

# The rational use of animal models in the evaluation of novel bone regenerative therapies

---

Perić, Mihaela; Dumić-Čule, Ivo; Grčević, Danka; Matijašić, Mario;  
Verbanac, Donatella; Paul, Ruth; Grgurević, Lovorka; Trkulja, Vladimir;  
Bagi, Čedo M.; Vukičević, Slobodan

Source / Izvornik: **Bone**, 2015, 70, 73 - 86

Journal article, Accepted version

Rad u časopisu, Završna verzija rukopisa prihvaćena za objavljivanje (postprint)

<https://doi.org/10.1016/j.bone.2014.07.010>

Permanent link / Trajna poveznica: <https://urn.nsk.hr/urn:nbn:hr:105:459882>

Rights / Prava: [In copyright](#) / [Zaštićeno autorskim pravom](#).

Download date / Datum preuzimanja: **2024-11-20**



Repository / Repozitorij:

[Dr Med - University of Zagreb School of Medicine  
Digital Repository](#)





## Središnja medicinska knjižnica

**Perić M., Dumić-Čule I., Grčević D., Matijašić M., Verbanac D., Paul R., Grgurević L., Trkulja V., Bagi Č. M., Vukičević S. (2015) *The rational use of animal models in the evaluation of novel bone regenerative therapies*. *Bone*, 70. pp. 73-86. ISSN 8756-3282**

<http://www.elsevier.com/locate/issn/87563282>

<http://www.sciencedirect.com/science/journal/87563282>

<http://dx.doi.org/10.1016/j.bone.2014.07.010>

<http://medlib.mef.hr/2330>

University of Zagreb Medical School Repository

<http://medlib.mef.hr/>

# The rational use of animal models in the evaluation of novel bone regenerative therapies

Mihaela Peric,<sup>b</sup> Ivo Dunic-Cule,<sup>a</sup> Danka Grcevic,<sup>c</sup> Mario Matijasic,<sup>b</sup> Donatella Verbanac,<sup>b</sup>  
Lovorka Grgurevic,<sup>a</sup> Vladimir Trkulja,<sup>d</sup> Cedo M. Bagi,<sup>e</sup> Slobodan Vukicevic<sup>a#</sup>

<sup>a</sup> University of Zagreb School of Medicine, Center for Translational and Clinical Research, Laboratory for Mineralized Tissues, Salata 11, Zagreb, Croatia

<sup>b</sup> Department for Intercellular communication, Salata 2, Zagreb, Croatia

<sup>c</sup> University of Zagreb School of Medicine, Department of Physiology and Immunology, Salata 3, Zagreb, Croatia <sup>d</sup> Department of Pharmacology, Salata 11, Zagreb, Croatia

<sup>e</sup> Pfizer Inc., Global Research and Development, Global Science and Technology, 100 Eastern Point Road, Groton, CT 06340, U.S.A.

# To whom correspondence should be addressed: e-mail: [vukicev@mef.hr](mailto:vukicev@mef.hr); tel. +385 1 4566812; fax. +385 1 4566822

**Keywords:** bone fracture, bone healing, animal model, regenerative therapy, tools for scoring, healing parameters

## **Abstract**

Bone has a high potential for endogenous self-repair. However, due to population aging, human diseases with impaired bone regeneration are on the rise. Current strategies to support bone healing include various biomolecules, cellular therapies, biomaterials and different combinations of these. Animal models for testing novel regenerative therapies remain the gold standard in pre-clinical phases of drug discovery and development. Despite improvements in animal experimentation, excessive poorly designed animal studies with inappropriate endpoints and inaccurate conclusions are being conducted. In this review, we discuss animal models, procedures, methods and technologies used in bone repair studies in an attempt to assist investigators in planning and performing scientifically sound experiments that respect the wellbeing of animals. In the process of designing an animal study for bone repair investigators should consider: skeletal characteristics of the selected animal species; a suitable animal model that mimics the intended clinical indication; an appropriate assessment plan with validated methods, markers, timing, endpoints and scoring systems; relevant dosing and statistically pre-justified sample sizes and evaluation methods; synchronization of the study with regulatory requirements and additional evaluations specific to cell-based approaches.

## **Highlights**

- Animal models in bone regeneration studies remain the golden standard of testing
- Advances in animal research are recommended to support the discovery of novel therapies
- Animal skeleton features, the study models, the assessment plan, dosing and statistics should be considered

## **1. Introduction**

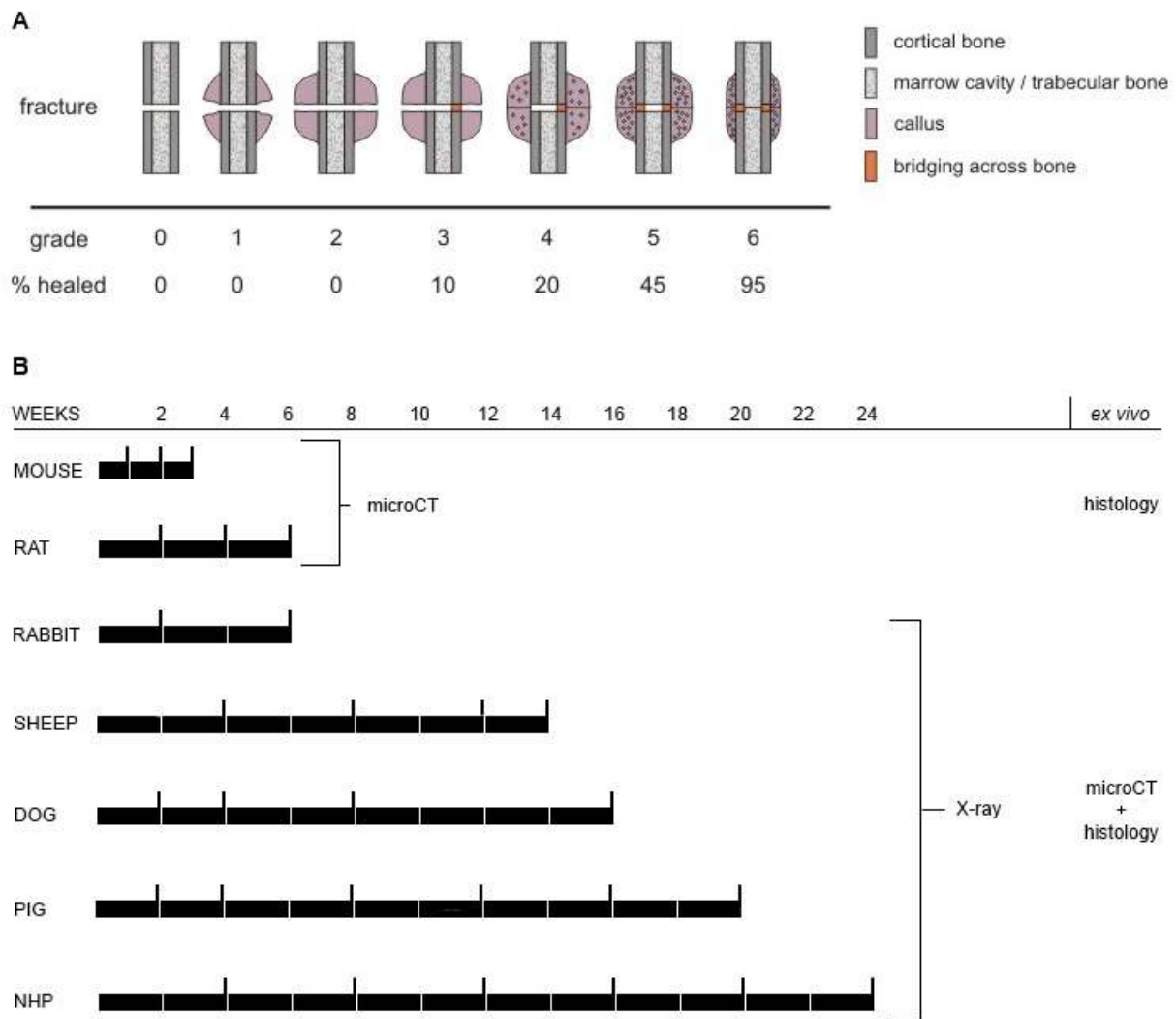
The already high incidence of bone trauma in the human population will inevitably increase as the human population ages. Osteoporosis as the major underlying condition makes approximately 27.6 million men and women in the EU (6 % of men and 21 % of women aged 50–84 years) to be susceptible to a bone fracture [1]. In 2010, approximately 3.5 million bone fractures were reported in the EU with direct healthcare costs of € 37 billion and 1.180.000 quality adjusted life years lost [2]; these costs expected to undergo a 25% increase by 2025. Large bone defects as well as non-unions and extensive bone loss after fractures still remain challenges for efficient clinical interventions and require additional support of the damaged site. Because present therapeutic approaches are often accompanied with prolonged treatments, pain and risk of infection, haemorrhage, nerve damage and loss of function, there is a significant unmet medical need for the development of new options for bone repair and the prevention of bone non-unions. Various animal models are available to study the efficacy, safety and tolerability of new therapies.

The objective of this review is to provide an overview of bone defect animal models and available tools for the assessment of bone healing, as well as to suggest guidelines for rational animal use in an attempt to advance bone research as well as to support the development of investigational products in bone regeneration.

## **2. Bone regenerative strategies: biomolecules, cells and biomaterials**

Bone healing is a precisely orchestrated regenerative process, which restores the bone quality by mimicking embryological cascade of events. Bone healing process is traditionally divided into three stages: an early inflammatory stage, a repair stage and late remodelling [3]. A schematic presentation of a long bone healing stages and grades are presented in Figure 1A.

Although bone possesses endogenous self-repair mechanisms [4-8], in conditions such as impaired blood supply, excessive damage to the periosteum, inadequate immobilization, infection at the affected area, mineral and vitamin deficiencies, underlying diseases and side effects of certain medications and radiation, the enhancement of the regenerative processes is necessary to ensure the rapid and adequate restoration of skeletal functions [9-11]. The standard therapy to treat bone fractures/defects includes mechanical support either via cast and/or mechanical devices (e.g. nails, plates and screws). Additional strategies being used and currently developed to further support bone healing are primarily based on the use of: (1) active ingredients (biomolecules), (2) cellular therapies and (3) biomaterials.



**Figure 1. A:** Tool for assessment and follow up of a long bone healing along the bone healing cascade expressed as a healing grade [12,13] and % of bone healed. The early phase of healing (grade 0-3) enables temporarily fracture stabilization and further endochondral bone formation, and is characterized by the recruitment of mesenchymal cells and successive chondrogenesis resulting in a soft callus formation [14]. The second stage (grade 4-5) is distinguished by deposition of the collagen and subsequent mineralization resulting in a woven bone formation. The last stage (grade 6) is characterized by the bone remodelling which restores the original bone structure and strength. The assessment of the healing process determines the sample size and end-points when planning an experiment. The scheme exemplifies a non-critical size defect healing. If one assumes that it depicts control animals, and that the grades “0” and “1” indicate average scores for an early process and grade “6” indicates an average score of a late process (6-8 weeks for a rat), then grades 0-1 or 6 would be biased towards “no difference” between a control and treatment intervention. Hence, “mid-time” evaluation points (grades 2-5) are of interest for a comparison (see section 5 for more details). **B:** Suggested time points for the assessment of bone fracture healing in the mouse, rat, rabbit, pig, sheep, dog and non-human primates (NHP). Overall duration of an experiment and assessment time points in critical size defect studies should be extended for at least 30-40% of time used for a bone fracture (see section 5, Figure 3A,B).

## 2.1. Biomolecules

Biomolecules used in the regenerative therapies for bone are mainly various growth factors [15]. Osteogenic factors primarily belong to the TGF- $\beta$  superfamily, and the most studied factors are bone morphogenetic protein BMP2, BMP4, BMP6 and BMP7 [16,17]. Because vascularization is essential for bone regeneration, angiogenic factors VEGF, PDGF, FGF and IGF are also being extensively tested for their usefulness in bone repair [18-24]. Immunomodulatory and anti-inflammatory agents, such as selective anti-cytokine therapies, corticosteroids and non-steroidal anti-inflammatory drugs, are used to direct specific effects on the regeneration and resorption pathways during bone healing [25,26]. Additionally, the use of parathyroidal hormone (PTH), growth hormone, steroids, calcitonin and vitamin D in systemic applications has also been shown to advance bone healing through stimulating osteogenesis, angiogenesis and osteoblast differentiation [27-30]. Various combinations of biomolecules have also been extensively evaluated in pre-clinical models with mostly positive results [27,31-37].

## 2.2. Cell-based therapy

Cell-based therapy utilizes stem/progenitor mesenchymal cells originally identified among bone marrow stromal cells [38]. Although most studies were conducted with bone marrow derived mesenchymal progenitor cells (MPCs), other tissues have been described to contain osteoprogenitor cells with similar regenerative potential including adipose tissue, muscle, umbilical cord blood, periosteum, dental pulp and periodontal ligament [4,39-45]. The multilineage differentiation ability, paracrine effects and immunomodulatory properties of MPCs make them an ideal for tissue engineering and regenerative purposes [5,7,46-48]. Under appropriate conditions MPCs could be differentiated into a variety of mesenchymal tissues such as bone, cartilage, tendon, ligament, marrow stroma, muscle, fat and dermis [4,49-53]. To induce fracture healing, MPCs are expanded *ex vivo* prior to their autologous grafting to the fracture site and differentiated into osteogenic lineages to promote bone regeneration [9,54]. Such cell-based strategy approaches have been used to demonstrate that autologous bone marrow-derived MPC transplantation was superior compared to unloaded scaffold [5,7]. Unique immunological characteristics of MPCs suggest that the implantation of allogenic or xenogenic MPCs could be successfully used for a cell-based therapy. Cells of non-mesenchymal origin such as endothelial progenitor cells may also enhance bone regeneration by secreting paracrine osteoinductive and angiogenic factors [7]. Furthermore, tissue engineering, a process of developing biological tissue substitutes for restoring, maintaining or improving tissue function [55], were used to construct a single device combining all of the important components of bone repair (osteoconductive scaffold, osteoinductive growth factors and osteogenic cells) [4,5,7,10,55-57]. Recent studies have attempted to improve the basic protocols of cell-based therapy via additional tissue engineering strategies, including alternative osteoprogenitor population, gene delivery modification of MPCs, growth factors or pharmacological compounds. Currently, more than

20 different clinical trials involving bone tissue engineering approaches using cell therapies are reported in Clinicaltrials.gov ([www.clinicaltrials.gov](http://www.clinicaltrials.gov)).

### **2.3. Biomaterials**

Starting from the natural materials such as bovine collagen which is currently raising safety concerns [58], the field of orthopaedic biomaterials has expanded to include an impressive array of materials that are currently being tested in preclinical models [59]. Biomaterials have a range of properties, from osteoinductive and osteoconductive to immunomodulatory. Hydroxyapatite and calcium phosphate as well as their composites such as HA/poly(DL-lactic-co-glycolic acid) (PLGA), in the form of ceramics, cements and coatings have shown osteoinduction in animal models [60-66]. Various hybrid materials combined as co-polymers, polymer blends and polymer-ceramic blends have also shown efficacy [67-73]. Advanced hydrogels, naturally derived collagen and gelatin gels as well as synthetic poly-ethylene glycol and poly-vinyl alcohol-based hydrogels, serve as matrices for other products and mimic the extracellular matrix topography [74-76]. Biomaterials with immunomodulatory strategies, such as artificial extracellular matrices (ECMs) (hydrogels, ECM coatings) and materials with surface property modulation, have the ability to modify the immune function and improve bone repair and regeneration [25,77].

### **3. Overview of Methodology and Animal Models for Bone-healing Studies**

Working with animals is a privilege and scientist as well as their institutional ethical boards should do their best to conform to the current animal care guidelines [78,79]. In recent years, the regulatory and scientific community imposed stringent rules to ensure that the wellbeing of laboratory animals is respected and that the 3R's paradigm implemented whenever possible without compromising the quality of the study and data analyses [80,81]. Despite copious improvements, too many animal studies are still being published without regard of their poor design, use of inadequate or insufficient biomarkers, inaccurate conclusions and/or producing insignificant data. The overall benefit of these studies to scientific community is negligible, with published studies being redundant and offering very little novelty. For instance, the BMP preclinical development is a telling example of the misuse of laboratory animals due to non-existent standard operating procedures for testing of novel compounds for bone regeneration. Large numbers of animal models, species and doses have been used to support the development of BMP2 and BMP7 devices, however majority of these studies yielded inconclusive results that were misleading and difficult to interpret. Between 1988 and 2004 hundreds of experiments were conducted on more than 17.000 animals (literature search was performed via PubMed using terms bone morphogenetic protein 7 and bone morphogenetic protein 2, revealing 157 and 421 articles, respectively). In our opinion, literature based data represent only a small fraction of the total number of animals used. Despite this, after years of use in clinics both BMP devices have



been confronted with major side-effects and their clinical use has been recently scrutinized [17,82-84].

### **3.1. Principles of Study Design**

The analysis of hard tissues requires a long, complex and expensive experimentation. Scientists, clinicians and regulatory authorities have recognized the necessity of using two laboratory species and several independent biomarkers when assessing the effect of novel bone and fracture-healing therapies [85,86]. The first step when preparing a study is to precisely determine the study goals and establish the criteria used to evaluate the overall success of the study. The following step is to plan the study design in which ten essentials should be selected: 1) Animal model (optimizing goals of the study); 2) Animal species; 3) Animal sex and age; 4) Study duration (allowing for the biological process to initiate and complete); 5) Number of animals per study group (sufficient for statistical analyses); 6) Dose and route of administration of the test article (mimicking anticipated clinical use); 7) Appropriate controls (including a sham, vehicle and/or a “positive” control group to ensure the credibility and reproducibility of the data and a standard of care drug with a well-known efficacy/safety should be used as a “positive” control); 8) Supply of the test article (sufficient for the entire study); 9) Optimal *in vivo* and *ex vivo* biomarkers, and 10) Tissue collection, storage and analyses planning. The common wisdom of *in vivo* experimentation is often ignored for various reasons, most frequently due to a lack of experience and poor planning, a lack of funds, a lack of in-house expertise, short timelines and the inadequate selection of biomarkers. The publication of poorly designed studies on animals should be restricted for both ethical and scientific reasons.

### **3.2. Methods and Technology**

All currently marketed drugs for the treatment of skeletal disorders, including fractures were successfully tested in preclinical models. The value of preclinical work involving animal models depends on two essentials. The first determinant depends on the availability of an animal model that mimics a human disease involving bone repair so that the data generated can be used to predict the drug efficacy and safety in patients. Examples of animal models with good predictability of clinical outcomes include models of postmenopausal osteoporosis [87-92], models of glucocorticoid induced bone loss [93-96], models of cancer metastasis to bone [97], disuse models [98,99], fracture healing models [100-103] and several others. The second determinant of successful experimentation relates to translational biomarkers of novel therapy efficacy and safety that can be accurately predicted and monitored in patients. Numerous methods that are thoroughly understood, extensively described and tested for predictability are available for testing the efficacy and safety of novel treatment targets (Table 1).

**Table 1.** The “toolbox” of methods and technologies that is available for *in vivo* and *ex vivo* assessment of bone physiology and pathology.

<b>IN VIVO ASSESSMENT</b>			
<b>Assessment</b>	<b>Process/Parameter</b>	<b>Assay/Technology</b>	<b>Translation</b>
<b>Biomarkers in serum and urine</b>	Bone formation, resorption, metabolism, cartilage formation, connective tissue degradation, calciotropic hormones	P1NP, Osteocalcin, BSAP, CTX, TRAP5b, Ca <sup>2+</sup> , P <sup>2-</sup> , PIIANP, ICTP, IGF-1, PTH, Vit. D, calcitonin, T3/T4	<b>High</b>
<b>Imaging technologies</b>	Bone anatomy, bone mass, geometry, and structure	Standard radiology, DEXA, pQCT, micro-CT, MRI, PET, use of contrast agents	<b>High</b>
<b>Functional tests</b>	Biomechanic/Biometric	Dynamic Weight Bearing System, Gait analyses (Digigait), some other	<b>High</b>
<b>Mechanical properties</b>	Bone strength: maximum load, stiffness, toughness, ultimate strength	BioDent	<b>Too early</b>
<b>EX VIVO ASSESSMENT</b>			
<b>Test</b>	<b>Activity/Parameter</b>	<b>Assay/Technology</b>	<b>Translation</b>
<b>Imaging technologies</b>	Local bone anatomy, bone geometry, bone mass, bone structure	Standard radiology, DEXA, pQCT, micro-CT, MRI, PET	<b>High</b>
<b>Bone Biomechanics (strength)</b>	Bone geometry, composition and strength of cortical and/or cancellous bone	Various methods (3- and 4-point bending methods, tensional test, compression test, Finite element modeling)	<b>High</b>
<b>Undecalcified bone histology and histomorphometry</b>	Bone remodeling and modeling at cortical bone envelopes, cancellous bone, Bone Formation Rate, Mineral Appositional Rate, osteoid, mineral	Requires <i>in vivo</i> labeling with fluorescent markers, embedding in methylmetacrylate, cutting and analyses (histomorphometry) or staining (von Kossa, Goldner trichrome, Toluidine blue). Cryosections should be considered.	<b>High (if bone biopsy is available)</b>
<b>Decalcified bone histology and histochemistry</b>	Bone cells (osteoblasts, osteoclasts, bone lining cells, osteocytes, bone marrow, chondrocytes)	Routine bone and joint stains (H&E, Toluidine blue, Safarin O) or immunostaining (TRAP, Factor VIII, PCNA, PGP9.5)	<b>High (if bone biopsy is available)</b>
<b>Microscopy</b>	Bone structure, lamellar bone, woven bone, cellular analyses	Polarized microscope, electron microscope	<b>High</b>

P1NP - serum type 1 procollagen (C-terminal/N-terminal); BSAP - serum bone-specific alkaline phosphatase; CTX - collagen type 1 cross-linked C-telopeptide; TRAP5b - tartrate-resistant acid phosphatase 5b; PIIANP - type I procollagen N-terminal propeptide; ICTP - carboxyterminal telopeptide of type I collagen; IGF-1 - insulin-like growth factor-1; PTH - parathyroid hormone; T3/T4 - thyroxine/triiodothyronine; PCNA - proliferating cell nuclear antigen; PGP9.5 - neuron cytoplasmic protein gene product also known as ubiquitin C-terminal hydrolase 1 (UCHL-1).

Radiological methods based on detecting bone minerals should always be used in combination with histology. Quantitative computed tomography (qCT), peripheral quantitative CT (pQCT) and micro CT ( $\mu$ CT) are additional techniques that are superior to X-ray in assessing bone geometry, mass and structure. X-ray with/without pQCT is the method of choice for *in vivo* follow-up due to its good correlation with the mechanical testing of bone strength [104]. The strength of bone and callus assessed via the 3- and/or 4-point bending method (or torsional testing performed *ex vivo*) supplements the assessment of bone regeneration quality and provides opportunities to correlate and accurately interpret radiologic and other data (serum biomarkers, histology). Bone histology and histochemistry performed *ex vivo* provide key and quantifiable methods to test bone cell activity during bone regeneration and should be an integral part of bone healing animal studies. Undecalcified bone histomorphometry based on the use of fluorescent dyes such as calcein, tetracycline and alizarin labelling mineralizing bone surfaces provides an accurate quantification of the bone formation and resorption processes in the callus and in the surrounding cortical and cancellous bone. Differential staining using the van Kossa method for minerals and counterstaining with toluidine blue enables the meticulous evaluation of cartilage and mineralized bone at the callus. The same metacrylate embedded bone samples can also be used for polarized light microscopy for determining the lamellar or woven bone structure in the newly formed bone. Examples of the healing process in long bones by using radiology methods and histology is depicted in Figure 1B. Both methodologies can be used separately or in combination to accurately score the healing cascade in order to assess efficacy and safety of tested therapies.

Serum and urine biomarkers of bone formation and resorption are highly desirable and recommended for the assessment of bone metabolism. Although serum biomarkers can be used to measure the overall activity of bone cells throughout the skeleton, and may not always detect local changes in bone activity around the fracture, these markers have a great translational value and as such provide an exceptional tool for accurately monitoring skeletal metabolism in live animals.

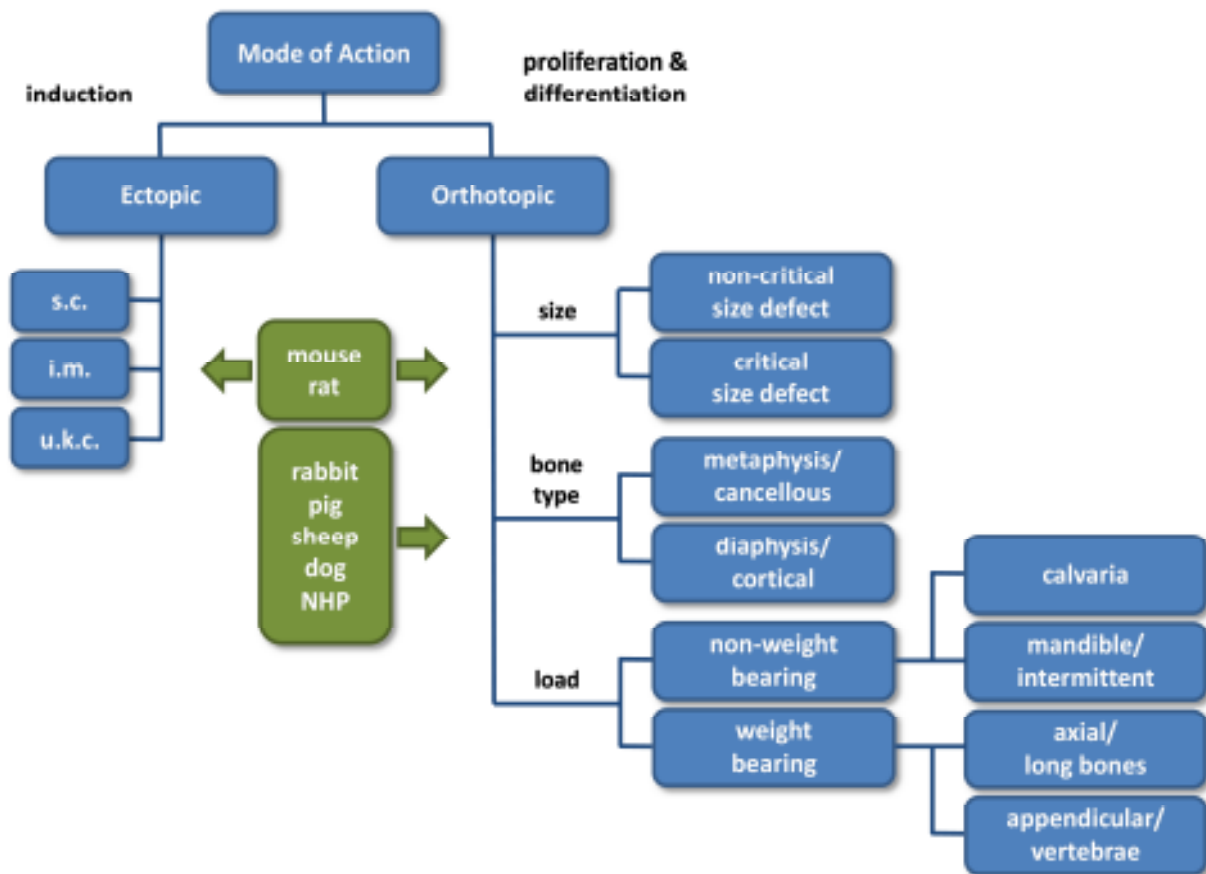
#### **4. 3R: Replacing, reducing and refining to improve animal welfare**

Although scientists are continually developing more complex alternative techniques, like engineered organs and *in vitro* tissue models, animal models are still the gold standard for fracture healing testing due to their ability to mimic complex human physiological processes and bone mechanics which cannot be simulated and replaced by even most advanced non-animal technologies. This is particularly true for testing of new medicinal products and during preclinical phases of drug development. Contemporary biomedical research projects are increasingly encountering efforts to apply the principles of the "3Rs" for conducting humane experimentation in animals focused on Replacement, Reduction and Refinement [80,81].

To align more firmly with the principles of the 3R, EU member states adopted the 2010/63/EU directive and the same processes are being implemented by other policy makers: EMA (<http://www.ema.europa.eu/ema/>), the OECD (<http://www.oecd.org/health/>) and the ICH (<http://www.ich.org/products/guidelines.html/>).

#### **4.1. Animal model considerations**

Animal models of bone regeneration fall into two categories: (1) ectopic models are primarily used to distinguish between the proliferative and inductive capacity of new products, while (2) orthotopic models are used to test the efficacy and safety of the new products and/or procedures. Ectopic models are relatively simple, less costly and less invasive. For example, ectopic bone formation models were recently used to elucidate whether the maturation status of implanted cells determines the origin of tissue-engineered bone [105] and to demonstrate new bone formation after the implantation of the OSTEOGROW device (rhBMP6 in autologous blood coagulum) without any signs of inflammation or fibrosis [17]. Although successfully used as a preliminary model for screening various formulations of osteogenic cells, scaffolds and growth factors, the ectopic model displays serious limitations including the eventual reabsorption of newly formed bone and the lack of effective mechanical stimulus required for bone remodelling [106]. Orthotopic models represent investigational procedures performed in or around the bone itself. The classification of the bone healing models is presented in Figure 2. The process of bone repair can also be studied in models of bone disuse under various loading conditions via animal models in which one limb is fully or only partially deprived of weight bearing activity. These models are more sophisticated because they require multiple procedures such as sciadic neurectomy, amputation or various immobilization techniques combined with bone defects [107,108]. The choice of the model should be based on scientific, ethical and practical merits of a particular study and reflect the human biology or disease and should accommodate for appropriate clinical settings with relevance to the product being tested [101,109-111].



**Figure 2.** Graphical presentation of animal bone models based on the investigated effect of the therapy. Ectopic models based on injection site are referenced as subcutaneous (s.c.), intramuscular (i.m.) and under the kidney capsule implantation (u.k.c.) models [16,112]. Based on the creation of the defect size, orthotopic models of bone healing are divided in a non-critical size defect models with the capacity to heal without an intervention, whereas in the critical size defect model, bone regeneration and restoration of the function will not occur without an intervention. Orthotopic models can also be grouped based on the anatomical location in the body (appendicular and axial skeleton) but also based on the bone structure and microanatomy of the bone being studied (cortical and/or cancellous bone). When based on mechanical loads imposed on the studied bone, animal models can resemble physiological and non-physiological conditions. For example, bone repair can be studied in non-loaded bones (such as calvarias), in bones that experience normal weight bearing loads (appendicular (vertebrae) or axial (long bones) skeleton) or in the mandible where non-weight bearing intermittent loads occur during chewing [113,114]. Bone defect studies can be performed in traditional laboratory animals (mice, rats, rabbits, dogs and non-human primates - NHP) as well as in domestic animals such as sheep and pigs, which are often used as viable substitutes for dogs and NHP.

Systematic factors discussed in the section 3.1 of this manuscript should be taken into account during the planning phase of the study. Models that are better understood and described in the literature often have proven records of being more predictive of clinical outcomes. The age of study animals as well as gender can influence the bone repair through the action of calciotropic hormones and thus merit careful consideration [115-119]. For example, aged, thyroparathyroidectomized and ovariectomized (OVX) animals are known for delayed fracture healing and reduced bone mineral density; therefore, OVX animals are frequently utilized to study osteoporotic fractures because these models mimic postmenopausal women [88,120,121]. Historic evidence suggests that rats, rabbits and mice are the most frequently used species to study bone physiology and drug efficacy and safety accounting for approximately 80% of all animals used to study bone repair, while other species including sheep, goats, pigs, dogs and non-human primates (NHP) make up for the remaining 20% [122,123]. The choice of a particular species is a critical step and is often based on the biochemical and microstructural characteristics of the bone tissue as well as on the similarities of the healing processes between the particular species and humans (Table 2, Figure 1). In addition, difference in gross anatomy as well as differences in distribution of the cortical and cancellous bone compartment within each bone should be taken into account when deciding which species to choose for the particular study since those differences reflect biomechanical properties that play a critical role in the bone repair processes [124]. The availability of serum biomarkers and the translatability of biomarkers to the clinical environment is a very important issue. Rats, dogs and NHP are routinely used in preclinical safety studies and serum biomarkers of bone metabolism are well established and validated, therefore using those species is advantageous from a biomarker standpoint; however, the use of dogs and NHP is restricted and those two species should only be used if necessary only for the late stage testing of efficacy and safety parameters. Clearly, no animal model entirely mimics human conditions because no animal species has a skeletal or biomechanical properties identical to human. Additional factors that could influence selection of the animal model include the size of the animal, the cost to acquire and care for animals, animal availability, ethical acceptability, tolerance to captivity, breeding cycles, ease of housing and handling, adequate facilities and qualified staff and familiarity with the model, technical capacities etc. [109,110,125].

**Table 2.** Bone research related characteristics of laboratory animals.

Laboratory animal	Life expectancy (months)	Bone maturation <sup>2</sup> (epiphyseal plate closure) (months)	Time to union of fractures (weeks)	Similarity to human bone (Ma+Mi+C+R) <sup>5</sup>	Ethical acceptance
Mouse	18-36	5	3	0+1+0+1 = 2	high
Rat (Sprague-Dawley)	30-48	11	4-6	1+1+0+1 = 3	high
Rabbit (New Zealand White)	84-96	6,8 tibia 5,3 femur	6-7	1+1+2+1 = 5	high
Dog (Grayhound)	108-168	7,5 tibia 7,3 femur	10-13	2+2+3+2 = 9	low
Sheep (Suffolk x Dorset)	180	17	10-14	3+1+2+2 = 8	medium
Pig (Gottingen)	180+	12-24 <sup>3</sup> 28 femur <sup>4</sup>	12-24	2+2+3+3 = 10	medium
Monkey (Rhesus)	240-360	75 femur <sup>5</sup>	16-24	3+3+3+3 = 12	low
Human	47-83 years <sup>1</sup>	240	18-24	-	-

<sup>1</sup> WHO Life expectancy at birth, both sexes, 2011; <sup>2</sup> ref [126]; <sup>3</sup> ref [127]; <sup>4</sup> ref [128]; <sup>5</sup> ref [129]; <sup>5</sup> adapted from [110] – comparison based on macrostructure (Ma), microstructure (Mi), composition (C) and remodelling (R) and scoring from 0 (not similar)-3 (very similar) resulting in the total sum representing similarity.

## 4.2. Species Skeleton Specification

### 4.2.1. Rodents

Of all laboratory animals mice and rats are considered to have skeletons and bone biologies that are least similar to humans since their skeletons are modelling-driven due to permanently open growth plates at the epiphyses of long bones, a lack of the Haversian system and low cancellous bone content at the epiphyses of the long bones [100,130-136]. However, studies in rodents are very informative and cost effective. Rodents, primarily mice are genetically very well defined and are best suited for studies with genetically modified strains to address specific molecular mechanisms of bone physiology and pathology. The advantages of using rodents, and particularly rats in early stage studies are numerous and include broad availability, inexpensive housing, easy handling, small size (relatively small quantities of test article needed), well-defined and described procedures and models and biomarker availability. Rats are most regularly used in safety toxicology studies and also to study the pharmacodynamic and pharmacokinetic properties of novel treatments [137]. Despite some deficiencies, rat models of skeletal diseases are very predictive of drug efficacy and safety in humans because bone cells, osteoblasts, osteocytes and osteoclasts in rats have similar receptors to human bone cells and therefore react to drug challenge in a similar fashion to human bone [85]. Some characteristics of rodent models, such as an open growth

plate, may be unfavourable for studies focusing on the adult skeleton, however, studies in rodents are very useful when investigating efficacy and safety of drugs targeting juveniles. ICH-harmonized guidelines for biotechnology-derived pharmaceuticals were recently updated to stress the importance of selecting the relevant species for the investigational product biological activity as well as for the safety [138].

#### **4.2.2. Rabbit**

Rabbits are the smallest commonly used non-rodent species in musculoskeletal research studies [139] because the rabbit skeleton does not include the two major drawbacks of rodent models; the lack of the Haversian system and permanently open growth plates. Rabbit bone also differs from that of humans in its size and shape [124]. Histologically, the skeleton of rabbits consists of primary lamellar bone while vascular canals parallel the long bone axis. Rabbits are commonly used models for bone healing studies due to the vast experience with rabbit handling, short duration necessary to reach the mature bone characteristics and bone densities that are similar to those of humans [103,140]. Rabbit models were used to study metaphyseal fractures [141], mandibular distraction osteogenesis [142], mandibular defect repair [143], investigational products in cranial models [144], critical size defects in animals of different age [145] and spinal fusion [146,147]. Rabbits are also widely used to study novel biomaterials, growth factors and stem-cells approaches [142-145,148,149].

#### **4.2.3. Dog**

Canine bone healing models are frequently used in musculoskeletal and dental research because significant amounts of information are available regarding the predictability of these models for human conditions [110,140,150]. Dog bones have a mixed microstructure with a primarily secondary osteonal bone and a plexiform bone in the vicinity of the periosteum and endosteum [140]. The bone composition in dogs is similar to that of humans and the structural properties of bones from several skeletal sites was recently summarized by Bagi et al. [124]. Dogs are often used in safety studies as a second species and biomarkers of bone metabolism are validated and are well-established for dogs. However, there are societal concerns regarding the use of dogs in biomedical research and the ethical framework related to their welfare [151] consistently restricts the use of dogs in preclinical testing.

#### **4.2.4. Pig**

Although pigs have been used for decades in bone studies this model was never widely deployed due to the fact that commercial breeds usually grow quickly and reach extreme body weights. With advances in the breeding of minipigs and micropigs, the use of pigs in biomedical and orthopaedic research has increased [152]. Regarding bone anatomy, microstructure, remodelling and healing, porcine bone closely resembles human bone [153,154]. Pigs were found to exhibit spontaneous vertebral fracture and their rates of bone



removal and deposition (trabecular and cortical bones) are similar to humans although porcine bone remodels slightly faster than human bone [152,155,156]. Limitations for the use of NHP in regulatory toxicology studies have opened discussions on the suitability of using minipigs in drug development studies [157]. Recently, it was shown that minipigs are comparable to NHP in immunogenicity testing [158,159] and their liver metabolism is similar to that of humans [160]. Additionally, models of porcine osteoporosis have been developed to expand the usefulness of this species for bone research [161-164].

#### **4.2.5. Sheep**

Sheep tibia models are considered to be valid and reliable for the evaluation of bone regeneration, with the advantage of having a maximal weight bearing scenario and long bone dimensions in adult animals that are suitable for the testing of human implants and prostheses [165-168]. However, sheep are seasonal breeders so their bone metabolism changes during the year which presents a significant hurdle for bone metabolism studies. The bone maturation period in sheep is long and the microstructure in the young animals is distinct (plexiform bone). In adult sheep, the bone structure is different from humans, consists primarily of primary bone and Haversian remodelling occurs during adulthood [110]. The bone mineral density and bone strength in sheep is increased relative to human. Moreover, sheep models of critical size defects are extensively applied to evaluate cell-based therapeutic approaches using autologues, allogeneic or xenogeneic MPCs in combination with growth factors and different types of scaffolds to enhance bone regeneration showing significant advantages compared with cell-unloaded (empty) scaffolds [43,53,166,169-176].

#### **4.2.6. Non-Human Primates**

NHP are the best characterized large animal model for skeletal research and their skeleton and posture as well as their bone structure, composition and remodelling patterns is similar to those of humans [124,177-179]. In addition to a high similarity between monkeys and humans regarding drug metabolism between monkey and human, the existence of validated serum and urine biomarkers with high translational value to human is of the utmost importance for regulatory studies using NHP. Although skeletal studies in Old World primates yield valuable data, the use of NHP are constrained by ethical and technical considerations, including high cost, limited availability and regulations (Directive 2010/63/EU) [80,180]. Although very useful in bone research for novel therapies, NHPs should only be used in situations when efficacy, safety and toxicity studies in other species could not provide appropriate answers i.e. human antibodies or indications such as hereditary non-union of the tibia or long bone fibrodysplasia. Marmosets were recently proposed as good alternative for skeleton studies because the adult marmoset skeleton has similar anatomical characteristics that are similar to those of adult humans, including the absence of growth plates, the presence of Haversian system and true remodeling of cancellous and cortical bone [181,182]. Compared with macaques, marmoset monkeys have an earlier puberty and sexual maturity and presumably achieve earlier peak bone mass. They

are easy to breed and to handle under controlled laboratory housing conditions. Similar to FDA guidelines [183], in the EMA Guideline on the evaluation of new medical products in the treatment of primary osteoporosis [184] states, that these substances should be tested in at least two species, one of which should be an ovariectomized rat and the other an animal with evaluable cortical bone remodeling. Primates, sheep and pigs are suggested as a second animal model by the EMA. Common marmoset monkeys (*Callithrix jacchus*) also fulfill these requirements because they show osteonal remodeling that is very similar to that of humans. It is therefore highly recommended that institutions involved in drug development request a scientific advice from regulatory agencies for opinions regarding non-clinical data requirements prior to firsts-in-human studies [184].

#### **4.3. Animal models in cell-based therapies**

To be able to utilize the major advantages of experimentation in laboratory animals, models of bone repair need to be optimized in accordance to the unique characteristics and requirements of different species. Different cell-based therapy approaches to treat segmental defects in weight-bearing long bones are given in Table 3 to illustrate the great variability of models regarding cell population and scaffold selection, protocols of intra-defect transplantation, species and defect localization, follow-up period and outcome measurements. Considering the wide diversity of conducted research, investigators should be extremely cautious to translate conclusion in-between models and species and pay special attention in designing the animal studies to draw valuable and reproducible results.

**Table 3.** Different cell-based therapeutic approaches used for the treatment of critical size segmental long bone defect in small and large animal models

Cell-based therapy	Species (study reference and details)	
	Rodent	Non-rodent
autologous (syngeneic) or allogeneic BM-derived MPCs	<b>Mouse:</b> [185], [186] <b>Rat:</b> [187] (DM), [188]	<b>Rabbit:</b> [189], [190], [191], [192] <b>Dog:</b> [193], [194] <b>Sheep/Goat:</b> [167], [170], [176], [195], [196], [197]
human BM-derived MPCs	<b>Mouse:</b> [198] <b>Rat:</b> [199], [200], [201]	<b>Rabbit:</b> [202] <b>Sheep:</b> [203]
non BM-derived MPCs or non-MPC cell-based therapy <sup>2</sup>	<b>Mouse:</b> [204] (hUCPVC), [205] (hSDF1/hBMP2-mFTG) <b>Rat:</b> [206] (rEPC), [207] (hADPVC)	<b>Rabbit:</b> [208] (rbPMPC), [209] (rbADPVC), [210] (rbDFAT) <b>Sheep:</b> [6] (sEPC)
genetically modified or labeled MPCs <sup>1</sup>	<b>Mouse:</b> [211], [212] (hBMP2-mMPC), [213] (hMPC/GFPCol1 $\alpha$ 1), [214] (mMPC/GFP), [215] (ShhN-mPDMPC) <b>Rat:</b> [216] (BMP7-rDF), [217] (hBMP2-rMPC), [45] (hBMP2-MDC or hBMP2-ADC)	<b>Rabbit:</b> [218] (bFGF-rbMPC), [149] (rbMPC/ferumoxide), [219] (hAng1-rbMPCs/PRP), [220] (hVEGF-rbMPC/PRP) <b>Minipig:</b> [221] (hBMP2/hVEGF-pADMPC), [222] (US2/US3-pADMPC)
MPCs with growth factors/compounds	<b>Mouse:</b> [223] (hVEGF/hMPC), [224] (hVEGF/hBMP2/hMPC) <b>Rat:</b> [225] (hBMP2/hMPC), [226] (hBMP7/hMPC)	<b>Rabbit:</b> [227], [228] (PRP/rbMPC), <b>Dog:</b> [229] (PRP/cMPC) <b>Sheep:</b> [43], [174] (sMPC/PRP), [230] (hBMP7/sMPC)

<sup>1</sup> Cells were genetically modified by viral or non-viral transfection to overexpress different growth factors or regulatory molecules. Cells transfected with US2/US3 genes downregulated MHC I expression. In some studies MPCs were fluorescently labeled for in vivo tracking.

<sup>2</sup> Non bone marrow-derived MPCs were isolated from adipose tissue, periosteum, muscle, articular cartilage, placenta, human umbilical cord blood, etc. Therapy with cells other than MPCs used endothelial progenitor cells or genetically modified fat cells.

Abbreviations: BM - bone marrow; h - human; m - mouse; r - rat; rb - rabbit, s - sheep; MPCs - mesenchymal progenitor cells; GFP - green fluorescent protein; DM - diabetes mellitus prone strain; BMP - bone morphogenetic protein; VEGF - vascular endothelial growth factor; PRP - platelet-rich plasma; ShhN - N-terminal sonic hedgehog peptide; UCPVC - umbilical cord perivascular cells; EPC - endothelial progenitor cells; bFGF - basic fibroblast growth factor; Ang1 - angiopoietin 1; MHC - major histocompatibility complex; SDF1 - stromal cell derived factor 1; FT - fat tissue graft; DFAT - adipocyte-derived dedifferentiated fat; Col1 $\alpha$ 1 - Collagen, type I, alpha 1; PDMPC - periosteal-derived mesenchymal progenitor cells; MDC - muscle-derived cells; ADC - adipose tissue-derived cells; DF - dermal fibroblasts; ADPVC - adipose tissue-derived perivascular cells, PMPC - placenta-derived mesenchymal progenitor cells

## 5. Experimental design and statistical considerations for animal fracture models

Here we describe a few possible modes for the standardization of the non-clinical efficacy evaluation of new treatments using the examples of critical and non-critical size defects of long bones in rat and rabbit models. We suggest that in both paradigms  $\mu$ CT (*in vivo* or *ex-vivo*; bone volume/tissue volume ratio), X-ray (rabbit *in vivo*) and histology are used; the latter two employing the elaborated scoring system shown in Figure 1. These methods and measures are reliable and reproducible with comparable mild-to-moderate variability in treated and control animals with relative standard deviation (RSD) in the range of 10-30% [231,232].

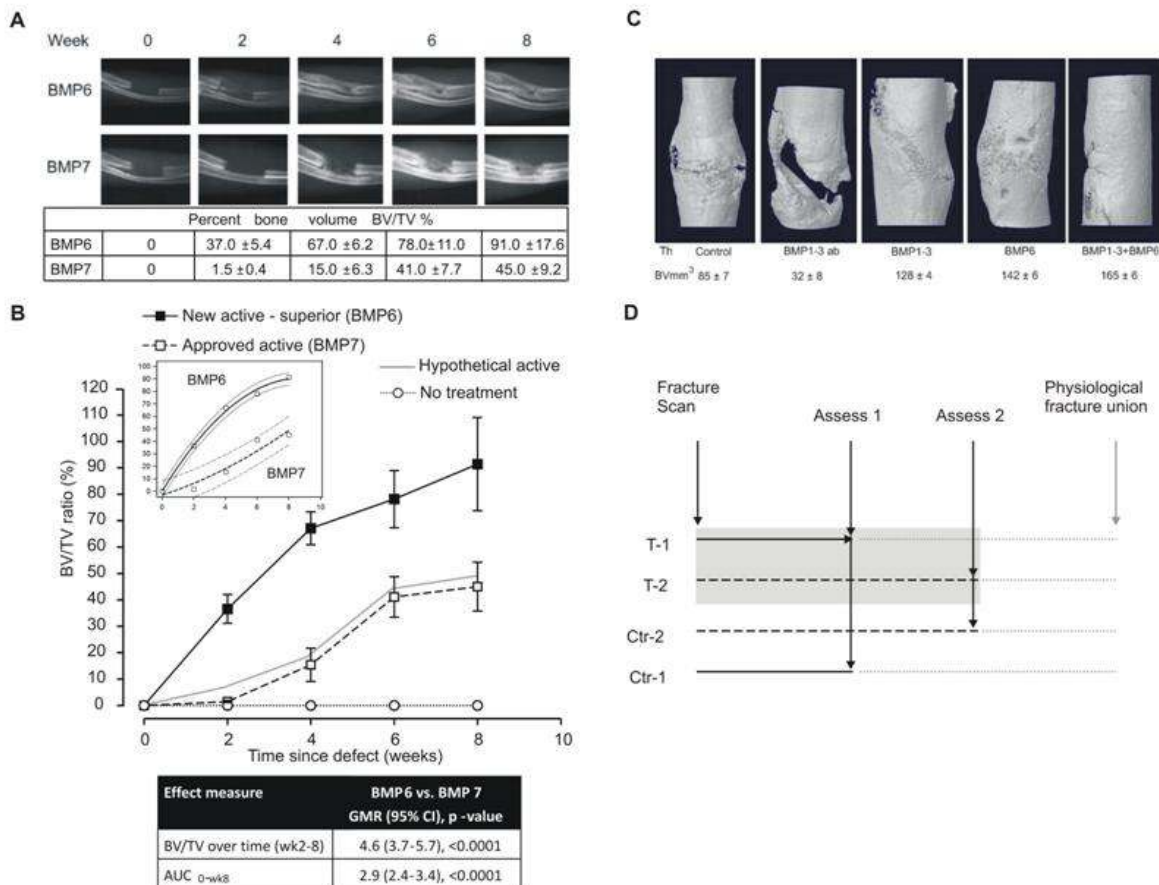
### 5.1. Critical size defect

Because the defect does not heal spontaneously and remains practically unchanged in control animals, evaluation at any time-point after the initial 1-2 weeks could indicate a bone-healing effect. However, we suggest that the evaluation of new treatments should be compared with the approved treatments, with main assessments based on repeated *in vivo* radiological measures over a period extending beyond the expected time of physiological union seconded in non-critical size fractures focusing on the overall process (Figure 1). Experiments should include only a few previously demonstrated inactive control animals (“placebo” to the new treatment). Figure 3A depicts four repeated radiograms and  $\mu$ CT scans (table in Figure 3A) taken over time in one such experiment. Data demonstrate low variability of values across the time point. We suggest data analysis by fitting general linear mixed models (GLMMs) with restricted maximum likelihood (REML) estimation (relaxed assumptions as compared to repeated measures ANOVA, units with individual missing values not excluded) to produce time-averaged difference between treatments. An alternative analysis would be based on integration of the response as the area under the curve (AUC) of change of the radiological measure (a difference between newly formed bone at each time point vs. 0) by fitting a general linear model (GLM). We suggest that the defect size is considered as a covariate. Figure 3A, B depicts a new treatment superior to an approved one (bone formation 4,6 fold greater based on time-averaged response and 2,9 fold greater based on AUC). We suggest that a new treatment indicates a potential superiority if any of these measures is at least 25% in its favour. With four repeated assessments, assuming variability as depicted in Figure 3 (or relative standard deviation (RSD) around 20% at each time point, as well as for AUC) autocorrelation 0.6 and autoregressive (1) [AR(1)] covariance structure, 7 animals per group per time-point provide 80% power to detect such a difference at a two-sided  $\alpha=0.05$ . The same sample size applies for 25% difference in AUC. Adding a treatment time interaction term in GLMM allows for comparisons at different time points, but requires adjustments of comparison-wise alpha level, resulting in a need for a larger sample (e.g. for pairwise comparison at four time points, under the above conditions 17-18 animals per group). To detect a time-averaged or AUC difference of at least 15% under the above conditions, 17-20 animals per group are needed. If such an experiment fails to reject null-hypothesis, it is reasonable to conclude that the test is comparable to the reference. We suggest that if the entire 95% confidence interval around the difference falls within the range -15% to +15%, it is plausible to conclude equivalence of two treatments. With 17-20 animals per group, the experiment provides >80% power to even formally demonstrate equivalence under the above conditions.

### 5.2. Non-critical size defect

The same approach with repeated assessment *in vivo* can be applied to the non-critical size defect model. We suggest that animals should be scanned immediately after the fracture – if non-zero values are present and an adjustment for baseline should be considered. Since the

defect heals spontaneously, assessment very early or late in the process are biased towards “no difference”. It could also be difficult to distinguish between different active treatments. We suggest that a test treatment (T) should be considered effective if it yields by at least 30% higher healing “scores” around mid-time of the spontaneous process as compared to an inactive control (Ctr). Figure 3C depicts an experiment in which several active and “disruptive” treatments were assessed along with the inactive control at the single time-point late in the healing process (*ex vivo* rat bone,  $\mu$ CT). Data analysis (GLM) requires adjustment for pairwise comparisons. Figure 3D depicts a hypothetical experiment in which a treatment is evaluated against an inactive control *ex vivo* at two time points around the mid-time of the process. It comprises one between-group factor with four levels (2 treatments x 2 time points) and should be analysed in a GLM with adjustments for two post-hoc comparisons of interest (T-1 vs. Ctr-1; T-2 vs. Ctr-2). With group RSD of 20%, 10 animals per group are needed (40 total) to detect a 30 % difference between T and Ctr at any of the two assessments.



**Figure 3. A.** X-ray of critical size defects of rabbit ulnae treated with a BMP6 in the modified whole blood coagulum (WBCD) and commercial BMP7 device and assessed at 0, 2, 4, 6 and 8 weeks after surgery. Tabulated data show corresponding BV/TV ratios obtained by  $\mu$ CT [233]. **B.** Graphical representation of BV/TV% over time (mean $\pm$ SD; n=8). A GLMM fitted to ln-transformed data to determine time-averaged difference (weeks 2-8) indicated around 4,6 times greater response with BMP6. A GLMM fitted to ln-transformed data to determine the time-averaged difference (weeks 2-8) indicate around 4,6 times greater response with BMP6. A GLM fitted to ln-transformed AUC of BV/TV% difference at weeks 2-8 vs. time 0 indicated around 2,9 times greater response (tabulated GMR). The insert shows a quadratic fit with 95%CI of prediction indicating that the effects of two treatments were not likely to “meet” even after 10+ weeks post-surgery. The intrapolated grey line indicates a hypothetical treatment not relevantly different vs. the approved one (<15% difference). **C.**  $\mu$ CT imaging of rat femurs ex vivo at 6 weeks after osteotomy in the proximal third of the femur. The rats were randomly assigned to one of the following groups: control; BMP1-3 antibody i.v. (50  $\mu$ g, 1x/wk); BMP1-3 i.v. (3  $\mu$ g, 3x7wk); BMP6 i.v. (250  $\mu$ g/kg, 2x/wk) and BMP1-3 (3  $\mu$ g, 3x/wk) +BMP6 i.v. (250  $\mu$ g, 2x/wk) therapy (mean $\pm$ SD, n=6) [234]. **D.** Outline of a hypothetical experiment in which tested treatment (T) and a control (Ctr) are assessed ex-vivo at two time points (1, 2) around midtime of the spontaneous healing process. Data are independent, i.e., there are four groups of animals (2 treatments x 2 time points). GLMM - general linear mixed models; GMR - geometric mean ratios; AUC - area under the curve; BV - bone volume; TV - tissue volume; GLM - general linear model.

## 6. Suggested guidelines for animal use in bone repair experimentation

To enable the collection of reliable data regarding the efficacy and safety of tested substances, animal models and methods should be carefully selected and combined to guide clinical studies so that the much-needed treatments aimed to facilitate the tissue regeneration process will ultimately reach the patient. Below are suggested guiding principles for conducting animal studies in bone repair scenarios.

1. The skeletal characteristics of each species must be considered when judging the translation of preclinical data to the human clinical situation. The rat model, despite its' limitations, remains the most informative small animal model and should be the first choice to initiate *in vivo* assessment. If studies in large animals are planned, the best option is to combine efficacy and safety study in the same species. Although NHPs are the best choice due to the high similarity to human outcomes, dogs or pigs are valid alternatives. To bridge the gap between rats and large animals, the use of "intermediary" models (marmoset or rabbit) could be considered with a caveat that these models may not provide sufficient new information to better guide studies in large animals.
2. The dosing and sample size must be based on previous *in vitro* studies as well as the healing biology of a specified indication and healing time point analyses, respectively. 3R principles should always be used in designing experimental protocols.
3. Efficacy testing in animal models should mimic the intended clinical indication.
4. Appropriate methods and markers must be chosen for the assessment of bone healing process as suggested in Table 1. Radiologic techniques in combination with histology, bone mechanics and serum and urine biomarkers are recommended.
5. The scanning of animals prior to recruitment will enable each animal to serve as its own control and ensure screening for potential fractures or malformations, establishing growth plate status and skeletal maturity to avoid biological variation errors.
6. A bone healing assessment plan should be carefully designed while bearing in mind the callus formation time course and the points of biomechanical bone restoration in different animal species. Primary and secondary end points of the study and scoring system should be carefully considered and specified in advance.
7. The chosen model, surgical and therapeutical procedures, variability, sample size and percent of expected outcomes should be supported by detailed and justified pre-experimental statistical analysis.
8. The safety and tolerability of the therapy should be monitored during the study and synchronized with regulatory documents whenever possible.
9. Isolated MPCs should be characterized by phenotype, gene expression profile and functional testing prior the grafting procedure. Markers for labelling the target osteoprogenitor population and lineage tracing approach are particularly useful for the *in vivo* tracking of the transplant. Cell-based engineered constructs could be harvested

at different time-points post-surgery and analyzed *ex vivo* to evaluate the viability, proliferation and differentiation of the transplanted cells.

10. Cell based therapies in combination with biomolecules and biomaterials require additional evaluation particularly regarding osteogenic differentiation of transplanted cells and graft-host integration. Moreover, construct vascularization, scaffold biodegradation and transplant-host integration are important predictors of skeletal tissue regeneration. Local and systemic immune reactions should also be monitored particularly in the case of allogeneic or xenogeneic grafts.

### **Acknowledgements**

The research leading to some of these results has received funding from the European Community's Seventh Framework Programme [FP7/2007-2013] under grant agreement n°HEALTH-F4-2011-279239.



## Reference List

- [1] E. Hernlund, A. Svedbom, M. Ivergard, J. Compston, C. Cooper, J. Stenmark, E.V. McCloskey, B. Jonsson, and J.A. Kanis, Osteoporosis in the European Union: medical management, epidemiology and economic burden. A report prepared in collaboration with the International Osteoporosis Foundation (IOF) and the European Federation of Pharmaceutical Industry Associations (EFPIA), *Arch. Osteoporos.* 8 (2013) 136.
- [2] A. Svedbom, E. Hernlund, M. Ivergard, J. Compston, C. Cooper, J. Stenmark, E.V. McCloskey, B. Jonsson, and J.A. Kanis, Osteoporosis in the European Union: a compendium of country-specific reports, *Arch. Osteoporos.* 8 (2013) 137.
- [3] I.H. Kalfas, Principles of bone healing, *Neurosurg. Focus.* 10 (2001) E1.
- [4] A. Keating, Mesenchymal stromal cells, *Curr. Opin. Hematol.* 13 (2006) 419-425.
- [5] Y. Homma, G. Zimmermann, and P. Hernigou, Cellular therapies for the treatment of non-union: the past, present and future, *Injury* 44 Suppl 1 (2013) S46-S49.
- [6] N. Rozen, T. Bick, A. Bajayo, B. Shamian, M. Schrift-Tzadok, Y. Gabet, A. Yayon, I. Bab, M. Soudry, and D. Lewinson, Transplanted blood-derived endothelial progenitor cells (EPC) enhance bridging of sheep tibia critical size defects, *Bone* 45 (2009) 918-924.
- [7] S. Khosla, J.J. Westendorf, and U.I. Modder, Concise review: Insights from normal bone remodeling and stem cell-based therapies for bone repair, *Stem Cells* 28 (2010) 2124-2128.
- [8] K. Schmidt-Bleek, H. Schell, N. Schulz, P. Hoff, C. Perka, F. Buttgereit, H.D. Volk, J. Lienau, and G.N. Duda, Inflammatory phase of bone healing initiates the regenerative healing cascade, *Cell Tissue Res.* 347 (2012) 567-573.
- [9] H.C. Fayaz, P.V. Giannoudis, M.S. Vrahas, R.M. Smith, C. Moran, H.C. Pape, C. Krettek, and J.B. Jupiter, The role of stem cells in fracture healing and nonunion, *Int. Orthop.* 35 (2011) 1587-1597.
- [10] J.E. Schroeder and R. Mosheiff, Tissue engineering approaches for bone repair: concepts and evidence, *Injury* 42 (2011) 609-613.
- [11] A.S. Shekkeris, P.K. Jaiswal, and W.S. Khan, Clinical applications of mesenchymal stem cells in the treatment of fracture non-union and bone defects, *Curr. Stem Cell Res. Ther.* 7 (2012) 127-133.
- [12] S.D. Cook, G.C. Baffes, M.W. Wolfe, T.K. Sampath, and D.C. Rueger, Recombinant human bone morphogenetic protein-7 induces healing in a canine long-bone segmental defect model, *Clin. Orthop. Relat Res.* (1994) 302-312.
- [13] V.M. Paralkar, F. Borovecki, H.Z. Ke, K.O. Cameron, B. Lefker, W.A. Grasser, T.A. Owen, M. Li, P. DaSilva-Jardine, M. Zhou, R.L. Dunn, F. Dumont, R. Korsmeyer, P. Krasney, T.A. Brown, D. Plowchalk, S. Vukicevic, and D.D. Thompson, An EP2 receptor-selective prostaglandin E2 agonist induces bone healing, *Proc. Natl. Acad. Sci. U. S. A* 100 (2003) 6736-6740.
- [14] R. Dimitriou, E. Tsiridis, and P.V. Giannoudis, Current concepts of molecular aspects of bone healing, *Injury* 36 (2005) 1392-1404.
- [15] T.N. Vo, F.K. Kasper, and A.G. Mikos, Strategies for controlled delivery of growth factors and cells for bone regeneration, *Adv. Drug Deliv. Rev.* 64 (2012) 1292-1309.
- [16] S. Vukicevic and T.K. Sampath, *Bone Morphogenetic Proteins: From Laboratory to Clinical Practice*, Birkhauser Verlag Basel-Boston-Berlin, 2002.

- [17] S. Vukicevic, H. Oppermann, D. Verbanac, M. Jankolija, I. Popek, J. Curak, J. Brkljacic, M. Pauk, I. Erjavec, I. Francetic, I. Dumic-Cule, M. Jelic, D. Durdevic, T. Vlahovic, R. Novak, V. Kufner, N.T. Bordukalo, M. Kozlovic, T.Z. Banic, J. Bubic-Spoljar, I. Bastalic, S. Vikić-Topic, M. Peric, M. Pecina, and L. Grgurevic, The clinical use of bone morphogenetic proteins revisited: a novel biocompatible carrier device OSTEOGROW for bone healing, *Int. Orthop.* 38 (2014) 635-647.
- [18] J.M. Kanczler and R.O. Oreffo, Osteogenesis and angiogenesis: the potential for engineering bone, *Eur. Cell Mater.* 15 (2008) 100-114.
- [19] H.P. Gerber, T.H. Vu, A.M. Ryan, J. Kowalski, Z. Werb, and N. Ferrara, VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation, *Nat. Med.* 5 (1999) 623-628.
- [20] H. Eckardt, M. Ding, M. Lind, E.S. Hansen, K.S. Christensen, and I. Hvid, Recombinant human vascular endothelial growth factor enhances bone healing in an experimental nonunion model, *J. Bone Joint Surg. Br.* 87 (2005) 1434-1438.
- [21] J.K. Leach, D. Kaigler, Z. Wang, P.H. Krebsbach, and D.J. Mooney, Coating of VEGF-releasing scaffolds with bioactive glass for angiogenesis and bone regeneration, *Biomaterials* 27 (2006) 3249-3255.
- [22] B.Y. Ozturk, I. Inci, S. Egri, A.M. Ozturk, H. Yetkin, G. Goktas, C. Elmas, E. Piskin, and D. Erdogan, The treatment of segmental bone defects in rabbit tibiae with vascular endothelial growth factor (VEGF)-loaded gelatin/hydroxyapatite "cryogel" scaffold, *Eur. J. Orthop. Surg. Traumatol.* 23 (2013) 767-774.
- [23] G.E. Friedlaender, S. Lin, L.A. Solchaga, L.B. Snel, and S.E. Lynch, The role of recombinant human platelet-derived growth factor-BB (rhPDGF-BB) in orthopaedic bone repair and regeneration, *Curr. Pharm. Des* 19 (2013) 3384-3390.
- [24] Y. Fei, G. Gronowicz, and M.M. Hurley, Fibroblast growth factor-2, bone homeostasis and fracture repair, *Curr. Pharm. Des* 19 (2013) 3354-3363.
- [25] P.M. Mountziaris, P.P. Spicer, F.K. Kasper, and A.G. Mikos, Harnessing and modulating inflammation in strategies for bone regeneration, *Tissue Eng Part B Rev.* 17 (2011) 393-402.
- [26] P.M. Mountziaris and A.G. Mikos, Modulation of the inflammatory response for enhanced bone tissue regeneration, *Tissue Eng Part B Rev.* 14 (2008) 179-186.
- [27] D.H. Kempen, L. Lu, T.E. Hefferan, L.B. Creemers, A. Heijink, A. Maran, W.J. Dhert, and M.J. Yaszemski, Enhanced bone morphogenetic protein-2-induced ectopic and orthotopic bone formation by intermittent parathyroid hormone (1-34) administration, *Tissue Eng Part A* 16 (2010) 3769-3777.
- [28] R.M. Neer, C.D. Arnaud, J.R. Zanchetta, R. Prince, G.A. Gaich, J.Y. Reginster, A.B. Hodsmann, E.F. Eriksen, S. Ish-Shalom, H.K. Genant, O. Wang, and B.H. Mitlak, Effect of parathyroid hormone (1-34) on fractures and bone mineral density in postmenopausal women with osteoporosis, *N. Engl. J. Med.* 344 (2001) 1434-1441.
- [29] J.D. Almeida, E.A. Arisawa, R.F. da Rocha, and Y.R. Carvalho, Effect of calcitonin on bone regeneration in male rats: a histomorphometric analysis, *Int. J. Oral Maxillofac. Surg.* 36 (2007) 435-440.
- [30] M. Bosetti, F. Boccafoschi, M. Leigheb, and M.F. Cannas, Effect of different growth factors on human osteoblasts activities: a possible application in bone regeneration for tissue engineering, *Biomol. Eng* 24 (2007) 613-618.

- [31] W. Zhang, C. Zhu, Y. Wu, D. Ye, S. Wang, D. Zou, X. Zhang, D.L. Kaplan, and X. Jiang, VEGF and BMP-2 promote bone regeneration by facilitating bone marrow stem cell homing and differentiation, *Eur. Cell Mater.* 27 (2014) 1-12.
- [32] M. Ramazanoglu, R. Lutz, P. Rusche, L. Trabzon, G.T. Kose, C. Prechtel, and K.A. Schlegel, Bone response to biomimetic implants delivering BMP-2 and VEGF: an immunohistochemical study, *J. Craniomaxillofac. Surg.* 41 (2013) 826-835.
- [33] A. Hernandez, R. Reyes, E. Sanchez, M. Rodriguez-Evora, A. Delgado, and C. Evora, In vivo osteogenic response to different ratios of BMP-2 and VEGF released from a biodegradable porous system, *J. Biomed. Mater. Res. A* 100 (2012) 2382-2391.
- [34] R. Reyes, B. De la Riva, A. Delgado, A. Hernandez, E. Sanchez, and C. Evora, Effect of triple growth factor controlled delivery by a brushite-PLGA system on a bone defect, *Injury* 43 (2012) 334-342.
- [35] Q. Cui, A.S. Dighe, and J.N. Irvine, Jr., Combined angiogenic and osteogenic factor delivery for bone regenerative engineering, *Curr. Pharm. Des* 19 (2013) 3374-3383.
- [36] B. Behr, M. Sorkin, M. Lehnhardt, A. Renda, M.T. Longaker, and N. Quarto, A comparative analysis of the osteogenic effects of BMP-2, FGF-2, and VEGFA in a calvarial defect model, *Tissue Eng Part A* 18 (2012) 1079-1086.
- [37] E.F. Morgan, Z.D. Mason, G. Bishop, A.D. Davis, N.A. Wigner, L.C. Gerstenfeld, and T.A. Einhorn, Combined effects of recombinant human BMP-7 (rhBMP-7) and parathyroid hormone (1-34) in metaphyseal bone healing, *Bone* 43 (2008) 1031-1038.
- [38] A.J. Friedenstein, K.V. Petrakova, A.I. Kurolesova, and G.P. Frolova, Heterotopic of bone marrow. Analysis of precursor cells for osteogenic and hematopoietic tissues, *Transplantation* 6 (1968) 230-247.
- [39] B.C. De, F. Dell'Accio, J. Vanlauwe, J. Eyckmans, I.M. Khan, C.W. Archer, E.A. Jones, D. McGonagle, T.A. Mitsiadis, C. Pitzalis, and F.P. Luyten, Mesenchymal multipotency of adult human periosteal cells demonstrated by single-cell lineage analysis, *Arthritis Rheum.* 54 (2006) 1209-1221.
- [40] D. Grcevic, S. Pejda, B.G. Matthews, D. Repic, L. Wang, H. Li, M.S. Kronenberg, X. Jiang, P. Maye, D.J. Adams, D.W. Rowe, H.L. Aguila, and I. Kalajzic, In vivo fate mapping identifies mesenchymal progenitor cells, *Stem Cells* 30 (2012) 187-196.
- [41] B.G. Matthews, D. Grcevic, L. Wang, Y. Hagiwara, H. Roguljic, P. Joshi, D.G. Shin, D.J. Adams, and I. Kalajzic, Analysis of alphaSMA-Labeled Progenitor Cell Commitment Identifies Notch Signaling as an Important Pathway in Fracture Healing, *J. Bone Miner. Res.* (2013).
- [42] D. Menicanin, K.M. Mrozik, N. Wada, V. Marino, S. Shi, P.M. Bartold, and S. Gronthos, Periodontal-Ligament-Derived Stem Cells Exhibit the Capacity for Long-Term Survival, Self-Renewal, and Regeneration of Multiple Tissue Types in Vivo, *Stem Cells Dev.* (2014).
- [43] P. Niemeyer, K. Fechner, S. Milz, W. Richter, N.P. Suedkamp, A.T. Mehlhorn, S. Pearce, and P. Kasten, Comparison of mesenchymal stem cells from bone marrow and adipose tissue for bone regeneration in a critical size defect of the sheep tibia and the influence of platelet-rich plasma, *Biomaterials* 31 (2010) 3572-3579.
- [44] H. Roguljic, B.G. Matthews, W. Yang, H. Cvija, M. Mina, and I. Kalajzic, In vivo identification of periodontal progenitor cells, *J. Dent. Res.* 92 (2013) 709-715.
- [45] C.H. Evans, F.J. Liu, V. Glatt, J.A. Hoyland, C. Kirker-Head, A. Walsh, O. Betz, J.W. Wells, V. Betz, R.M. Porter, F.A. Saad, L.C. Gerstenfeld, T.A. Einhorn, M.B. Harris, and

- M.S. Vrahas, Use of genetically modified muscle and fat grafts to repair defects in bone and cartilage, *Eur. Cell Mater.* 18 (2009) 96-111.
- [46] V.B. Fernandez Vallone, M.A. Romaniuk, H. Choi, V. Labovsky, J. Otaegui, and N.A. Chasseing, Mesenchymal stem cells and their use in therapy: what has been achieved?, *Differentiation* 85 (2013) 1-10.
- [47] P. Bianco, P.G. Robey, I. Saggio, and M. Riminucci, "Mesenchymal" stem cells in human bone marrow (skeletal stem cells): a critical discussion of their nature, identity, and significance in incurable skeletal disease, *Hum. Gene Ther.* 21 (2010) 1057-1066.
- [48] A. Harichandan and H.J. Buhning, Prospective isolation of human MSC, *Best. Pract. Res. Clin. Haematol.* 24 (2011) 25-36.
- [49] B. Sacchetti, A. Funari, S. Michienzi, C.S. Di, S. Piersanti, I. Saggio, E. Tagliafico, S. Ferrari, P.G. Robey, M. Riminucci, and P. Bianco, Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment, *Cell* 131 (2007) 324-336.
- [50] M.F. Pittenger, A.M. Mackay, S.C. Beck, R.K. Jaiswal, R. Douglas, J.D. Mosca, M.A. Moorman, D.W. Simonetti, S. Craig, and D.R. Marshak, Multilineage potential of adult human mesenchymal stem cells, *Science* 284 (1999) 143-147.
- [51] A.I. Caplan, New era of cell-based orthopedic therapies, *Tissue Eng Part B Rev.* 15 (2009) 195-200.
- [52] K. Lee, C.K. Chan, N. Patil, and S.B. Goodman, Cell therapy for bone regeneration--bench to bedside, *J. Biomed. Mater. Res. B Appl. Biomater.* 89 (2009) 252-263.
- [53] R.C. McCarty, S. Gronthos, A.C. Zannettino, B.K. Foster, and C.J. Xian, Characterisation and developmental potential of ovine bone marrow derived mesenchymal stem cells, *J. Cell Physiol* 219 (2009) 324-333.
- [54] E. Gomez-Barrena, P. Rosset, I. Muller, R. Giordano, C. Bunu, P. Layrolle, Y.T. Konttinen, and F.P. Luyten, Bone regeneration: stem cell therapies and clinical studies in orthopaedics and traumatology, *J. Cell Mol. Med.* 15 (2011) 1266-1286.
- [55] U.A. Stock and J.P. Vacanti, Tissue engineering: current state and prospects, *Annu. Rev. Med.* 52 (2001) 443-451.
- [56] E.J. Harvey, J.E. Henderson, and S.T. Vengallatore, Nanotechnology and bone healing, *J. Orthop. Trauma* 24 Suppl 1 (2010) S25-S30.
- [57] A. Arthur, A. Zannettino, and S. Gronthos, The therapeutic applications of multipotential mesenchymal/stromal stem cells in skeletal tissue repair, *J. Cell Physiol* 218 (2009) 237-245.
- [58] Notices from European Union Institutions Bodies Offices and Agencies, Note for guidance on minimising the risk of transmitting animal spongiform encephalopathy agents via human and veterinary medicinal products (EMA/410/01 rev.3), *Official Journal of the European Union* 2011/C 73/01 (2011).
- [59] M. Pilia, T. Guda, and M. Appleford, Development of composite scaffolds for load-bearing segmental bone defects, *Biomed. Res. Int.* 2013 (2013) 458253.
- [60] U. Ripamonti, The induction of bone in osteogenic composites of bone matrix and porous hydroxyapatite replicas: an experimental study on the baboon (*Papio ursinus*), *J. Oral Maxillofac. Surg.* 49 (1991) 817-830.
- [61] U. Ripamonti, Osteoinduction in porous hydroxyapatite implanted in heterotopic sites of different animal models, *Biomaterials* 17 (1996) 31-35.

- [62] P. Habibovic, C.M. van der Valk, C.A. van Blitterswijk, G.K. de, and G. Meijer, Influence of octacalcium phosphate coating on osteoinductive properties of biomaterials, *J. Mater. Sci. Mater. Med.* 15 (2004) 373-380.
- [63] P. Habibovic, U. Gbureck, C.J. Doillon, D.C. Bassett, C.A. van Blitterswijk, and J.E. Barralet, Osteoconduction and osteoinduction of low-temperature 3D printed bioceramic implants, *Biomaterials* 29 (2008) 944-953.
- [64] A.K. Gosain, L. Song, P. Riordan, M.T. Amarante, P.G. Nagy, C.R. Wilson, J.M. Toth, and J.L. Ricci, A 1-year study of osteoinduction in hydroxyapatite-derived biomaterials in an adult sheep model: part I, *Plast. Reconstr. Surg.* 109 (2002) 619-630.
- [65] A.K. Gosain, P.A. Riordan, L. Song, M.T. Amarante, B. Kalantarian, P.G. Nagy, C.R. Wilson, J.M. Toth, and B.L. McIntyre, A 1-year study of osteoinduction in hydroxyapatite-derived biomaterials in an adult sheep model: part II. Bioengineering implants to optimize bone replacement in reconstruction of cranial defects, *Plast. Reconstr. Surg.* 114 (2004) 1155-1163.
- [66] F. Barrere, C.M. van der Valk, R.A. Dalmeijer, G. Meijer, C.A. van Blitterswijk, G.K. de, and P. Layrolle, Osteogenicity of octacalcium phosphate coatings applied on porous metal implants, *J. Biomed. Mater. Res. A* 66 (2003) 779-788.
- [67] H. Cao and N. Kuboyama, A biodegradable porous composite scaffold of PGA/beta-TCP for bone tissue engineering, *Bone* 46 (2010) 386-395.
- [68] T.M. Chu, S.J. Warden, C.H. Turner, and R.L. Stewart, Segmental bone regeneration using a load-bearing biodegradable carrier of bone morphogenetic protein-2, *Biomaterials* 28 (2007) 459-467.
- [69] F. Jegoux, E. Goyenvalle, R. Cognet, O. Malard, F. Moreau, G. Daculsi, and E. Aguado, Reconstruction of irradiated bone segmental defects with a biomaterial associating MBCP+(R), microstructured collagen membrane and total bone marrow grafting: an experimental study in rabbits, *J. Biomed. Mater. Res. A* 91 (2009) 1160-1169.
- [70] D. Lickorish, L. Guan, and J.E. Davies, A three-phase, fully resorbable, polyester/calcium phosphate scaffold for bone tissue engineering: Evolution of scaffold design, *Biomaterials* 28 (2007) 1495-1502.
- [71] A.A. Ignatius, O. Betz, P. Augat, and L.E. Claes, In vivo investigations on composites made of resorbable ceramics and poly(lactide) used as bone graft substitutes, *J. Biomed. Mater. Res.* 58 (2001) 701-709.
- [72] C. Xu, P. Su, X. Chen, Y. Meng, W. Yu, A.P. Xiang, and Y. Wang, Biocompatibility and osteogenesis of biomimetic Bioglass-Collagen-Phosphatidylserine composite scaffolds for bone tissue engineering, *Biomaterials* 32 (2011) 1051-1058.
- [73] A.R. Amini, C.T. Laurencin, and S.P. Nukavarapu, Bone tissue engineering: recent advances and challenges, *Crit Rev. Biomed. Eng* 40 (2012) 363-408.
- [74] K.Y. Lee and D.J. Mooney, Hydrogels for tissue engineering, *Chem. Rev.* 101 (2001) 1869-1879.
- [75] B.V. Slaughter, S.S. Khurshid, O.Z. Fisher, A. Khademhosseini, and N.A. Peppas, Hydrogels in regenerative medicine, *Adv. Mater.* 21 (2009) 3307-3329.
- [76] J.B. Park, The use of hydrogels in bone-tissue engineering, *Med. Oral Patol. Oral Cir. Bucal.* 16 (2011) e115-e118.
- [77] A.R. Amini, D.J. Adams, C.T. Laurencin, and S.P. Nukavarapu, Optimally porous and biomechanically compatible scaffolds for large-area bone regeneration, *Tissue Eng Part A* 18 (2012) 1376-1388.

- [78] Institute for laboratory Animal Research, Guide for the care and use of laboratory animals, National Academic Press, Washington, DC, 1996.
- [79] B. Howard, T. Nevalainen, and G. Perretta, The COST Manual of Laboratory Animal Care and Use – Refinement, Reduction, and Research. 2010. CRC Press .
- [80] Animal research: a balancing act, *Nat Med* 19 (2013) 1191.
- [81] W.M.S. Russell and R.L. Burch, The principles of humane experimental technique , Methuen & Co. Ltd, London, 1954.
- [82] C. Ferretti, U. Ripamonti, E. Tsiroidis, C.J. Kerawala, A. Mantalaris, and M. Heliotis, Osteoinduction: translating preclinical promise into clinical reality, *Br. J. Oral Maxillofac. Surg.* 48 (2010) 536-539.
- [83] R. Fu, S. Selph, M. McDonagh, K. Peterson, A. Tiwari, R. Chou, and M. Helfand, Effectiveness and harms of recombinant human bone morphogenetic protein-2 in spine fusion: a systematic review and meta-analysis, *Ann. Intern. Med.* 158 (2013) 890-902.
- [84] M.C. Simmonds, J.V. Brown, M.K. Heirs, J.P. Higgins, R.J. Mannion, M.A. Rodgers, and L.A. Stewart, Safety and effectiveness of recombinant human bone morphogenetic protein-2 for spinal fusion: a meta-analysis of individual-participant data, *Ann. Intern. Med.* 158 (2013) 877-889.
- [85] D.D. Thompson, H.A. Simmons, C.M. Pirie, and H.Z. Ke, FDA Guidelines and animal models for osteoporosis, *Bone* 17 (1995) 125S-133S.
- [86] W.S. Jee and W. Yao, Overview: animal models of osteopenia and osteoporosis, *J. Musculoskelet. Neuronal. Interact.* 1 (2001) 193-207.
- [87] T.J. Wronski, M. Cintron, and L.M. Dann, Temporal relationship between bone loss and increased bone turnover in ovariectomized rats, *Calcif. Tissue Int.* 43 (1988) 179-183.
- [88] M. Cesnjaj, A. Stavljenic, and S. Vukicevic, In vivo models in the study of osteopenias, *Eur. J. Clin. Chem. Clin. Biochem.* 29 (1991) 211-219.
- [89] E. Bonucci and P. Ballanti, Osteoporosis--Bone Remodeling and Animal Models, *Toxicol. Pathol.* (2013).
- [90] V. Rinotas, A. Niti, R. Dacquin, N. Bonnet, M. Stolina, C.Y. Han, P. Kostenuik, P. Jurdic, S. Ferrari, and E. Douni, Novel genetic models of osteoporosis by overexpression of human RANKL in transgenic mice, *J. Bone Miner. Res.* (2013).
- [91] K.M. Melville, N.H. Kelly, S.A. Khan, J.C. Schimenti, F.P. Ross, R.P. Main, and M.C. van der Meulen, Female mice lacking estrogen receptor-alpha in osteoblasts have compromised bone mass and strength, *J. Bone Miner. Res.* 29 (2014) 370-379.
- [92] B. Cortet, Bone repair in osteoporotic bone: postmenopausal and cortisone-induced osteoporosis, *Osteoporos. Int.* 22 (2011) 2007-2010.
- [93] P. Govindarajan, T. Khassawna, M. Kampschulte, W. Bocker, B. Huerter, L. Durselen, M. Faulenbach, and C. Heiss, Implications of combined ovariectomy and glucocorticoid (dexamethasone) treatment on mineral, microarchitectural, biomechanical and matrix properties of rat bone, *Int. J. Exp. Pathol.* 94 (2013) 387-398.
- [94] K.J. Zhang, J. Zhang, Z.K. Kang, X.M. Xue, J.F. Kang, Y.W. Li, H.N. Dong, and D.G. Liu, Ibandronate for prevention and treatment of glucocorticoid-induced osteoporosis in rabbits, *Rheumatol. Int.* 32 (2012) 3405-3411.

- [95] L. Baofeng, Y. Zhi, C. Bei, M. Guolin, Y. Qingshui, and L. Jian, Characterization of a rabbit osteoporosis model induced by ovariectomy and glucocorticoid, *Acta Orthop.* 81 (2010) 396-401.
- [96] M. Ding, L. Cheng, P. Bollen, P. Schwarz, and S. Overgaard, Glucocorticoid induced osteopenia in cancellous bone of sheep: validation of large animal model for spine fusion and biomaterial research, *Spine (Phila Pa 1976. )* 35 (2010) 363-370.
- [97] T.J. Rosol, S.H. Tannehill-Gregg, B.E. LeRoy, S. Mandl, and C.H. Contag, Animal models of bone metastasis, *Cancer* 97 (2003) 748-757.
- [98] X.J. Li, W.S. Jee, S.Y. Chow, and D.M. Woodbury, Adaptation of cancellous bone to aging and immobilization in the rat: a single photon absorptiometry and histomorphometry study, *Anat. Rec.* 227 (1990) 12-24.
- [99] X. Tian, W.S. Jee, X. Li, C. Paszty, and H.Z. Ke, Sclerostin antibody increases bone mass by stimulating bone formation and inhibiting bone resorption in a hindlimb-immobilization rat model, *Bone* 48 (2011) 197-201.
- [100] P.S. Gomes and M.H. Fernandes, Rodent models in bone-related research: the relevance of calvarial defects in the assessment of bone regeneration strategies, *Lab Anim* 45 (2011) 14-24.
- [101] L.A. Mills and A.H. Simpson, In vivo models of bone repair, *J. Bone Joint Surg. Br.* 94 (2012) 865-874.
- [102] E.A. Horner, J. Kirkham, D. Wood, S. Curran, M. Smith, B. Thomson, and X.B. Yang, Long bone defect models for tissue engineering applications: criteria for choice, *Tissue Eng Part B Rev.* 16 (2010) 263-271.
- [103] J. Reifenrath, N. Angrisani, M. Lalk, and S. Besdo, Replacement, refinement, and reduction: Necessity of standardization and computational models for long bone fracture repair in animals, *J. Biomed. Mater. Res. A* (2013).
- [104] C.M. Bagi, N. Hanson, C. Andresen, R. Pero, R. Lariviere, C.H. Turner, and A. Laib, The use of micro-CT to evaluate cortical bone geometry and strength in nude rats: correlation with mechanical testing, pQCT and DXA, *Bone* 38 (2006) 136-144.
- [105] F. Tortelli, R. Tasso, F. Loiacono, and R. Cancedda, The development of tissue-engineered bone of different origin through endochondral and intramembranous ossification following the implantation of mesenchymal stem cells and osteoblasts in a murine model, *Biomaterials* 31 (2010) 242-249.
- [106] J. Eyckmans and F.P. Luyten, Species specificity of ectopic bone formation using periosteum-derived mesenchymal progenitor cells, *Tissue Eng* 12 (2006) 2203-2213.
- [107] W.S. Jee and W. Yao, Overview: animal models of osteopenia and osteoporosis, *J. Musculoskelet. Neuronal. Interact.* 1 (2001) 193-207.
- [108] W.S. Jee and Y. Ma, Animal models of immobilization osteopenia, *Morphologie.* 83 (1999) 25-34.
- [109] J.A. Auer, A. Goodship, S. Arnoczky, S. Pearce, J. Price, L. Claes, R.B. von, M. Hofmann-Antenbrinck, E. Schneider, R. Muller-Terpitz, F. Thiele, K.P. Rippe, and D.W. Grainger, Refining animal models in fracture research: seeking consensus in optimising both animal welfare and scientific validity for appropriate biomedical use, *BMC. Musculoskelet. Disord.* 8 (2007) 72.
- [110] A.I. Pearce, R.G. Richards, S. Milz, E. Schneider, and S.G. Pearce, Animal models for implant biomaterial research in bone: a review, *Eur. Cell Mater.* 13 (2007) 1-10.

- [111] C. Vannabouathong, S. Sprague, and M. Bhandari, Guidelines for fracture healing assessments in clinical trials. Part I: definitions and endpoint committees, *Injury* 42 (2011) 314-316.
- [112] A.H. Reddi and C. Huggins, Biochemical sequences in the transformation of normal fibroblasts in adolescent rats, *Proc. Natl. Acad. Sci. U. S. A* 69 (1972) 1601-1605.
- [113] T.M. Van Eijden, Biomechanics of the mandible, *Crit Rev. Oral Biol. Med.* 11 (2000) 123-136.
- [114] M.W. Bidez and C.E. Misch, Issues in bone mechanics related to oral implants, *Implant. Dent.* 1 (1992) 289-294.
- [115] R.J. Bergman, D. Gazit, A.J. Kahn, H. Gruber, S. McDougall, and T.J. Hahn, Age-related changes in osteogenic stem cells in mice, *J. Bone Miner. Res.* 11 (1996) 568-577.
- [116] B.M. Bowman and S.C. Miller, Skeletal mass, chemistry, and growth during and after multiple reproductive cycles in the rat, *Bone* 25 (1999) 553-559.
- [117] B.M. Bowman, C.C. Siska, and S.C. Miller, Greatly increased cancellous bone formation with rapid improvements in bone structure in the rat maternal skeleton after lactation, *J. Bone Miner. Res.* 17 (2002) 1954-1960.
- [118] K.E. Naylor, P. Iqbal, C. Fledelius, R.B. Fraser, and R. Eastell, The effect of pregnancy on bone density and bone turnover, *J. Bone Miner. Res.* 15 (2000) 129-137.
- [119] E.G. Vajda, B.M. Bowman, and S.C. Miller, Cancellous and cortical bone mechanical properties and tissue dynamics during pregnancy, lactation, and postlactation in the rat, *Biol. Reprod.* 65 (2001) 689-695.
- [120] P.P. Lelovas, T.T. Xanthos, S.E. Thoma, G.P. Lyritis, and I.A. Dontas, The laboratory rat as an animal model for osteoporosis research, *Comp Med.* 58 (2008) 424-430.
- [121] I. Dumic-Cule, N. Draca, A.T. Luetic, D. Jezek, D. Rogic, L. Grgurevic, and S. Vukicevic, TSH Prevents Bone Resorption and with Calcitriol Synergistically Stimulates Bone Formation in Rats with Low Levels of Calcitropic Hormones, *Horm. Metab Res.* (2014) doi:10.1055/s-0033-1363989.
- [122] L. Martini, M. Fini, G. Giavaresi, and R. Giardino, Sheep model in orthopedic research: a literature review, *Comp Med.* 51 (2001) 292-299.
- [123] P.F. O'Loughlin, S. Morr, L. Bogunovic, A.D. Kim, B. Park, and J.M. Lane, Selection and development of preclinical models in fracture-healing research, *J. Bone Joint Surg. Am.* 90 Suppl 1 (2008) 79-84.
- [124] C.M. Bagi, E. Berryman, and M.R. Moalli, Comparative bone anatomy of commonly used laboratory animals: implications for drug discovery, *Comp Med.* 61 (2011) 76-85.
- [125] J.H. Schimandle and S.D. Boden, Spine update. The use of animal models to study spinal fusion, *Spine (Phila Pa 1976.)* 19 (1994) 1998-2006.
- [126] S.H. Kilborn, G. Trudel, and H. Uthoff, Review of growth plate closure compared with age at sexual maturity and lifespan in laboratory animals, *Contemp. Top. Lab Anim Sci.* 41 (2002) 21-26.
- [127] M.M. Swindle and A.C. Smith, Comparative anatomy and physiology of the pig, *Lab Anim Sci.* 25 (1998) 11-21.
- [128] H. Tsutsumi, K. Katagiri, S. Takeda, T. Nasu, S. Igarashi, M. Tanigawa, and K. Mamba, Standardized data and relationship between bone growth and bone metabolism in female Gottingen minipigs, *Exp. Anim* 53 (2004) 331-337.
- [129] J.M. Cheverud, Epiphyseal union and dental eruption *Macaca mulatta*, *Am. J. Phys. Anthropol.* 56 (1981) 157-167.



- [130] D.H. ENLOW, Functions of the Haversian system, *Am. J. Anat.* 110 (1962) 269-305.
- [131] H.M. Frost and W.S. Jee, On the rat model of human osteopenias and osteoporoses, *Bone Miner.* 18 (1992) 227-236.
- [132] D.N. Kalu, The ovariectomized rat model of postmenopausal bone loss, *Bone Miner.* 15 (1991) 175-191.
- [133] S.C. Miller, B.M. Bowman, and W.S. Jee, Available animal models of osteopenia--small and large, *Bone* 17 (1995) 1175-1235.
- [134] H.M. Frost, Human Haversian system measurements, *Henry. Ford. Hosp. Med. Bull.* 9 (1961) 145-147.
- [135] R.G. Erben, Trabecular and endocortical bone surfaces in the rat: modeling or remodeling?, *Anat. Rec.* 246 (1996) 39-46.
- [136] A. Vignery and R. Baron, Dynamic histomorphometry of alveolar bone remodeling in the adult rat, *Anat. Rec.* 196 (1980) 191-200.
- [137] J. Tibbitts, J.A. Cavagnaro, C.A. Haller, B. Marafino, P.A. Andrews, and J.T. Sullivan, Practical approaches to dose selection for first-in-human clinical trials with novel biopharmaceuticals, *Regul. Toxicol. Pharmacol.* 58 (2010) 243-251.
- [138] ICH harmonized tripartite guideline, Preclinical safety evaluation of biotechnology-derived pharmaceuticals, International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use, 2011.
- [139] J.G. Neyt, J.A. Buckwalter, and N.C. Carroll, Use of animal models in musculoskeletal research, *Iowa Orthop. J.* 18 (1998) 118-123.
- [140] X. Wang, J.D. Mabrey, and C.M. Agrawal, An interspecies comparison of bone fracture properties, *Biomed. Mater. Eng* 8 (1998) 1-9.
- [141] N. Han, P.X. Zhang, W.B. Wang, D.C. Han, J.H. Chen, H.B. Zhan, and B.G. Jiang, A new experimental model to study healing process of metaphyseal fracture, *Chin Med. J. (Engl. )* 125 (2012) 676-679.
- [142] I.S. Kim, T.H. Cho, Z.H. Lee, and S.J. Hwang, Bone regeneration by transplantation of human mesenchymal stromal cells in a rabbit mandibular distraction osteogenesis model, *Tissue Eng Part A* 19 (2013) 66-78.
- [143] Z.L. Tang, W.J. Zhang, D.X. Wang, J.M. Chen, H. Ma, and D.R. Wu, An Experimental Study Addressing the Promotion of Mandibular Defect Repair Through the Intermittent Subcutaneous Injection of Parathyroid Hormone, *J. Oral Maxillofac. Surg.* (2013).
- [144] H.P. Lim, A.E. Mercado-Pagan, K.D. Yun, S.S. Kang, T.H. Choi, J. Bishop, J.T. Koh, W. Maloney, K.M. Lee, Y.P. Yang, and S.W. Park, The effect of rhBMP-2 and PRP delivery by biodegradable beta-tricalcium phosphate scaffolds on new bone formation in a non-through rabbit cranial defect model, *J. Mater. Sci. Mater. Med.* 24 (2013) 1895-1903.
- [145] A.H. Hassanein, R.A. Couto, A. Nedder, E.R. Zielins, and A.K. Greene, Critical-size defect ossification: effect of leporid age in a cranioplasty model, *J. Craniofac. Surg.* 22 (2011) 2341-2343.
- [146] J.H. Roling and C. Bunger, Recommendations regarding the rabbit posterolateral spinal fusion model, *Journal of Orthopaedic Research* 31 (2013) 1860.
- [147] A.M. Riordan, R. Rangarajan, J.W. Balts, W.K. Hsu, and P.A. Anderson, Reliability of the rabbit postero-lateral spinal fusion model: A meta-analysis, *Journal of Orthopaedic Research* 31 (2013) 1261-1269.

- [148] T. Sato, H. Shimizu, M. Beppu, and M. Takagi, Effects on bone union and prevention of tendon adhesion by new porous anti-adhesive poly L-lactide-co-epsilon-caprolactone membrane in a rabbit model, *Hand Surg.* 18 (2013) 1-10.
- [149] S. Oshima, M. Ishikawa, Y. Mochizuki, T. Kobayashi, Y. Yasunaga, and M. Ochi, Enhancement of bone formation in an experimental bony defect using ferumoxide-labelled mesenchymal stromal cells and a magnetic targeting system, *J. Bone Joint Surg. Br.* 92 (2010) 1606-1613.
- [150] J.L. Kuhn, S.A. Goldstein, M.J. Ciarelli, and L.S. Matthews, The limitations of canine trabecular bone as a model for human: a biomechanical study, *J. Biomech.* 22 (1989) 95-107.
- [151] N. Hasiwa, J. Bailey, P. Clausing, M. Daneshian, M. Eileraas, S. Farkas, I. Gyertyan, R. Hubrecht, W. Kobel, G. Krummenacher, M. Leist, H. Lohi, A. Miklosi, F. Ohl, K. Olejniczak, G. Schmitt, P. Sinnett-Smith, D. Smith, K. Wagner, J.D. Yager, J. Zurlo, and T. Hartung, Critical evaluation of the use of dogs in biomedical research and testing in Europe, *ALTEX.* 28 (2011) 326-340.
- [152] J.C. Litten-Brown, A.M. Corson, and L. Clarke, Porcine models for the metabolic syndrome, digestive and bone disorders: a general overview, *Animal.* 4 (2010) 899-920.
- [153] L. Mosekilde, J. Kragstrup, and A. Richards, Compressive strength, ash weight, and volume of vertebral trabecular bone in experimental fluorosis in pigs, *Calcif. Tissue Int.* 40 (1987) 318-322.
- [154] D.M. Raab, T.D. Crenshaw, D.B. Kimmel, and E.L. Smith, A histomorphometric study of cortical bone activity during increased weight-bearing exercise, *J. Bone Miner. Res.* 6 (1991) 741-749.
- [155] J.C. Teo, K.M. Si-Hoe, J.E. Keh, and S.H. Teoh, Relationship between CT intensity, micro-architecture and mechanical properties of porcine vertebral cancellous bone, *Clin. Biomech. (Bristol. , Avon. )* 21 (2006) 235-244.
- [156] M.P. Walsh, C.A. Wijdicks, J.B. Parker, O. Hapa, and R.F. LaPrade, A comparison between a retrograde interference screw, suture button, and combined fixation on the tibial side in an all-inside anterior cruciate ligament reconstruction: a biomechanical study in a porcine model, *Am. J. Sports Med.* 37 (2009) 160-167.
- [157] R. Forster, G. Bode, L. Ellegaard, and J.W. van der Laan, The RETHINK project--minipigs as models for the toxicity testing of new medicines and chemicals: an impact assessment, *J. Pharmacol. Toxicol. Methods* 62 (2010) 158-159.
- [158] G.J. van Mierlo, N.H. Cnubben, C.F. Kuper, J. Wolthoorn, A.P. van Meeteren-Kreikamp, M.M. Nagtegaal, R. Doornbos, N.C. Ganderup, and A.H. Penninks, The Gottingen minipig(R) as an alternative non-rodent species for immunogenicity testing: a demonstrator study using the IL-1 receptor antagonist anakinra, *J. Immunotoxicol.* 10 (2013) 96-105.
- [159] G.J. van Mierlo, N.H. Cnubben, D. Wouters, G.J. Wolbink, M.H. Hart, T. Rispens, N.C. Ganderup, C.F. Kuper, L. Aarden, and A.H. Penninks, The minipig as an alternative non-rodent model for immunogenicity testing using the TNFalpha blockers adalimumab and infliximab, *J. Immunotoxicol.* 11 (2014) 62-71.
- [160] E. Anzenbacherova, P. Anzenbacher, Z. Svoboda, J. Ulrichova, J. Kvetina, J. Zoulova, F. Perlik, and J. Martinkova, Minipig as a model for drug metabolism in man: comparison of in vitro and in vivo metabolism of propafenone, *Biomed. Pap. Med. Fac. Univ Palacky. Olomouc. Czech. Repub.* 147 (2003) 155-159.

- [161] R.W. Boyce, D.C. Ebert, T.A. Youngs, C.L. Paddock, L. Mosekilde, M.L. Stevens, and H.J. Gundersen, Unbiased estimation of vertebral trabecular connectivity in calcium-restricted ovariectomized minipigs, *Bone* 16 (1995) 637-642.
- [162] S.W. Kim, K.S. Kim, C.D. Solis, M.S. Lee, and B.H. Hyun, Development of osteoporosis animal model using micropigs, *Lab Anim Res.* 29 (2013) 174-177.
- [163] L. Mosekilde, S.E. Weisbrode, J.A. Safron, H.F. Stills, M.L. Jankowsky, D.C. Ebert, C.C. Danielsen, C.H. Sogaard, A.F. Franks, M.L. Stevens, and ., Calcium-restricted ovariectomized Sinclair S-1 minipigs: an animal model of osteopenia and trabecular plate perforation, *Bone* 14 (1993) 379-382.
- [164] L. Mosekilde, S.E. Weisbrode, J.A. Safron, H.F. Stills, M.L. Jankowsky, D.C. Ebert, C.C. Danielsen, C.H. Sogaard, A.F. Franks, M.L. Stevens, and ., Evaluation of the skeletal effects of combined mild dietary calcium restriction and ovariectomy in Sinclair S-1 minipigs: a pilot study, *J. Bone Miner. Res.* 8 (1993) 1311-1321.
- [165] E. Newman, A.S. Turner, and J.D. Wark, The potential of sheep for the study of osteopenia: current status and comparison with other animal models, *Bone* 16 (1995) 277S-284S.
- [166] J.C. Reichert, A. Cipitria, D.R. Epari, S. Saifzadeh, P. Krishnakanth, A. Berner, M.A. Woodruff, H. Schell, M. Mehta, M.A. Schuetz, G.N. Duda, and D.W. Hutmacher, A tissue engineering solution for segmental defect regeneration in load-bearing long bones, *Sci. Transl. Med.* 4 (2012) 141ra93.
- [167] E. Kon, A. Muraglia, A. Corsi, P. Bianco, M. Marcacci, I. Martin, A. Boyde, I. Ruspantini, P. Chistolini, M. Rocca, R. Giardino, R. Cancedda, and R. Quarto, Autologous bone marrow stromal cells loaded onto porous hydroxyapatite ceramic accelerate bone repair in critical-size defects of sheep long bones, *J. Biomed. Mater. Res.* 49 (2000) 328-337.
- [168] K. Klaue, U. Knothe, C. Anton, D.H. Pfluger, M. Stoddart, A.C. Masquelet, and S.M. Perren, Bone regeneration in long-bone defects: tissue compartmentalisation? In vivo study on bone defects in sheep, *Injury* 40 Suppl 4 (2009) S95-102.
- [169] R. Cancedda, P. Giannoni, and M. Mastrogiacomo, A tissue engineering approach to bone repair in large animal models and in clinical practice, *Biomaterials* 28 (2007) 4240-4250.
- [170] V. Viateau, G. Guillemin, V. Bousson, K. Oudina, D. Hannouche, L. Sedel, D. Logeart-Avramoglou, and H. Petite, Long-bone critical-size defects treated with tissue-engineered grafts: a study on sheep, *J. Orthop. Res.* 25 (2007) 741-749.
- [171] J.C. Reichert, M.E. Wullschleger, A. Cipitria, J. Lienau, T.K. Cheng, M.A. Schutz, G.N. Duda, U. Noth, J. Eulert, and D.W. Hutmacher, Custom-made composite scaffolds for segmental defect repair in long bones, *Int. Orthop.* 35 (2011) 1229-1236.
- [172] J.C. Reichert, M.A. Woodruff, T. Friis, V.M. Quent, S. Gronthos, G.N. Duda, M.A. Schutz, and D.W. Hutmacher, Ovine bone- and marrow-derived progenitor cells and their potential for scaffold-based bone tissue engineering applications in vitro and in vivo, *J. Tissue Eng Regen. Med.* 4 (2010) 565-576.
- [173] J.C. Reichert, D.R. Epari, M.E. Wullschleger, S. Saifzadeh, R. Steck, J. Lienau, S. Sommerville, I.C. Dickinson, M.A. Schutz, G.N. Duda, and D.W. Hutmacher, Establishment of a preclinical ovine model for tibial segmental bone defect repair by applying bone tissue engineering strategies, *Tissue Eng Part B Rev.* 16 (2010) 93-104.
- [174] A. Berner, J.C. Reichert, M.A. Woodruff, S. Saifzadeh, A.J. Morris, D.R. Epari, M. Nerlich, M.A. Schuetz, and D.W. Hutmacher, Autologous vs. allogenic mesenchymal

- progenitor cells for the reconstruction of critical sized segmental tibial bone defects in aged sheep, *Acta Biomater.* 9 (2013) 7874-7884.
- [175] M. Mastrogiacomo, A. Papadimitropoulos, A. Cedola, F. Peyrin, P. Giannoni, S.G. Pearce, M. Alini, C. Giannini, A. Guagliardi, and R. Cancedda, Engineering of bone using bone marrow stromal cells and a silicon-stabilized tricalcium phosphate bioceramic: evidence for a coupling between bone formation and scaffold resorption, *Biomaterials* 28 (2007) 1376-1384.
- [176] P. Giannoni, M. Mastrogiacomo, M. Alini, S.G. Pearce, A. Corsi, F. Santolini, A. Muraglia, P. Bianco, and R. Cancedda, Regeneration of large bone defects in sheep using bone marrow stromal cells, *J. Tissue Eng Regen. Med.* 2 (2008) 253-262.
- [177] C.P. Jerome, C.H. Turner, and C.J. Lees, Decreased bone mass and strength in ovariectomized cynomolgus monkeys (*Macaca fascicularis*), *Calcif. Tissue Int.* 60 (1997) 265-270.
- [178] C.P. Jerome, Primate models of osteoporosis, *Lab Anim Sci.* 48 (1998) 618-622.
- [179] C.P. Jerome and P.E. Peterson, Nonhuman primate models in skeletal research, *Bone* 29 (2001) 1-6.
- [180] U. Sauer, B. Phillips, K. Reid, V. Schmit, and M. Jennings, Ethical review of projects involving non-human primates funded under the European Union's 7th Research Framework Programme, *Altern. Lab Anim* 41 (2013) 271-306.
- [181] C.M. Bagi, M. Volberg, M. Moalli, V. Shen, E. Olson, N. Hanson, E. Berryman, and C.J. Andresen, Age-related changes in marmoset trabecular and cortical bone and response to alendronate therapy resemble human bone physiology and architecture, *Anat. Rec. (Hoboken.)* 290 (2007) 1005-1016.
- [182] D.H. Abbott, D.K. Barnett, R.J. Colman, M.E. Yamamoto, and N.J. Schultz-Darken, Aspects of common marmoset basic biology and life history important for biomedical research, *Comp Med.* 53 (2003) 339-350.
- [183] Food and Drug Administration, *Guidelines for Preclinical and Clinical Evaluation of Agents Used in the Prevention or Treatment of Postmenopausal Osteoporosis.* Washington, DC: FDA Division of Metabolism and Endocrine Drug Products, FDA Division of Metabolism and Endocrine Drug Products, Washington, DC, 1994.
- [184] European Medicines Agency, Guideline on the evaluation of medicinal products in the treatment of primary osteoporosis, 2006.
- [185] M.D. Hoffman and D.S. Benoit, Emerging ideas: Engineering the periosteum: revitalizing allografts by mimicking autograft healing, *Clin. Orthop. Relat Res.* 471 (2013) 721-726.
- [186] T. Long, Z. Zhu, H.A. Awad, E.M. Schwarz, M.J. Hilton, and Y. Dong, The effect of mesenchymal stem cell sheets on structural allograft healing of critical sized femoral defects in mice, *Biomaterials* 35 (2014) 2752-2759.
- [187] E.A. Breitbart, S. Meade, V. Azad, S. Yeh, L. Al-Zube, Y.S. Lee, J. Benevenia, T.L. Arinze, and S.S. Lin, Mesenchymal stem cells accelerate bone allograft incorporation in the presence of diabetes mellitus, *J. Orthop. Res.* 28 (2010) 942-949.
- [188] S. Srouji and E. Livne, Bone marrow stem cells and biological scaffold for bone repair in aging and disease, *Mech. Ageing Dev.* 126 (2005) 281-287.
- [189] X.H. Zou, H.X. Cai, Z. Yin, X. Chen, Y.Z. Jiang, H. Hu, and H.W. Ouyang, A novel strategy incorporated the power of mesenchymal stem cells to allografts for segmental bone tissue engineering, *Cell Transplant.* 18 (2009) 433-441.

- [190] L. Pang, W. Hao, M. Jiang, J. Huang, Y. Yan, and Y. Hu, Bony defect repair in rabbit using hybrid rapid prototyping polylactic-co-glycolic acid/beta-tricalciumphosphate collagen I/apatite scaffold and bone marrow mesenchymal stem cells, *Indian J. Orthop.* 47 (2013) 388-394.
- [191] C.R. Rathbone, T. Guda, B.M. Singleton, D.S. Oh, M.R. Appleford, J.L. Ong, and J.C. Wenke, Effect of cell-seeded hydroxyapatite scaffolds on rabbit radius bone regeneration, *J. Biomed. Mater. Res. A* (2013).
- [192] S.H. Kang, Y.G. Chung, I.H. Oh, Y.S. Kim, K.O. Min, and J.Y. Chung, Bone regeneration potential of allogeneic or autogeneic mesenchymal stem cells loaded onto cancellous bone granules in a rabbit radial defect model, *Cell Tissue Res.* 355 (2014) 81-88.
- [193] S.P. Bruder, K.H. Kraus, V.M. Goldberg, and S. Kadiyala, The effect of implants loaded with autologous mesenchymal stem cells on the healing of canine segmental bone defects, *J. Bone Joint Surg. Am.* 80 (1998) 985-996.
- [194] T.L. Arinzech, S.J. Peter, M.P. Archambault, C. van den Bos, S. Gordon, K. Kraus, A. Smith, and S. Kadiyala, Allogeneic mesenchymal stem cells regenerate bone in a critical-sized canine segmental defect, *J. Bone Joint Surg. Am.* 85-A (2003) 1927-1935.
- [195] C. Wang, Z. Wang, A. Li, F. Bai, J. Lu, S. Xu, and D. Li, Repair of segmental bone-defect of goat's tibia using a dynamic perfusion culture tissue engineering bone, *J. Biomed. Mater. Res. A* 92 (2010) 1145-1153.
- [196] J.R. Field, M. McGee, R. Stanley, G. Ruthenbeck, T. Papadimitrakis, A. Zannettino, S. Gronthos, and S. Itescu, The efficacy of allogeneic mesenchymal precursor cells for the repair of an ovine tibial segmental defect, *Vet. Comp Orthop. Traumatol.* 24 (2011) 113-121.
- [197] G. Liu, L. Zhao, W. Zhang, L. Cui, W. Liu, and Y. Cao, Repair of goat tibial defects with bone marrow stromal cells and beta-tricalcium phosphate, *J. Mater. Sci. Mater. Med.* 19 (2008) 2367-2376.
- [198] C.S. Bahney, D.P. Hu, A.J. Taylor, F. Ferro, H.M. Britz, B. Hallgrímsson, B. Johnstone, T. Miclau, and R.S. Marcucio, Stem Cell Derived Endochondral Cartilage Stimulates Bone Healing by Tissue Transformation, *J. Bone Miner. Res.* (2013).
- [199] S.P. Bruder, A.A. Kurth, M. Shea, W.C. Hayes, N. Jaiswal, and S. Kadiyala, Bone regeneration by implantation of purified, culture-expanded human mesenchymal stem cells, *J. Orthop. Res.* 16 (1998) 155-162.
- [200] L. Meinel, O. Betz, R. Fajardo, S. Hofmann, A. Nazarian, E. Cory, M. Hilbe, J. McCool, R. Langer, G. Vunjak-Novakovic, H.P. Merkle, B. Rechenberg, D.L. Kaplan, and C. Kirker-Head, Silk based biomaterials to heal critical sized femur defects, *Bone* 39 (2006) 922-931.
- [201] L.F. Amorosa, C.H. Lee, A.B. Aydemir, S. Nizami, A. Hsu, N.R. Patel, T.R. Gardner, A. Navalgund, D.G. Kim, S.H. Park, J.J. Mao, and F.Y. Lee, Physiologic load-bearing characteristics of autografts, allografts, and polymer-based scaffolds in a critical sized segmental defect of long bone: an experimental study, *Int. J. Nanomedicine.* 8 (2013) 1637-1643.
- [202] P. Niemeyer, K. Szalay, R. Luginbuhl, N.P. Sudkamp, and P. Kasten, Transplantation of human mesenchymal stem cells in a non-autogenous setting for bone regeneration in a rabbit critical-size defect model, *Acta Biomater.* 6 (2010) 900-908.
- [203] P. Niemeyer, T.S. Schonberger, J. Hahn, P. Kasten, J. Fellenberg, N. Suedkamp, A.T. Mehlhorn, S. Milz, and S. Pearce, Xenogenic transplantation of human mesenchymal

- stem cells in a critical size defect of the sheep tibia for bone regeneration, *Tissue Eng Part A* 16 (2010) 33-43.
- [204] W.P. Tsang, Y. Shu, P.L. Kwok, F. Zhang, K.K. Lee, M.K. Tang, G. Li, K.M. Chan, W.Y. Chan, and C. Wan, CD146+ human umbilical cord perivascular cells maintain stemness under hypoxia and as a cell source for skeletal regeneration, *PLoS. One.* 8 (2013) e76153.
- [205] S. Zwingenberger, Z. Yao, A. Jacobi, C. Vater, R.D. Valladares, C. Li, C. Nich, A.J. Rao, J.E. Christman, J.K. Antonios, E. Gibon, A. Schambach, T. Maetzig, S.B. Goodman, and M. Stiehler, Enhancement of BMP-2 Induced Bone Regeneration by SDF-1alpha Mediated Stem Cell Recruitment, *Tissue Eng Part A* 20 (2014) 810-818.
- [206] K. Atesok, R. Li, D.J. Stewart, and E.H. Schemitsch, Endothelial progenitor cells promote fracture healing in a segmental bone defect model, *J. Orthop. Res.* 28 (2010) 1007-1014.
- [207] A.W. James, J.N. Zara, M. Corselli, M. Chiang, W. Yuan, V. Nguyen, A. Askarinam, R. Goyal, R.K. Siu, V. Scott, M. Lee, K. Ting, B. Peault, and C. Soo, Use of human perivascular stem cells for bone regeneration, *J. Vis. Exp.* (2012) e2952.
- [208] Z.X. Fan, Y. Lu, L. Deng, X.Q. Li, W. Zhi, J. Li-Ling, Z.M. Yang, and H.Q. Xie, Placenta-versus bone-marrow-derived mesenchymal cells for the repair of segmental bone defects in a rabbit model, *FEBS J.* 279 (2012) 2455-2465.
- [209] A. Kim, D.H. Kim, H.R. Song, W.H. Kang, H.J. Kim, H.C. Lim, D.W. Cho, and J.H. Bae, Repair of rabbit ulna segmental bone defect using freshly isolated adipose-derived stromal vascular fraction, *Cytotherapy.* 14 (2012) 296-305.
- [210] S. Kikuta, N. Tanaka, T. Kazama, M. Kazama, K. Kano, J. Ryu, Y. Tokuhashi, and T. Matsumoto, Osteogenic effects of dedifferentiated fat cell transplantation in rabbit models of bone defect and ovariectomy-induced osteoporosis, *Tissue Eng Part A* 19 (2013) 1792-1802.
- [211] D. Gazit, G. Turgeman, P. Kelley, E. Wang, M. Jalenak, Y. Zilberman, and I. Moutsatsos, Engineered pluripotent mesenchymal cells integrate and differentiate in regenerating bone: a novel cell-mediated gene therapy, *J. Gene Med.* 1 (1999) 121-133.
- [212] C. Xie, D. Reynolds, H. Awad, P.T. Rubery, G. Pelled, D. Gazit, R.E. Guldberg, E.M. Schwarz, R.J. O'Keefe, and X. Zhang, Structural bone allograft combined with genetically engineered mesenchymal stem cells as a novel platform for bone tissue engineering, *Tissue Eng* 13 (2007) 435-445.
- [213] D.J. Corn, Y. Kim, M.D. Krebs, T. Mounts, J. Molter, S. Gerson, E. Alsberg, J.E. Dennis, and Z. Lee, Imaging early stage osteogenic differentiation of mesenchymal stem cells, *J. Orthop. Res.* 31 (2013) 871-879.
- [214] M.D. Hoffman, C. Xie, X. Zhang, and D.S. Benoit, The effect of mesenchymal stem cells delivered via hydrogel-based tissue engineered periosteum on bone allograft healing, *Biomaterials* 34 (2013) 8887-8898.
- [215] C. Huang, M. Tang, E. Yehling, and X. Zhang, Overexpressing sonic hedgehog Peptide restores periosteal bone formation in a murine bone allograft transplantation model, *Mol. Ther.* 22 (2014) 430-439.
- [216] R.B. Rutherford, M. Moalli, R.T. Franceschi, D. Wang, K. Gu, and P.H. Krebsbach, Bone morphogenetic protein-transduced human fibroblasts convert to osteoblasts and form bone in vivo, *Tissue Eng* 8 (2002) 441-452.

- [217] H. Tsuchida, J. Hashimoto, E. Crawford, P. Manske, and J. Lou, Engineered allogeneic mesenchymal stem cells repair femoral segmental defect in rats, *J. Orthop. Res.* 21 (2003) 44-53.
- [218] X. Guo, Q. Zheng, I. Kulbatski, Q. Yuan, S. Yang, Z. Shao, H. Wang, B. Xiao, Z. Pan, and S. Tang, Bone regeneration with active angiogenesis by basic fibroblast growth factor gene transfected mesenchymal stem cells seeded on porous beta-TCP ceramic scaffolds, *Biomed. Mater.* 1 (2006) 93-99.
- [219] L. Cao, X. Liu, S. Liu, Y. Jiang, X. Zhang, C. Zhang, and B. Zeng, Experimental repair of segmental bone defects in rabbits by angiopoietin-1 gene transfected MSCs seeded on porous beta-TCP scaffolds, *J. Biomed. Mater. Res. B Appl. Biomater.* 100 (2012) 1229-1236.
- [220] P. Kasten, M. Beverungen, H. Lorenz, J. Wieland, M. Fehr, and F. Geiger, Comparison of platelet-rich plasma and VEGF-transfected mesenchymal stem cells on vascularization and bone formation in a critical-size bone defect, *Cells Tissues. Organs* 196 (2012) 523-533.
- [221] Q. Chen, Z. Yang, S. Sun, H. Huang, X. Sun, Z. Wang, Y. Zhang, and B. Zhang, Adipose-derived stem cells modified genetically in vivo promote reconstruction of bone defects, *Cytherapy.* 12 (2010) 831-840.
- [222] M.L. Ren, W. Peng, Z.L. Yang, X.J. Sun, S.C. Zhang, Z.G. Wang, and B. Zhang, Allogeneic adipose-derived stem cells with low immunogenicity constructing tissue-engineered bone for repairing bone defects in pigs, *Cell Transplant.* 21 (2012) 2711-2721.
- [223] J.M. Kanczler, P.J. Ginty, J.J. Barry, N.M. Clarke, S.M. Howdle, K.M. Shakesheff, and R.O. Oreffo, The effect of mesenchymal populations and vascular endothelial growth factor delivered from biodegradable polymer scaffolds on bone formation, *Biomaterials* 29 (2008) 1892-1900.
- [224] J.M. Kanczler, P.J. Ginty, L. White, N.M. Clarke, S.M. Howdle, K.M. Shakesheff, and R.O. Oreffo, The effect of the delivery of vascular endothelial growth factor and bone morphogenic protein-2 to osteoprogenitor cell populations on bone formation, *Biomaterials* 31 (2010) 1242-1250.
- [225] C. Kirker-Head, V. Karageorgiou, S. Hofmann, R. Fajardo, O. Betz, H.P. Merkle, M. Hilbe, R.B. von, J. McCool, L. Abrahamsen, A. Nazarian, E. Cory, M. Curtis, D. Kaplan, and L. Meinel, BMP-silk composite matrices heal critically sized femoral defects, *Bone* 41 (2007) 247-255.
- [226] G. Burastero, S. Scarfi, C. Ferraris, C. Fresia, N. Sessarego, F. Fruscione, F. Monetti, F. Scarfo, P. Schupbach, M. Podesta, G. Grappiolo, and E. Zocchi, The association of human mesenchymal stem cells with BMP-7 improves bone regeneration of critical-size segmental bone defects in athymic rats, *Bone* 47 (2010) 117-126.
- [227] P. Kasten, J. Vogel, F. Geiger, P. Niemeyer, R. Luginbuhl, and K. Szalay, The effect of platelet-rich plasma on healing in critical-size long-bone defects, *Biomaterials* 29 (2008) 3983-3992.
- [228] R.M. El Backly, S.H. Zaky, A. Muraglia, L. Tonachini, F. Brun, B. Canciani, D. Chiapale, F. Santolini, R. Cancedda, and M. Mastrogiacomo, A platelet-rich plasma-based membrane as a periosteal substitute with enhanced osteogenic and angiogenic properties: a new concept for bone repair, *Tissue Eng Part A* 19 (2013) 152-165.
- [229] D. Brodke, H.A. Pedrozo, T.A. Kapur, M. Attawia, K.H. Kraus, C.E. Holy, S. Kadiyala, and S.P. Bruder, Bone grafts prepared with selective cell retention technology heal

- canine segmental defects as effectively as autograft, *J. Orthop. Res.* 24 (2006) 857-866.
- [230] B.C. Di, N.N. Aldini, E. Lucarelli, B. Dozza, T. Frisoni, L. Martini, M. Fini, and D. Donati, Osteogenic protein-1 associated with mesenchymal stem cells promote bone allograft integration, *Tissue Eng Part A* 16 (2010) 2967-2976.
- [231] P. Armitage, G. Barry, and J.N.S. Matthews, *Statistical methods in medical research*, Blackwell Science, 2002.
- [232] R.C. Littell, G.A. Milliken, W.W. Stroup, R.D. Wolfinger, and O. Schabenberger, *SAS for mixed models*, SAS Institute Inc., Cary, NC, USA, 2006.
- [233] K. Song, C. Krause, S. Shi, M. Patterson, R. Suto, L. Grgurevic, S. Vukicevic, M. Van Dinther, D. Falb, P. Ten Dijke, and M.H. Alaoui-Ismaili, Identification of a key residue mediating bone morphogenetic protein (BMP)-6 resistance to noggin inhibition allows for engineered BMPs with superior agonist activity, *Journal of Biological Chemistry* 285 (2010) 12169-12180.
- [234] L. Grgurevic, B. Macek, M. Mercep, M. Jelic, T. Smoljanovic, I. Erjavec, I. Dumic-Cule, S. Prgomet, D. Durdevic, D. Vnuk, M. Lipar, M. Stejskal, V. Kufner, J. Brkljacic, D. Maticic, and S. Vukicevic, Bone morphogenetic protein (BMP)1-3 enhances bone repair, *Biochemical and Biophysical Research Communications* 408 (2011) 25-31.