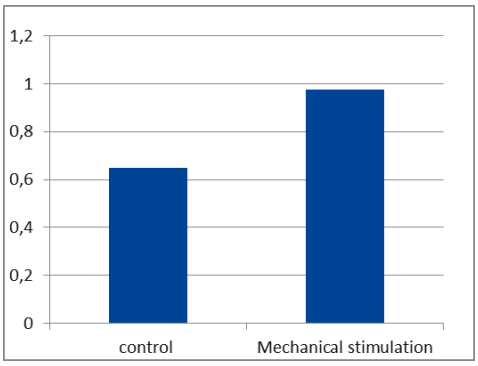

Fig. 12: Calcification assay. Samples without mechanical stimulation compared to mechanically stimulated samples.

Induction of angiogenesis

Within the tasks of WP 7 a test method that allows quantitative characterization of a growth factor gradient regarding its angiogenic potential was developed. This method allows to assess the effect of growth factor gradients on the migratory behavior of investigated cells (Fig. 13).

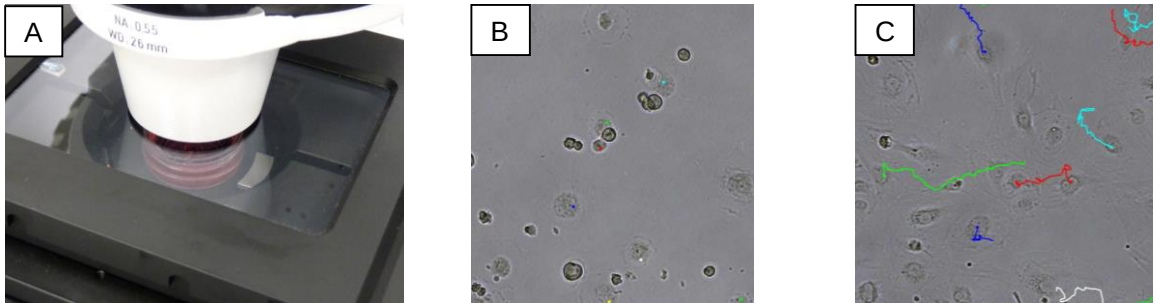

Fig. 13: Trajectory tracking. (A) Microvascular endothelial cells are cultured and monitored via life cell imaging. (B) At t=0, a growth factor gradient is applied. (C) By tracking the cell, a trajectory can be generated.

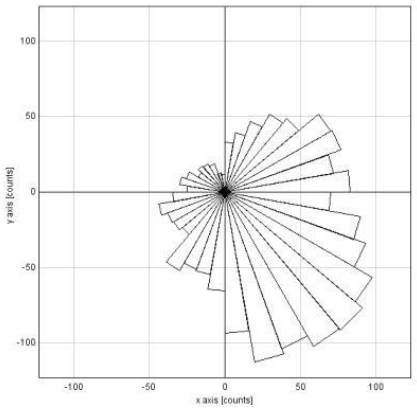

Fig. 14: Trajectory analysis.

Based on the trajectory, the angiogenic potential of a growth factor gradient can be determined. The figure shows a preferred migration direction, which is introduced by the growth factor VEGF (

Fig. 14).

Optimization of 3D dynamical co culture

For the characterization of the commercially-available β-TCP scaffolds and the scaffolds developed in the project (partner KTH and PPP), dynamic culture in the bioreactor system was performed. Therefore, a suitable seeding protocol has been developed. Scaffold cylinders were seeded under dynamic condition in the perfusion bioreactor. Scaffolds were placed in the custom-fit notch of the bioreactor cartridge and perfused by a peristaltic pump for one hour with cell culture medium. hMSCs were harvested and suspended in growth medium to a concentration of 5 x 10^5 hMSCs/ mL. 10 mL of cell suspension was transferred to a 10 mL syringe and was injected air-bubble-free through the sterile sampling port with a syringe pump with a flow rate of 0.5 mL/minute. Applied pressure was monitored by the pressure sensor and controlled to 10—20 mmHg. Then, cell suspension was pumped through the bioreactor chamber in alternating cycles for 10 seconds forward and 3 seconds backward at total of 1 hour at 1.6 mL/minute. Following, the pump was stopped for 30 minutes to allow cell adhesion. The protocol allowed distributing MSCs in the scaffolds.

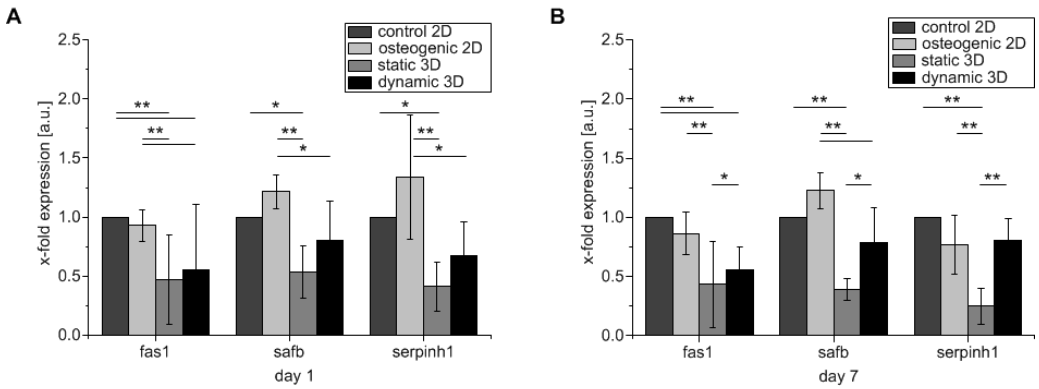

Fig. 15: Evaluation of stress related genes within the scaffolds. (A, B) Samples were analyzed by quantitative real time

polymerase chain reaction (qRT-PCR) for detection of genes related to stress markers (fas1, safb, serpinh1). Housekeeping genes gapdh and rplp0 were used for normalization. Furthermore, time-fold gene expressions were normalized to control cells in standard two-dimensional (2D) culture conditions. Legend: Control 2D: human mesenchymal stem cells (hMSCs) on standard tissue culture polystyrene using proliferative medium; Osteogenic 2D: hMSCs on standard tissue culture polystyrene using osteogenic differentiation medium; Static 3D: hMSCs in the 3D polymer scaffold using proliferative medium; Dynamic 3D: hMSCs in the 3D polymer scaffold exposed to shear stress employing the bioreactor system (n = 4). * Denotes a significant difference in gene level expression between culture conditions (*$p < 0.05$; **$p < 0.01$), error bars represent standard deviation.

Following cell seeding, impact of shear stress on differentiation was characterized. Therefore, stress marker gene level, measured by qRT-PCR (Fig. 15A, B) revealed that the stress marker genes fas and safb in dynamic culture conditions were up-regulated compared to static culture conditions on day 1. The expression rate of safb and fas turned to be significantly up-regulated after 7 days of dynamic culture in comparison to static culture conditions. On day 7, also serpinh1 exposed a significantly higher up-regulation in dynamic conditions compared to static culture. For comparison, neither safb, fas, nor serpin showed significant differences between proliferation and osteogenic medium on day 1 and day 7. Interestingly, in the 3D culture system, except from serpinh1 on day 7, all measured stress markers were lower compared to the standard two-dimensional (2D) culture conditions for all experimental settings.

Transfer of pre-vascularized bone implants to GMP conform manufacturing

Additionally, Evonik developed a medium using a defined platelet lysate conform to GMP standards (MSC 18). Although this medium formulation cannot be regarded as animal origin free it offers the opportunity to eventually make use of autologous platelet lysate for the expansion of autologous hMSC for the production and reimplantation of bone implants.

The whole process was transferred into a GMP-compliant environment. Bioreactor technology was modified towards GMP by introducing redundant components for process-critical step. Specifications and protocols meet GMP-requirements as does the overall documentation system of the process.

Based on the pre-vascularized implant from this WP, two animals trails were designed and carried out, which are object to WP 8. The general process operations it depicted in Fig. 16.

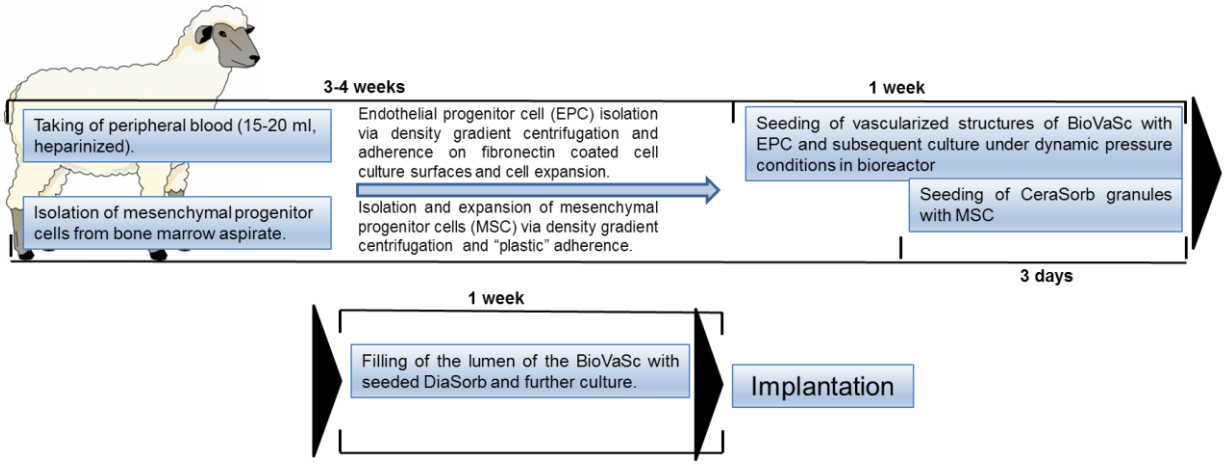

Fig. 16: Timeline for the construction of the pre-vascularized bone implant.

WP 8: Preclinical trials

Work package 8 deals with preclinical trials and the preclinical precondition for clinical trials. The VascuBone concept consists of different components that had to be tested separately, namely cells, biomaterials and a vascular bed. Therefore, distinct animal models had to be developed and used.

Cellular components:

The evaluation of cells for osseous regeneration was performed in several animal models. In WP 8 the FHN sheep study in Würzburg by partners UWü-KLH and MRB which was started in reporting period 4 was performed and finished in reporting period 5. MRI studies of all animals were performed at the MRB. In addition, ex vivo µCT imaging of all treated femoral heads was also performed at the MRB (Fig. 17). Even though the femoral heads of the sheep were very small, MRI was able to visualise the necrotic region with high quality images within reasonable scan time in the time course as planned. The combination of different image contrasts allowed a good differentiation between different stages of the necrosis in this animal model.

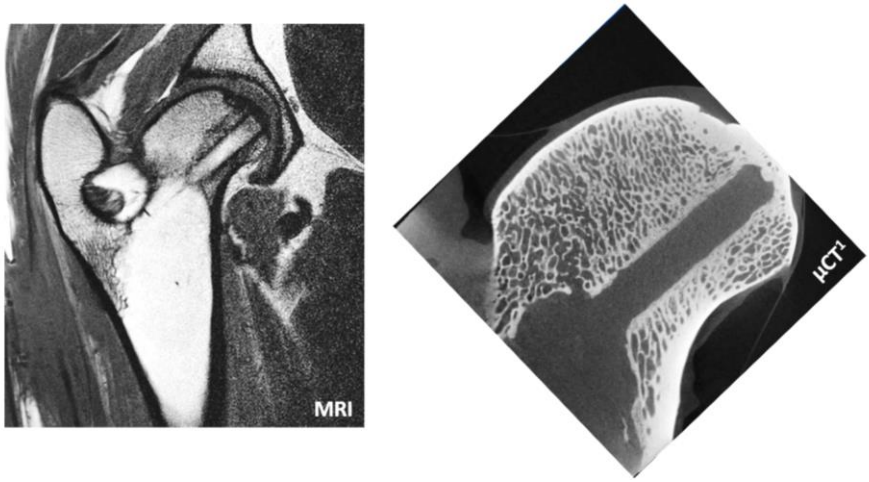

Fig. 17: In vivo MRI and ex vivo µCT: Comparison of single slices from 3D MRI and µCT datasets.

[1] Collaboration with Dr. Zabler, Physics Dept. (X-ray microscopy), University Würzburg.

MRI monitoring in a preclinical study is thus valuable tool because of the obtained additional results, which provide important additional information, which would otherwise be ignored.

The acquired data showed a good correlation of the MR signal and bone marrow fraction, even though MRI was not able to resolve the bone microstructure, thus, allowing to quantitatively characterise the bone marrow fraction in the time course without the necessity to sacrifice the animals at every time point.

MRI and µCT results are in accordance and provide complementary information in many cases and also allow to combine in vivo image information with high resolution ex vivo information leading to a much more complete characterisation of the animal model in the time course, cf. Fig. 18.

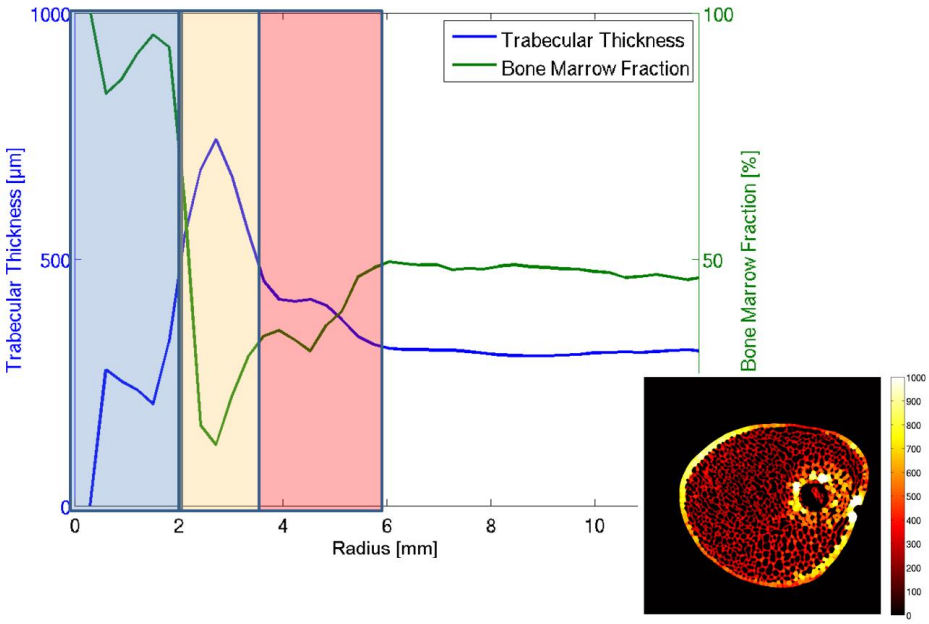

Fig. 18: Analysis of Trabecular Thickness and Bone Marrow Fraction (BMF) from µCT and MRI datasets.

In a rat study in Innsbruck, the enchondral ossification process was simulated by inducing chondrogenic differentiation (the first step in bone development). As chondrocytes are bradytrophic cells and not depending on a distinct blood supply we hypothesized an improved cell survival rate within the defect and by inducing chondrogenic differentiation – an early stage of enchondral bone formation – an enhanced ossification. As scaffold the biodegradable, non- toxic poloxamer Pluronic F-127 was used, that is already approved by the FDA for human use. The thermoreversible gelation properties of Pluronic F-127 (fluid at low temperatures and solid at high temperatures) are very interesting for this purpose. Bone marrow derived mesenchymal stem cells from fisher rats were isolated, cultivated and suspended in two different hydrogels.

For group I a 25% (w/w) Pluronic hydrogel containing a standard culture medium was used as solvent, while for group II Pluronic was solved in a chondroinductive differentiation medium. After 7 days of culture, cell migration within both gels can be observed and the cells form cluster as they do in chondrogenic differentiation. Thus, the differentiation capacity was not affected by the hydrogel, as similar Col2a expression after 3 weeks could demonstrate, but the percentage of viable cells was higher in the group II.

Critical size defects in the mandible of Fisher F344 rats were performed for in vivo testing. A trephine drill with an outer diameter of 5 mm was used. One side was filled with Pluronic, the other side with chondroinductive Pluronic F-127 and MSCs. The animals were sacrificed after 6 weeks. µCT data showed improved bone formation when the differentiation hydrogel was applied with cells. Consecutive histological and immunohistochemical analyses confirmed the results showing enhanced collagen production and a higher cellularity compared to the empty defect. Immunohistochemical exposure of osteopontin was also enhanced in the treated defects.

Bone replacement materials

Another component of artificial vascularized bone transplants are biomaterials. Beside the development of new polymers, existing biomaterials were functionalized by surface modifications. One possibility tested in VascuBone was coating of β-TCP with nanocristalline diamond particles. These particles do not change the surface topography but change via their sidechains the electrochemical behaviour. These modified surfaces show distinct possibilities for binding different growth factors (via physisorption or covalent binding). This strategy allows inducing vascular ingrowth and improves the blood circulation within the regeneration process. Therefore, Cerasorb cylinders were coated with nanocristalline diamonds and Angiopoietin-1 (Ang-1) was bound. This leads to an enhanced vascular ingrowth and neovascularisation. The same samples with physisorbed BMP-2 instead of Ang-1 improved the bone formation significantly.

The nanocristalline diamond particles by itself modify the hydrophilicity of the biomaterials. To evaluate the effect of nanocristalline particles on bone replacement materials a new animal model simulating lateral bone augmentation in mandibles was developed. This experimental series shows that higher concentration of nanocristalline diamonds induce more bone formation than low concentrations.

Toxicity testing for nanocristalline diamonds was performed in established and accepted animal models in Bergen.

New promising technologies were also developed and evaluated in preclinical trails. Individualized 3D printed copolymers were developed for treatment of segmental femoral defects in rats (Fig. 19).

Fig. 19: 3D printed scaffold for femur reconstruction.

Besides the precise reconstructed shape of the scaffold the internal structure was differently printed and the impact of these structures histologically and radiologically evaluated.

BioVaSc

The BioVaSc was tested in two different sheep models. Once the construct was tested in tibia defects and secondary with microvascular support in mandibular continuity defects.

The BioVaSc consists of bone replacement material (diamond coated β-TCP), a decellularized and reseeded (EPCs) porcine gut segment and mesenchymal stem cells.

In Autralia, tibial defects were performed and the BioVaSc was used for bridging. Stabilization of the tibia defect was guaranteed by strong reconstruction plates.

Fig. 20 illustrates the immunohistochemical staining of alpha smooth muscle actin (red) as an indicator for blood vessels. The red color clearly shows the presence of vessel structures in the regenerated bone (light green) as well as in the region of diamond coated β-TCP.

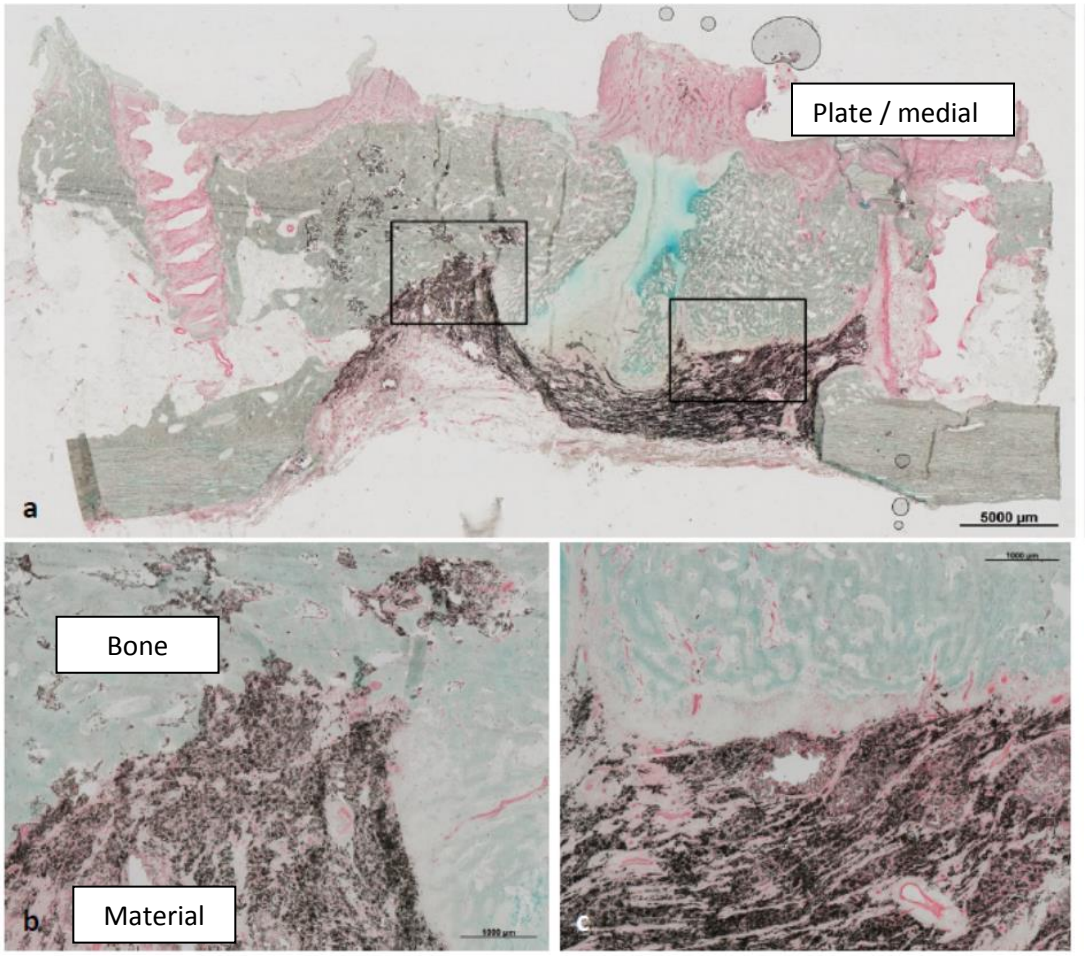

Fig. 20: Anti-alpha smooth muscle actin staining (red) with methyl green counter staining. The image shows a fibrous and cartilaginous area in the center of the defect. The dark area represents the bone replacement material (diamond coated β-TCP) inside the BioVaSc. Blood vessels are visible in the region of the defect as well as in the bone replacement material.

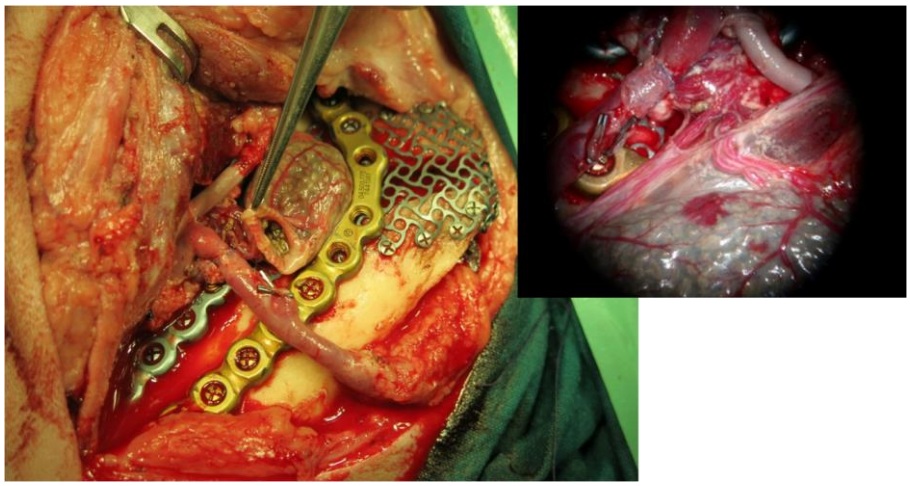

Fig. 21: Microvascular transplant after anasomosis in the mandibular angle, small picture shows revascularized vessels.

In Austria continuity defects of the mandible were used for evaluating the BioVaSc. As sheep are ruminants with strong masticatory forces, a preliminary biomechanical test of different osteosynthesis configurations was performed, so that this animal model could be performed successfully. The BioVaSc was implanted in continuity defects of the mandibular angle and the vessels were anastomosed to the facial artery and vein. Successful blood circulation was achieved and after 6 month the BioVaSc treated defect showed bridging bone formation (Fig. 21).

4. Description of the potential impact, the main dissemination activities and the exploitation of results

Socio-economic impact assessment will lead to information how VascuBone-development influences and will influence social and economic well-being of the community. Development impacts are generally evaluated in terms of changes in demographics, physical environment, income and social status, education, gender, public/health services, health qualities/genetics of the community.

For VascuBone a combination of all these factors are relevant. The therapeutic strategies addressed in this project will reduce the morbidity as well as reduce disability and therefore, also their social costs. The direct social benefits for the patients and health services include

- increased patient life expectancy
- improved patient quality of life
- shorter time of convalescence
- faster re-entry into work
- less patients with a partial disablement due to donor site morbidities
- saving health economic resources by reducing the hospitalisation costs.

Morbidity and disability have severe negative impacts on patients and their families. They also require considerable resources from healthcare systems and their professional staff. VascuBone aims to make a substantial step by developing regenerative therapeutic strategies that will constitute a major societal advance in addressing the health and well-being of the European population. The reduction of the anxiety and stress on family members and other care providers is also of great importance.

The new biomaterials of the tool box will influence the industry, creating new markets and new employment opportunities, notably in the area of tissue engineering.

Other socio-economic impacts include increased education and employment:

Education: PhD and post-doc students have been hired within the VascuBone framework allowing expansion of their skills and capacities in various disciplines e.g. chemistry, biology, physics, diagnostics and medical sciences. A training platform was established for the young scientists in the project since a close collaboration between the inter- and transdisciplinary fields is one important step towards educating young scientists. During the project the PhD-students received a broad knowledge and understanding of the possibilities and the needs in tissue engineering, both from a material, process and clinical perspective. This

will in the end improve their attractiveness to industry.

Employment: PhD students, young scientists as well as experienced scientists have been recruited by the partners to help executing the VascuBone project. They came into contact with challenging opportunities in material science and in regenerative medicine. Alongside the necessary Quality Assurance, Quality Control and Manufacturing personnel was hired and educated contributing to the growth of the necessary ATMP translational competences. Investment in education and training systems, anticipation of skills needs are thus fundamental in order to raise productivity, competitiveness, economic growth and ultimately employment.

Following the commercialisation strategy of VascuBone it is aimed to gain market shares even in new applications. This may help to increase the project portfolio of the industrial partners in a longer perspective.

In general, advanced therapeutic strategies pursue the goal to provide patients with improved and innovative findings from research and development. This was one of the declared goals of the VascuBone EU project. Beside this desirable and ambitious aim, patients' safety has to be addressed at any stage of the development. The legislator has given an extensive set of regulation on national and international level to reveal a maximum possible safety for patients and to reveal high quality of the study results. In order to successfully implement these objectives, pre-clinical trial on small and large animals were performed within VascuBone. The results allow minimizing the risk of our advanced therapies in human. Moreover, these pre-clinical results serve as a fundamental basis to rapidly overcome regulatory hurdles in defining risk/benefit ratio for patients treated with our innovative therapies.

The high demand on innovation in VascuBone has led to a broad platform on pre-clinical results, which facilitate the performance of new musculoskeletal therapies strategies (e.g. avascular necrosis of the femoral head, large size bone defects). Together with our acquired and specialized knowledge on advanced therapies, future trials can be implemented more rapidly and can be made available to the public.

In general, our medicinal products base on an individual design containing autologous cells. Besides all advantages like maximum potential compatibility in human, manufacturing of an advanced medicinal product is time consuming, requires high personal cost, and demands high infrastructural environments. Consequently, medium size and large-scale industry show only limited interest in these early phases of clinical development. Only with a sufficient financial support as given in the large scale EU funding of VascuBone, our innovative therapeutic options have the possibility to evaluate their safety and efficacy. Therefore, our results provide the basis for accessibility of advanced therapies to a border population within the European Union.

The number of aging people is steadily increasing both in industrialized and developing countries. In due course of these societal changes, the growing incidence rate of falls-associated injuries is posing one major challenge to societies and health care systems. Individuals and families may become exposed to a continuously compromised quality of life. After traumatic events persons not only suffer from immobilization, destruction and pain but also from social isolation.

Research and development of VascuBone was indirectly concerned with this issue by directly addressing technical difficulties associated with regeneration and repair of bodily functions after trauma. In this respect and following the approach of devising a toolbox provision of a bundle of products, methods and innovations progress could be achieved. Initially, Vascubone was concerned with clinically approved biomaterials with distinct limitations and restraints. Albeit therapeutically applied, vested interests were to improve and replace biomaterials for better solutions regarding biofunctionalization, cell-loading and vascularization. Working along these lines, the technical expertise could be greatly expanded by creating novel enabling technologies which could be implemented to warrantably test biocompatibility of materials, to reliably functionalize biomaterials with growth factors that shall promote osseous regeneration and healing as well as to stably cellularize biomaterials by means of novel reactor technologies permitting the growth of tissues ex vivo for subsequent implantation. The latter is particularly important in scenarios of traumatic injuries where parts of the body become deficient in appropriate healing because of loss of integrative anatomical structures.

Vascubone also provided synthetic materials which are now to being tested clinically to substitute grafted living bone that needs to be explanted from healthy body parts. Due to the technical advancements made by VascuBone it is now foreseeable that macroscopic bone precursors derived from one’s own cells can be produced in bioreactors already in the near future. As these aspects are in most instances single most important for elderly patients, VascuBone also addressed this pertinent issue by specifying culture methods and monitoring devises in order to maintain cells from elderly individuals for subsequent use in tissue engineering approaches in optimal shape, while at the same time vastly propagating them in culture.

Currently technology for quality control requires lysis of cellular materials in order to make critical assessments. Thereby cellular material is lost for good. Analyses are tedious and time consuming; VasuBone also addressed this issue by designing noninvasive monitoring technology for fast and reliable quality assessment. Conclusively implementation of VascuBone technology and services provides viable solutions for personalized medicinal approaches and therapies after traumatic injuries where otherwise individuals will suffer from life-long bodily deficiency and/or social exclusion.

Within the 5 year duration of Vascubone the participating companies and universities cooperatively contributed to generate a tool-box of novel methodologies. Thereby these focused activities increased the stock of useful knowledge in the field of bone regeneration. Skilled graduates were trained that will expand acquired Vasubone knowledge and will transfer abilities to solve complex problems in their future career to other working groups. Several quality control assays were developed that are useful to successfully evaluate tissue engineered cell-containing constructs. These novel assays and products in the future may have wider

implications for tissue engineering in our aged population. Activities of the consortium, e.g. flyers and presentations beyond the scientific community additionally were part of knowledge transfer mechanisms aimed to raise public awareness for the importance of tissue engineering in the future within the human health care system.

As already mentioned, one of the main goals of Vascubone was the development of an artificial bone transplant with a microvascular system for the regeneration of large bone defects. In many surgical disciplines large bone defects have to be handled after ablative tumor surgery, severe trauma or infections. For restoration of the patients integrity microvascular bone transplants from e.g.: the iliac crest, fibular bone, scapula,… are used for reconstruction. This means an additional operation field, longer operation times, and more comorbidities. The developed BioVaSc provides for the first time an adequate artificial scaffold. The harvesting of cells is minimal invasive and presents an big relief for the patient compared to the operative harvesting of a vascularized transplants. The clinical handling showed that that the scaffold is feasible for a routine clinical situation and has the potential to replace many microvascular transplants.

The developed, modified and tested bone replacement materials as polymers, diamond coated tricalcium phosphate or 3D printed scaffolds are promising materials in oral surgery and can facilite the dental restoration with implants. All in all, the patient will benefit from individualised solutions, shorter healing and osseoformation periodes.

Main activities of dissemination and exploitation of results (1/2010 – 3/2015)

Communication, dissemination and exploitation in VascuBone have been realized in various activities to bring the research to the attention of as many relevant people as possible. These activities are divided into four main categories:

- dissemination of scientific project results
- early establishment of an exploitation and dissemination plan to create and support the commercialization of VascuBone related results and products
- training/education – training and secondments: young researchers (clinicians, SMEs).
- training of all project partners with respect to the exploitation and dissemination plan

In work package 9 “Dissemination and Exploitation” these activities are realized by performing five tasks:
Task 9.1 Creation of IPR-related part of CA
Task 9.2 Protection of project results
Task 9.3 Training/Education
Task 9.4 Dissemination of project results
Task 9.5 Development of an exploitation strategy

A plan for dissemination and exploitation including appropriate protection strategy was developed within the consortium. The plan is structured in three parts:

i. strategy and realization of dissemination of scientific results and developments
ii. advertisement, visibility, public relation of VascuBone for broader public
iii. strategy for IP regulation, agreement of all partners for patent application procedure during and after project time

ad i.)

Dissemination of the scientific results was an important part of the work during the entire period of the project. The following media have been utilized to inform scientific community about the progress and findings of the project:

- scientific press
- EC's own press – e.g. CORDIS focus, euro abstracts, RTD info, etc.
- scientific publications, master/PhD thesis – through e-journals as well as traditional paper journals
- scientific conferences and trade shows – including MEDICA
- workshops – for partners, end users/clinicians and interested groups
- organisation of lunch symposia, conference/fair contributions
- etc.

ad ii.)

To reach industry and public audience the following instruments have been used:

- corporate identity: Website: www.vascubone.eu, VascuBone-logo
- project flyers
- conference booklet: serves as tool to demonstrate the activities and outcome of the research and work realized by the partners in VascuBone. On six pages the aim, expertise and contribution of each organisation is described. Additionally a blank template is available as insert. Each partner can show his current or updated news supplementary.
- The VascuBone project has been chosen by the European Commission for a special coverage on YOURIS in form of a video and an article. The VascuBone partners will utilize this as dissemination tool and link the video/article to their websites. For more information follow this two links:
  http://www.youris.com/Health/Smart_Devices/Human-Bones-From-The-Lab.kl
  http://www.youris.com/Health/Smart_Devices/Heike-Walles--A-Diverse-Toolbox-For-Regenerating-Bones.kl

ad iii.)

Protection+ of IP and how to exploit the results commercially during and after the project period was an important objective of VascuBone. In the beginning the Exploitation Manager (EM) was responsible for identifying and assessing the project results and reporting this to all project members throughout the entire project duration. Together with the Exploitation Board (EB) an appropriate protection strategy resulting in an

exploitation plan in addition to the CA should be developed. However, in the third year of the project the consortium decided to transfer the duties and responsibility of the EM and EB to the newly founded spin-off company DiaCoating GmbH. A crucial advantage of this line of action was the strong commitment of the shareholders to achieve marketable and commercialisable medical products for the VascuBone partners. From the knowledge developed so far, a number of different types of IPR are likely to be developed, which may include patents, copyright, software and database rights, and secret knowledge. The management of these different types of IPR was allocated at the spin-off DIA as part of the overall Exploitation Strategy.

The Exploitation/Dissemination (ED) Team managed by DIA worked closely with all industrial partners to identify the optimal route to market for the project results and identified all potential regulatory barriers to the development of each product or process and provide recommendations.

Professional support was given by external consultants in qualified quality management sessions to both train and implement quality management among the VascuBone partners:

- TÜV Süd: Regulatory Affairs (Dr. Runge), Clinical data for medical device certification (Dr. Höppfner), (2012, Hamburg)
- TÜV Süd: Requirements for market access, medical device (Prof. Kloth), (2014, Stuttgart)

Hicham Abghay, beneficiary in the EC-funded coordination and support action “Fit for health (http://www.fitforhealth.eu) explained within a workshop in the frame of the bi-annual project meeting (2011, Berlin) how to create IP from scientific and technical inventions, the pitfalls, the procedures and the different patent-related procedures in various nations and continents.

A SWOT-analysis of the project related results has been elaborated with the following outcomes:

Potential products/exploitables:

- medical products/scaffolds/implants and intermediates, cell therapy
- research products
- procedures

Prioritization with respect to

- patient benefit
- commercial success
- time-to-market, ROI
- patentability

To structure the efforts of the consortium members for exploitation of results, transfer into new products and possible joint patent applications, DIA has authored a Dynamic Agreement that describes regulations like:

- how to deal with the exploitables within the consortium or when involving any type of Third Party
- defines responsibilities and timelines for the process e.g. individual negotiations between the IP owners and potentially interested parties
- defines rights for use (cost-free) for consortial partners during project duration
- organized priority rights for Consortium Partners (right of first offer) during project duration

This document was legally reviewed by all partner organisations and a final version was drafted. All partners agreed that the document will be signed when needed. The final Dissemination and Exploitation plan is a dynamic document and will be adapted in case of requirement.

At the end of the project the patent situation in VascuBone turns out to be as follows:
DiaCoating/Fraunhofer have applied for two patents in order to have the Freedom to Operate with novel DiaSorb scaffolds based on their developments.
PPPolymer intends to file a patent application within 2015 focussing on their novel polymer-based scaffolds.

Training and Education:
The interaction between consortial partners to promote cross-fertilization between industry and academia was realized focusing on two aspects: education/training of clinicians and PhD/postgraduate exchange to broaden the research competences and career possibilities of participating scientists and clinicians. Focus was on training for VascuBone members:
short-term and medium-term exchange schemes promote training and technology transfer, maximize opportunities for data comparison and project development within the consortium especially for PhD and postgraduated scientists, and broaden the research competences and career possibilities of participants.
In addition workshops for scientific writing, PhD-workshops (presentation, self-expression, etc.) are carried out as education actions.
However, exchange with leading international laboratories in the fields of cell therapy, Bone Tissue Engineering as well as clinicians was performed successfully during the entire period of the project.

Dissemination of the results after VascuBone was discussed and should be mentioned here:

- Würzburg Inititative on Tissue Engineering (WITE) Date: 10.-12.06.2015, Place: Würzburg http://www.wite.org/ - lunch symposium
- utilization of network Fraunhofer Gesellschaft (exhibitions, fairs, etc. – distribution of Conference Booklet)
- partners will distribute the Conference Booklet with updated inlay-information
- submission of papers to peer-reviewed scientific journals ongoing.