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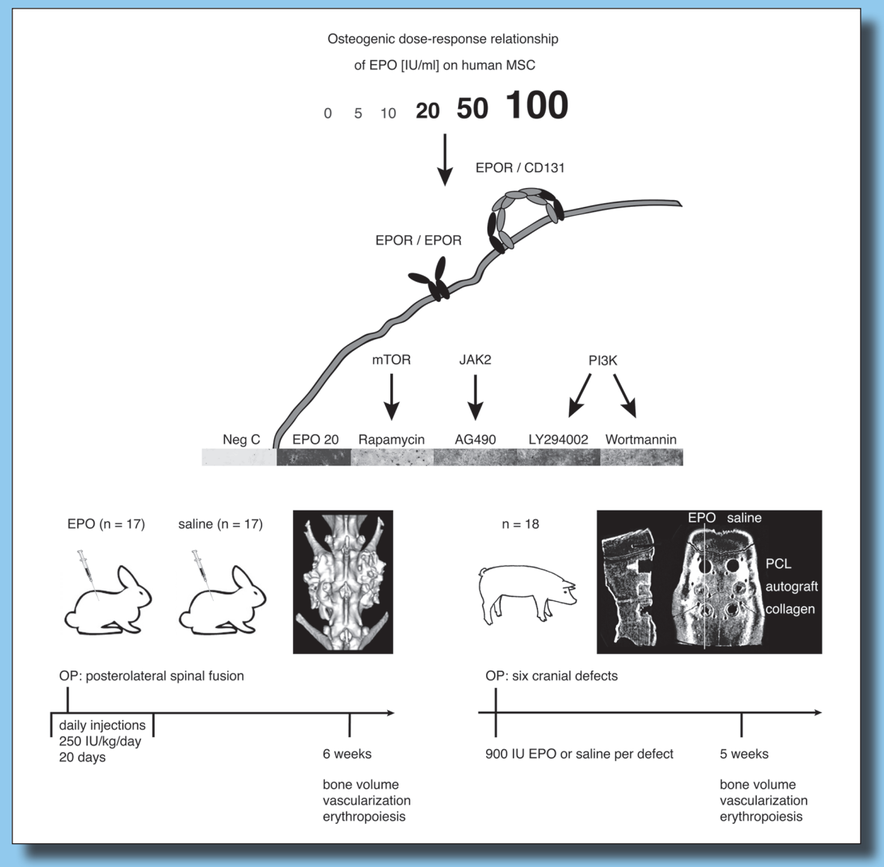
The effect of erythropoietin on bone

Jan Hendrik Duedal Rölfing



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Preface

This PhD thesis consists of three papers and an overview, which will be presented in the following. It introduces the scientific background and purpose of the PhD project, presents the coherence of the papers, thoroughly discusses the choice of methodology, shortly presents the main findings, discusses the results and, finally, provides an outlook and discusses the perspective of the new discoveries presented.

The presented work is based on research projects carried out at the Orthopaedic Research Laboratory at Aarhus University Hospital and the Department of Clinical Medicine at Aarhus University. At Aarhus University, the Department of Forensic Medicine, the FACS Core Facility and the Department of Animal Science in Foulum played a pivotal part in conducting the experimental studies during my enrolment as a PhD student at the Faculty of Health Sciences at Aarhus University.

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Finally, I would like to thank the people most important to me my family: Louise, Johannes, and *lille pigen,* my mother, family-in-law, and friends, who have always supported my endeavors.

List of Papers

This PhD disseration is based on the following papers:

Paper 1: Rölfing JHD, Bendtsen M, Jensen J, Stiehler M, Foldager CB, Hellfritzsch MB, Bünger C.
Erythropoietin augments bone formation in a rabbit posterolateral spinal fusion model.
J Orthop Res. 2012. 30(7): 1083-1088.
doi: 10.1002/jor.22027.

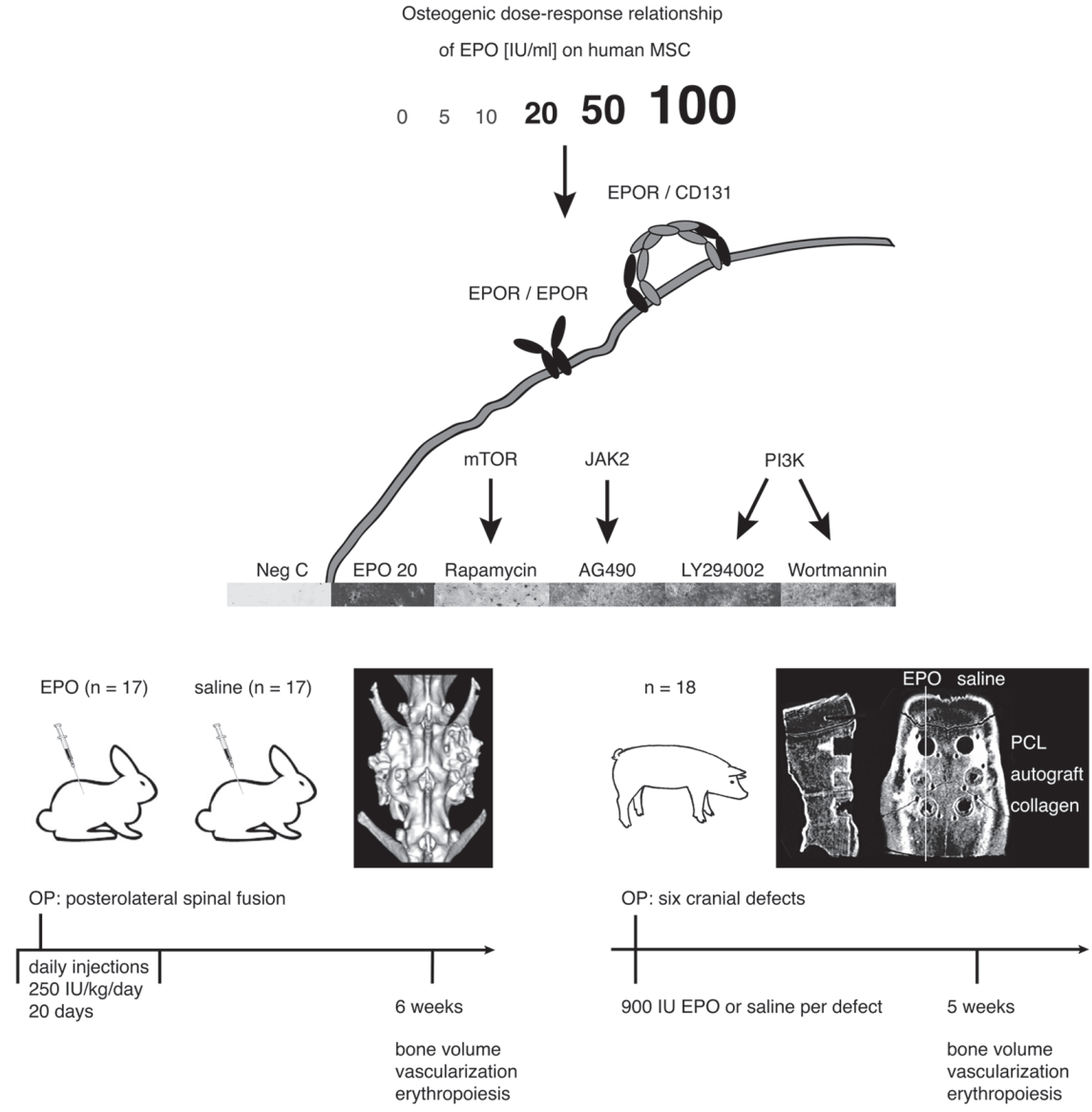
Paper 2: Rölfing JHD, Baatrup A, Stiehler M, Jensen J, Lysdahl H, Bünger C.
The osteogenic effect of erythropoietin on human mesenchymal stromal cells is dose-dependent and involves non-hematopoietic receptors and multiple intracellular signaling pathways.
Stem Cell Rev. 2013 Sep 20. [Epub ahead of print].
doi: 10.1007/s12015-013-9476-x.

Paper 3: Rölfing JHD, Jensen J, Jensen JN, Greve AS, Lysdahl H, Chen M, Rejnmark L, Bünger C.
A single topical dose of erythropoietin applied on a collagen carrier enhances calvarial bone healing in pigs.
Acta Orthop. In press.

Abbreviations and Mesh terms

ALP	Alkaline phosphatase activity
AZR	Alizarin red S staining
BMP	"Bone morphogenetic proteins"[Mesh]
BV/TV	Bone volume fraction
CD131	"Cytokine receptor common beta subunit"[Mesh]
CI	Confidence interval
CT	Computed tomography
EPO	"Erythropoietin"[Mesh]
EPOR	"Receptors, erythropoietin"[Mesh]
ESAs	Erythropoiesis-stimulating agents, "Hematinics" [Mesh]
FDA	U.S. Food and Drug Administration
HCT	"Hematocrit"[Mesh]
HGB	Hemoglobin, "Hemoglobins"[Mesh]
HSC	"Hematopoietic stem cells"[Mesh]
JAK	"Janus kinases"[Mesh]
MAPK	"Mitogen-activated protein kinase kinases"[Mesh]
Mesh	Medical subject headings
MSCs	"Mesenchymal stromal cells"[Mesh]
mTOR	"TOR serine-threonine kinases"[Mesh]
PCL	"Polycaprolactone"[Supplementary Concept]
PI3K	"Phosphatidylinositol 3-kinase"[Mesh]
PLT	"Blood platelets"[Mesh] OR "platelet count"[Mesh]
RBC	"Erythrocytes"[Mesh] OR "erythrocyte count" [Mesh]
rhEPO	recombinant human erythropoietin
STAT	"STAT transcription factors"[Mesh]
TNFα	"Tumor necrosis factor-alpha"[Mesh]
WBC	"Leukocytes"[Mesh] OR "leukocyte count"[Mesh]

Summary



Erythropoietin (EPO) is a hematopoietic growth factor stimulating the formation of red blood cells. EPO is notoriously known as a doping substance in high-performance sports, and in cycling in particular. In the clinical setting, this erythropoiesis-stimulating agent is utilized to treat anemia, especially if caused by a lack of endogenous EPO production due to chronic renal failure.

In recent years, the non-hematopoietic functions of EPO, also known as pleiotropic functions, have been intensively investigated. Of interest for orthopedics and musculoskeletal tissue engineering, the non-hematopoietic capabilities of EPO include osteogenic and angiogenic potencies.

The objectives of the present thesis were to address and investigate the efficacy of EPO in regenerating bone and facilitating bone healing.

The first paper investigated the effectiveness of continuous low-dose systemic EPO administration to enhance bone formation in an autograft posterolateral spinal fusion model in rabbits. We observed an increased bone volume and neovascularization compared with saline-treated controls after six weeks of observation.

The second paper set out to investigate the cellular mechanisms of the osteogenic action of EPO and to describe the dose-response relationship in vitro. Human mesenchymal

stromal cells (hMSCs) were exposed to a wide range of EPO-concentrations for up to three weeks. The lowest effective dose was 20 IU/ml EPO, and a proportional dose-response relationship was observed. Hence, the highest tested concentration of 100 IU/ml EPO yielded the most pronounced osteogenic effect. Regarding the cellular ways of action, two cell membrane receptors were observed, namely the EPO receptor (EPOR) and the cytokine receptor common beta subunit (CD131). Furthermore, the osteogenic effect was mediated via three intracellular signaling pathways: TOR serine-threonine kinase (mTOR), Janus kinase 2 (JAK2), and phosphatidylinositol 3-kinase (PI3K).

The third paper was designed to accelerate clinical progress. Before the clinical implementation of EPO it was necessary to test EPO in a large-animal model. Systemic EPO administration can cause severe adverse events such as thromboembolisms. A single, locally administered, low-dose approach was therefore chosen. Bone formation was assessed in a porcine calvarial defect model. The defects were treated with EPO or placebo and in combination either with autologous bone graft, a commercially available collagen carrier, or a polycaprolactone scaffold. After five weeks of observation, an increased bone volume after EPO treatment was observed in the collagen carrier group. The excellent regenerative potential of the autograft was underlined by the fact that the bone volume did not significantly differ from that of the healthy reference bone. At the other end of the spectrum, bony ingrowth into the PCL scaffold was sparse both with and without EPO, which suggests the need to investigate other types of scaffold material or modified PCL constructs.

In conclusion, bony ingrowth and vascularization of three-dimensional scaffolds for bone tissue regeneration remains a challenge. The described pleiotropic functions of EPO may overcome this limitation of skeletal tissue engineering in the future. EPO could potentially facilitate neovascularization, and the migration of cells that are directed into the core of the scaffold will facilitate bony ingrowth. Moreover, EPO promotes a direct and indirect osteogenic stimulation of hMSCs. A clinically safe dose enhanced bone healing in a large-animal model. This is encouraging news for the potential direct clinical utilization of EPO. EPO is therefore a promising growth factor in regenerative medicine.

Introduction

Erythropoietin (EPO)

Characterization, production, function and degradation

In 1906, Carnot and Deflandre proclaimed the existence of a humeral factor ("hemopoietine") that regulates red blood cell production. After a century of extensive research in erythropoietin (EPO), its structure, production and hematopoietic way of action have been described in detail, while the clearance and degradation of EPO is not yet entirely understood (Jelkmann 1992; 2007).

EPO consists of 60% protein and 40% carbohydrates. A chain of 165 amino acids constitutes the protein core. The peptide terminals of the core mediate functionality via binding to the receptor, while the four carbohydrate side chains protect EPO from degradation in the blood (Jelkmann 1992).

In 1985, the discovery of the nucleotide sequence of EPO made it possible to produce recombinant human EPO (rhEPO) for clinical use (Jacobs et al. 1985). Endogenous EPO and rhEPO are identical apart from minor differences in glycosylation (Koury and Bondurant 1992).

In 1989, the U.S. Food and Drug Administration (FDA) approved the use of rhEPO for the treatment of anemia caused by insufficient endogenous EPO production due to chronic renal failure. The approval has since been extended to a wide range of indications, e.g. anemia induced by chemotherapy or HIV, and to decrease the need for transfusion in patients scheduled for certain types of surgery (Jenkins 2007). It is also used in patients who refuse blood transfusions, e.g. Jehovah's Witnesses.

The kidney is the primary production site of EPO. Upon hypoxia, renal peritubular fibroblasts increase EPO gene expression via hypoxia-inducible transcription factor and subsequently release EPO into the circulation. Before birth, the liver is the primary production site and it still accounts for about 10% of production in adult life (Jelkmann 2007). In the past decades, it has become well established that EPO is also expressed locally in many tissues and that it acts in a paracrine fashion. For more information, see *Pleiotropic effects of EPO*.

Erythroid progenitor cells in the bone marrow express the homodimeric EPO receptor (EPOR) on the cell surface. Binding of EPO activates Janus kinase 2 (JAK2) and downstream phosphatidylinositol 3-kinase (PI3K)/Akt, MAP kinase, protein kinase C and STAT signaling pathways (Jelkmann 2007). In the absence of EPO, most erythroid progenitor cells undergo apoptosis. A rise in the EPO concentration salvages these cells and thereby facilitates the production of red blood cells.

In health, about 1% of the red blood cells are renewed on a daily basis and the plasma level of EPO varies from 10–25 IU/l, but it can rise up to 20,000 IU/l in anemic patients (Hellebostad et al. 1988; Jelkmann 2007). Despite the fact that only a small fraction of hematopoietic progenitor cells express EPOR at any time, only about 3% of the highly affinitive EPOR receptors are occupied (Brines and Cerami 2008). Notably, the bioavailability of subcutaneous EPO increases from 30% at low doses to 70% when high doses are administered (Olsson-Gisleskog et al. 2007).

The clearance of EPO from the blood is not completely understood, but both renal, hepatic and receptor-mediated clearance via internalization and degradation of the EPO-EPOR complex have been described (Jelkmann 2002; 2007; Krzyzanski and Wyska 2007). The last mechanism plays a vital role in the clearance of EPO. The half-life of EPO therefore depends upon the affinity of EPO to its receptor and the level of receptor expression. Degradation in the liver depends on the composition of the sugar side chains of EPO. These carbohydrates prevent recognition by the galactose receptor on hepatocytes and thereby block intracellular uptake and degradation. However, the side chains are slowly degraded in the blood stream by glycosidases and proteinases, and EPO is subsequently degraded in the liver.

Of interest for pre-clinical animal experiments, the DNA homology of EPO among mammals is high. For instance, human EPO is 80–82% identical to porcine EPO, and a mounting body of evidence describes that rhEPO is able to elicit effects in mammals (Wen et al. 1993).

Erythropoiesis-stimulating agents (ESAs)

ESAs are similar to the endogenous EPO and likewise increase the production of red blood cells. Epoetin alpha (Eprex®, Epogen®, Procrit®) and epoetin beta (NeoRecormon®, Recormon®) are commonly available and were chosen for the present studies because they are the most investigated types of ESAs and because their non-hematopoietic function supposedly does not need repetitive stimulation to be maintained (Brines and Cerami 2008). They are produced in Chinese hamster ovary cells and differ from endogenous EPO with regards to carbohydrates. Their half-life is approximately 8 hours (Macdougall et al. 1991; Wu et al. 2012). Because of the relatively short half-life of first-generation rhEPO, e.g. epoetin alpha and beta, anemic patients need EPO injections several times a week.

Later, the clinical feasibility of EPO administration was increased by developing ESAs with longer half-lives. Alternations of glycosylation influence both bioavailability, affinity for EPOR and half-lives. Epoetin delta (Dynepo®) is produced in human cell lines and epoetin omega (Epomax®) in

baby hamster kidney cells. The carbohydrate composition of epoetin zeta (Retacrit®, EPO biosimilar) is similar to that of endogenous EPO. The second-generation ESA, darbepoetin alpha (Aranesp®), has a three times longer half-life because of hyperglycosylation. Third-generation ESAs have half-lives up to 130 hours. This extreme long-lasting half-life of Continuous-Erythropoiesis-Receptor-Activator (Mircera®) is due to PEGylation of epoetin beta. Furthermore, ESAs also contain non rhEPOs such as EPO-mimetic peptides, GATA antagonists and HIF stabilizers (Sølling 2011) .

Units

The bioactivity of ESAs varies; hence, the amount of EPO is expressed in EPO units rather than in SI units of mole or kilogram. International units (IU) of EPO have been defined by the National Institute for Biological Standards and Controls and by the WHO (Storring and Gaines Das 1992). One IU EPO elicits the same erythropoiesis-stimulating response in rodents as 5 μM cobalt measured with an exhypoxic polycythemic mouse assay or similar in vivo assays (Jelkmann 2009). EPO is usually calibrated in bioassays against WHO international biological reference preparations, in particular the Second International Reference Preparation of Human Urinary EPO, and the newer International Standard for Recombinant DNA-Derived EPO.

Notably, Krzyzanski et al. suggest that 7.7 μg rhEPO (molecular weight 30.4 kDa) is equivalent to 1,000 IU when conversion from IU/l into pM is needed (Krzyzanski and Wyska 2007).

Safety of EPO and ESAs

In 2007, alarming data from the Correction of Hemoglobin and Outcomes in Renal Insufficiency (CHOIR) study and the Normal Hematocrit Study prompted an FDA revision of ESA-labeling to include a boxed warning (Besarab et al. 1998; Singh et al. 2006).

In 2012, the Cochrane Haematological Malignancies Group published a meta-analysis based on 93 randomized controlled trials enrolling 20,102 cancer patients receiving either ESA treatment or placebo (Tonia et al. 2012). As intended, ESAs reduced the need for blood transfusions and a trend towards improved quality of life was observed. However, severe adverse effects were documented. Evidence was presented that ESAs increase both mortality during the study period (hazard ratio (HR) 1.17, 95% confidence interval (CI) 1.06–1.29), long-term mortality (HR 1.05; 95% CI 1.00–1.11), thromboembolic complications such as stroke and myocardial infarction (risk ratio (RR) 1.52, 95% CI 1.34–1.74) and possibly hypertension. The authors concluded that insufficient data exist to support an effect of ESAs on tumor progression (RR 1.02, 95% CI 0.98–1.06) and warrant further research into cellular mechanisms and pathways (Tonia et al. 2012). Hence, the matter of debate whether EPO can promote tumor growth and induces cancer-therapy resistance continues (Jelkmann 2007). Another meta-analysis of the safety and efficacy of "erythropoiesis-stimulating agents for myelodysplasic syndromes (Protocol)" is currently being performed (Acevedo et al. 2012).

In off-label use in non-cancer patients, severe adverse effects have also been reported. For instance, an attempt was made to exploit the tissue-protective action of EPO in a phase II/III clinical trial, the German Multicenter EPO Stroke Trial, with 522 patients suffering from acute ischemic stroke. However, a cumulative dose of 120,000 IU EPO intravenously administered over 48 hours, did not show favorable effects. On the contrary, the death rate was substantially increased in the EPO group compared with the control group, 16.4 vs. 9.0% (odds ratio (OR) 1.98; 95% CI 1.16–3.38) (Ehrenreich et al. 2009).

The current opinion of the FDA has not significantly changed since the director of the Office of New Drugs pointed out in 2007 that: "Drug safety is a risk-to-benefit balance. […] Evaluating the benefits and risks of all drug products is a dynamic process – and FDA's ongoing evaluation of ESAs is no exception. […] At this time, FDA continues to believe that ESAs are safe and effective when used according to the recently revised product labeling, at the recommended dose and approved indication." (Jenkins 2007).

Pleiotropic effects of EPO

In 1990, Anagnostou et al. were the first to observe a non-hematopoietic effect of EPO (Anagnostou et al. 1990). The past two decades have seen mounting evidence that EPO exerts pleiotropic effects in various tissues. Today, it is well established that EPOR is expressed in endothelium and vascular smooth muscle and that this causes angiogenesis, faster wound healing and vascular protection (Jaquet et al. 2002; Heeschen et al. 2003). Furthermore, tissue-protective and anti-inflammatory effects have been described in several tissues. Among other functions, EPO elicits neuroprotection after vascular insult, cardioprotection after myocardial infarction, and renoprotection after kidney injury (Figure 1) (Prunier et al. 2007; Arcasoy 2010; Chateauvieux et al. 2011).

It has been suggested that inflammation is inhibited by EPO. The proposed underlying mechanism is that EPO antagonizes pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNFα) (Brines and Cerami 2008). For instance, TNFα is released in the center of injury and diffuses into the surrounding healthy tissue, causing apoptosis and amplification of inflammation. This initially viable area is therefore also at risk of dying. In this area, EPO could interact with its non-hematopoietic receptor and salvage the tissue. However, it has recently been shown that the inhibition of inflammation is likely to be indirect and secondary to tissue protection because EPO did not directly affect TNFα or IL-6 production (Cervellini et al. 2013).

In 2004, Brines et al. proposed that the pleiotropic actions of EPO are mediated via a heterodimeric EPOR/CD131 receptor instead of the homodimeric EPOR (Brines et al. 2004; Leist et

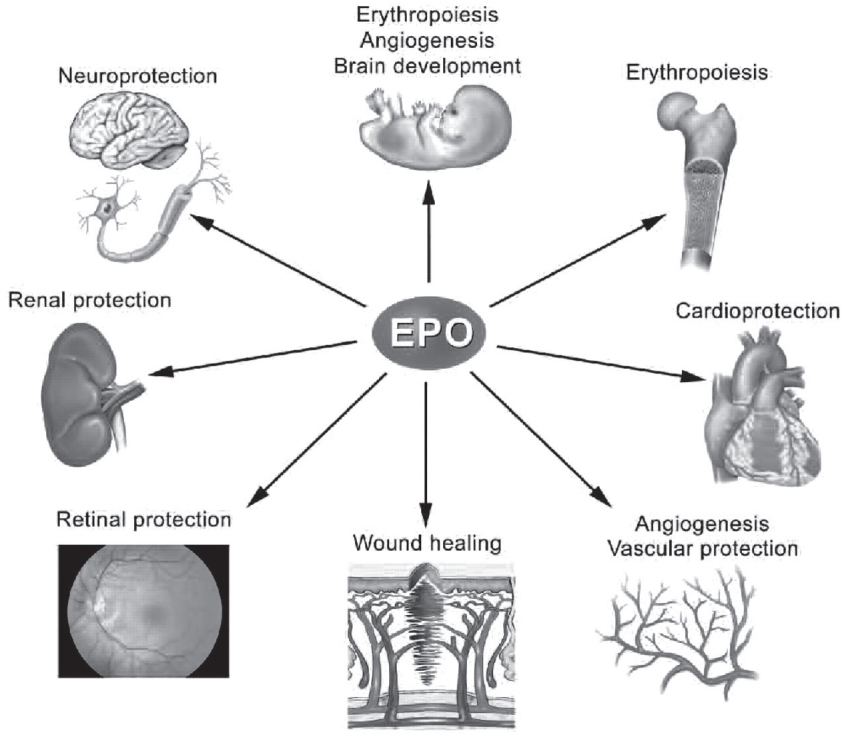


Figure 1. Non-erythroid effects of erythropoietin. Copyright: Arcasoy MO, Haematologica 2010;95:1803-1805. Obtained from Haematologica/the Hematology Journal website http://www.haematologica.org

al. 2004; Brines and Cerami 2008). Their experiments showed that EPO-related molecules, which were unable to bind to the homodimeric EPOR, could exert tissue protection, but not erythropoiesis. Details about this receptor were later published showing that two EPOR bind to two intertwined, antiparallel CD131 in a covalent fashion via cysteine linkage (Brines and Cerami 2008). Compared with the homodimeric EPOR known from the hematopoietic system, this receptor has a low affinity for its ligand, EPO. Tissue-protective and other pleiotropic actions require therefore a higher dosage of EPO than is needed to elicit erythropoiesis-stimulating actions (Brines and Cerami 2008). The EPOR/CD131 receptor hypothesis was tested in several tissues. The most convincing studies document pleiotropic effects of EPO-derived components which are incapable of binding to the homodimeric EPOR, but can only mediate their action through EPOR/CD131 (Leist et al. 2004; Brines et al. 2008; Bohr et al. 2013).

Non-hematopoietic intracellular signaling pathways include nitric oxide production and signaling via JAK2, STAT3/5, PI3K/Akt as well as MAPK pathways (Brines and Cerami 2008; Burger et al. 2009; Maiese et al. 2012). As reviewed by Brines, while some pleiotropic functions have been assigned to specific pathways, the activation of multiple of these intracellular signaling pathways is often required for the full pleiotropic effect (Brines and Cerami 2008). The role of these pathways and their contribution to the net-pleiotropic effect needs further elucidation. Of particular interest are JAK2, PI3K, and mTOR signaling (Kim et al. 2012; Maiese et al. 2012).

Reports questioning the pleiotropic potencies of EPO have also been published. These papers postulate that EPOR antibodies are non-specific and the papers hence question the expression of EPOR on non-hematopoietic cells (Sinclair et al. 2010; Singbrant et al. 2011). However, this doubt was opposed by a collaboration of leading EPO experts who published a paper entitled "Erythropoietin: not just about erythropoiesis" in the Lancet in 2010 (Ghezzi et al. 2010). These EPO experts argue that ample evidence exists and they underline their argument presenting a PubMed-search for "erythropoietin AND protect*" retrieving 863 hits, whereof 346 papers report a non-hematopoietic or tissue-protective action and only 10 papers report the lack of pleiotropic efficacy. Despite the ongoing debate about antibody specificity and EPOR expression in non-hematopoietic tissue, this is compelling evidence of the pleiotropic functions of EPO.

EPO and bone

When this PhD project was commenced, only two reports concerning EPO and bone had been published. In 2006, Bozlar et al. described increased initial bone healing and angiogenesis in a tibia fracture model in rats (Bozlar et al. 2006). The following year, Holstein et al. described that EPO improves early endochondral ossification and mechanical strength

after a fracture of the femur in mice (Holstein et al. 2007). Daily intraperitoneal injections of 5000 IU/kg EPO for 6 days increased torsional stiffness and callus density after 2 weeks. Notably, this effect was no longer evident after 5 weeks of observation. Later and concurrently with this PhD project, these authors reported that daily, systemic injections of 500 IU/kg/day resulted in a larger bone volume and union rate after fracture, which is of clinical relevance (Holstein et al. 2011). The observation and treatment time was 10 weeks. Furthermore, increased angiogenesis after 2 weeks was observed which indicates a potentially indirect way of osteogenic action. Moreover, direct cellular mechanisms were investigated, but the expression of endothelial and inducible nitric oxide synthases as well as the angiopoietin receptor revealed no statistically significant difference between EPO-treated and placebo-treated animals. In a third murine femoral fracture model, all animals receiving 500 IU/kg EPO daily obtained fracture union, while the fusion rate was only 50% in the control group after 5 weeks (Garcia et al. 2011). In contrast to their previous study, the authors rejected the hypothesis of increased cell proliferation as a means of facilitating the osteogenic action of EPO. However, increased levels of circulating endothelial progenitor cells were observed along with decreased levels of the pro-inflammatory regulator NF-kB.

In conclusion, this group believes that the osteogenic potency of EPO is rooted in its ability to decrease inflammation and downregulate NF-kB in particular (Garcia et al. 2011). In contrast to their first study, no decline in the osteogenic effect of EPO was observed in their latest study; however, a serious drawback for clinical translation was the extremely high hemoglobin levels (see below).

In a rabbit mandibular distraction model of osteogenesis, four injections of a physiological dose of 150 IU/kg EPO increased the number of osteoblasts and blood vessels 30 days after the operation (Mihmanli et al. 2009). The number of osteoclasts was also reduced.

The described net bone-forming capacity of EPO is not unopposed. Singbrant et al. raised the intuitive hypothesis that EPO primarily increases erythropoiesis in the bone marrow. It follows that expansion of the bone marrow cavity at the expense of trabecular bone is a plausible consequence. Accordingly, their published results show a decrease in trabecular bone and an increase in bone remodeling in mice. They also found that EPOR is restricted to the hematopoietic linage and that osteoblast progenitors, Kusa4b10 cells, did not differentiate into osteoblasts when stimulated with EPO. Finally, they reported that because of the lack of EPOR on mesenchymal cells, osteoclastogenesis only took part in vivo, but not in vitro. They conclude that EPO negatively regulates skeletal homeostasis (Singbrant et al. 2011).

A Canadian research group, led by Taichman, has also published several papers regarding the effect of EPO on bone. In 2008, they showed that hematopoietic stem cells (HSCs) are capable of inducing osteogenic differentiation of murine MSCs via paracrine stimulation with bone morphogenetic protein 2 and 6 (BMP-2, BMP-6) (Jung et al. 2008). This finding was substantiated in a subsequent paper which demonstrated that EPO activates JAK/STAT signaling in HSCs leading to BMP secretion (Shiozawa et al. 2010). In that study, daily EPO injections of 1500–6000 IU/kg for 28 days increased bone volume and the number of osteoblasts and osteoclasts in mice. Opposing the results of Singbrant et al., EPO increased the bone volume and decreased bone marrow cavity in the examined murine vertebra. Furthermore, these authors were the first to describe a direct osteogenic effect on MSCs and osteoclasts. While EPO increased the number of osteoclasts, it impaired their function via down-regulation of cathepsin K which resulted in net bone formation (Shiozawa et al. 2010; Kim et al. 2012). In their setting, 20 IU/ml EPO increased the osteogenic differentiation of hMSCs about 1.7-fold compared with the positive control group estimated with alizarin red staining (AZR) after 21 days (Kim et al. 2012). Regarding the intracellular signaling pathway, rapamycin, a specific inhibitor of mTOR, reduced the osteogenic differentiation of hMSCs independently of EPO stimulation (Kim et al. 2012). Discrepancies between mice and human stem cells were also reported in that paper. In another study in which a murine cranial defect was used, the very osteoinductive BMP-2 response was further augmented after repetitive, site-specific injection of 1000 IU/kg EPO. Here, EPO was administered every other day for up to 2 weeks (Sun et al. 2012). Besides improved bone formation, increased calcification and vessel ingrowth were observed. Interestingly, the cranial bone healing occurred via endochondral ossification rather than via intramembranous ossification (Sun et al. 2012). This treatment regime significantly increased erythropoiesis in spite of the authors' effort to limit the erythropoiesis-stimulating action and the potential side effects by decreasing the EPO dose compared with their prior study. The clinical feasibility of this approach is therefore limited. In conclusion, the current working hypothesis of Taichman's group is that "osteoclastogenesis is the primary means for EPO-improved bone regeneration" (Sun et al. 2012).

Sufficient blood supply is critical for bone regeneration in fracture healing and for musculoskeletal tissue engineering (Hankenson et al. 2011). Two main mechanisms are responsible for the formation of new blood vessels, also known as neovascularization. In angiogenesis, new vessels sprout from existing blood vessels. In vasculogenesis, on the other hand, blood vessels are formed from circulating endothelial progenitor cells without initial connection to pre-existing vessels (Isner and Asahara 1999). Regarding EPO, both of these mechanisms have been observed (Ribatti et al. 1999; Joshi et al. 2010). In this light, EPO-induced neovascularization could potentially resemble an indirect way of promoting bone regeneration via an improved microenvironment and nutrient supply.

Chemotaxis; EPO has been shown to attract MSCs and endothelial progenitor cells. Although increased cell migra-

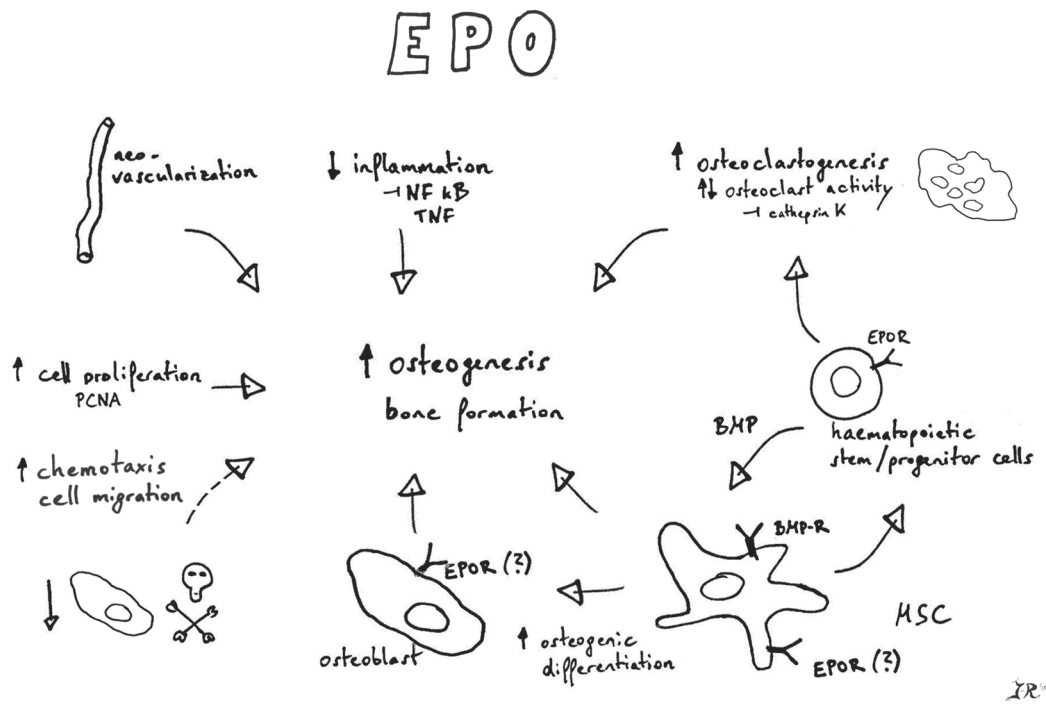


Figure 2. The effect of EPO on bone. Mechanisms leading to increased bone formation are illustrated.

tion was observed at EPO doses ranging from 1 IU/ml to 1000 IU/ml, the chemotactic effect peaked at 100 IU/ml EPO (Anagnostou et al. 1990; Chen et al. 2008; Koh et al. 2009; Yu et al. 2011; Nair et al. 2013). Both this chemotactic potency and the angiogenic and osteogenic potential of EPO are of utmost interest for musculoskeletal tissue engineering. Notably, EPO may be utilized to overcome the major limitation of large-scaled, three-dimensional cell-based tissue regeneration, which is that it fosters vascularization and bony ingrowth in large 3D scaffolds.

In 2012, a summary was published presenting the state of knowledge at that time about the effects of EPO on bone and the mechanisms involved (Figure 2) (McGee et al. 2012). This review was published before the results of Papers 1-3 were presented and before the writing of the present thesis. Since publication of that review, it has been shown that osteoblasts produce EPO, which acted in paracrine and systemic fashion (Rankin et al. 2012). In fact, the production of EPO by osteoblasts was sufficient to drive erythropoiesis.

In conclusion, several research groups have intensively investigated the osteogenic potency of EPO. Hallmarks on the road to clinical translation: firstly, adequate dosing preventing adverse effects and excessive erythropoiesis; secondly, a deeper understanding of the receptors and signaling pathways involved; and, thirdly, testing of the efficacy of EPO in large-animal models, must be accomplished. The aims and hypotheses of this PhD thesis are therefore highly relevant and have not been answered before.

Aims and hypotheses

The general aim of this PhD project was to investigate the efficacy of EPO in promoting bone regeneration in pre-clinical experiments. Furthermore, the aim was to pave the way for clinical translation through the investigation of the cellular ways of action, the establishment of a dose-response relationship in vitro, and the application of a clinically safe dose in a large-animal study.

The hypotheses of the three papers were:

Paper 1: Continuous, systemically administered low-dose EPO increases bone volume and bone quality after spinal fusion.

Paper 2: EPO stimulates the osteogenic differentiation of hMSCs in a dose-dependent manner through direct, receptor-mediated signaling that triggers multiple intracellular pathways.

Paper 3: Site-specific, single low-dose EPO can augment bone defect healing.

Methodological considerations

Study design

Paper 1

In Paper 1, the following reasoning governed the choice of the rabbit as an animal to study the efficiency of EPO in the setting of a posterolateral spinal fusion. First, the fusion rate in rabbit is comparable to that in man, the model has been much used in the past, and the standard deviation of the primary outcome evaluation was well defined which allowed the performance of an accurate sample size calculation prior to the investigation (Khan and Lane 2004; Choi et al. 2007). Second, compared with large-animals it is a cost-effective screening tool and its implementation at our institution was feasible.

The systemic administration of EPO hindered a paired within-subject study design. Hence, an unpaired study design that does not eliminate biological variability between animals was chosen. The power was 80% and the significance level 5%. According to past literature, the relative standard deviation was 16% when the same methodology was applied to assess the primary outcome measure, bone volume (Choi et al. 2007). The estimated effect size was a 20% increase in bone formation. According to these parameters, at least 13 animals needed to be available for analysis in both the control group and the treatment group. Taking safety precautions, we included 17 animals in each group. This sample size is large compared with the median sample size of eight rabbits (range 2–25) used in this model (Riordan et al. 2013). However, many animal studies are underpowered and that may lead to false conclusions about the efficacy of a studied intervention (van der Worp et al. 2010).

At the time of the experiment, we were concerned about potential limitations of this model which included the subjective evaluation of the fusion rate by manual palpation, the potentially negative influence of daily handling when injecting EPO, and, last, the generalizability of the results. However, the validity of our conscious choices when designing the rabbit spinal fusion study was confirmed in a recently published meta-analysis (Riordan et al. 2013). We chose to blind the surgeons who used a paramedian approach to implant 2.00 g autogenous iliac crest bone graft per side in treatment-randomized, female New Zealand white rabbits weighing above 3.5 kg. In comparison, the meta-analysis recommends the use of skeletally mature rabbits (>6-months old, >3 kg) and the use of 1–2 cm^3 autograft in addition to blinding and treatment randomization. Furthermore, the feasibility of the subjective, but validated evaluation method of manual palpation for the assessment of spinal fusion was stressed. However, computed tomography (CT) and other analyses should be employed to study other outcome measures (Riordan et al. 2013). The primary outcome measure of Paper 1 was bone volume assessed with CT (Rölfing et al. 2012).

The pharmacokinetics and pharmacodynamics of EPO in animals may differ from those seen in humans. The higher metabolism of small animals could potentially imply a faster elimination of EPO from the body. Furthermore, the dose-response relationship could vary. However, this remains speculation and needs further investigations in other studies.

Paper 2

The aim of Paper 2 was to elucidate the cellular mechanisms of EPO. We chose commercially available cells (Lonza Inc., Allendale, NJ, USA) because the burden of proof to confirm that the cells were hMSCs was lifted by Lonza Inc. itself. According to Keating, the term human mesenchymal stromal cells (hMSCs) should be used if cells exhibit plastic adherence, differentiation to adipogenic, chondrogenic and osteogenic cells and if they express the surface markers CD105, CD73, and CD90, while hematopoietic markers are absent (Dominici et al. 2006; Keating 2012). The term mesenchymal stem cell instead of mesenchymal stromal cell should only be used if the cells have documented self-renewal and differentiation characteristics. Certificates of analysis are available from www.lonza.com. Furthermore, the use of commercially available cells allows other researchers to repeat our experiments. The biological variability and hence the external validity of the results was increased by testing hMSCs from two different donors. In order to maximize the internal validity, technical variability was accounted for by multiple technical replicates and by conducting all experiments two or three times. The study design secured the comparability of the effect size of 20 IU/ml EPO with the results published by Kim et al. (Kim et al. 2012). In fact, they also used cells from the same source, and they used a similar positive control and an observation time of 21 days. Because the dose-response relationship of the non-hematologic effects of EPO remains undefined, a wide range of concentrations (0-100 IU/ml) was applied.

Paper 3

In Paper 3, a large-animal model was chosen to pave the way for clinical translation. The swine compares favorably with man in terms of bone healing rate (Schweiberer and Büchner 1977; Hönig and Merten 1993; Bouxsein et al. 2010; Dempster et al. 2013). The porcine animal model also fulfills many of the requirements of an optimal model as it allows a paired within-subject study design with multiple testing sites. Furthermore, the required facilities and animals were locally

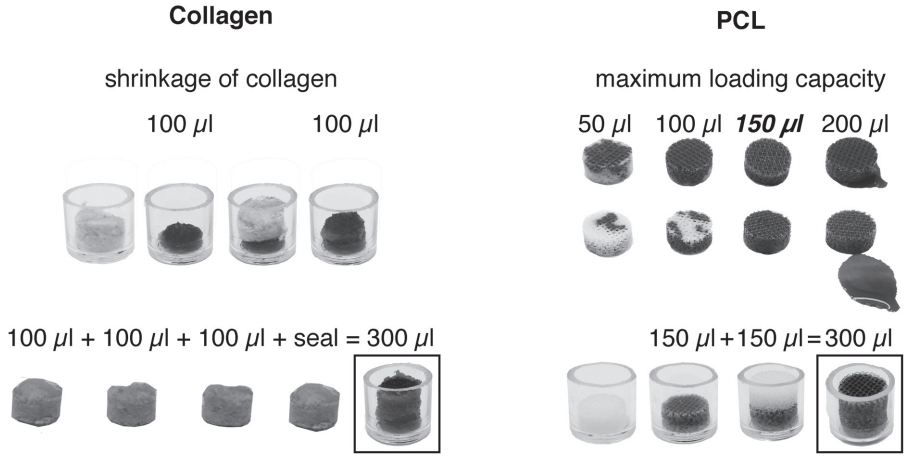


Figure 3. Insertion and loading of collagen carrier and PCL scaffold.
Left panel: The maximum loading capacity of the collagen scaffold was 100 µl, which caused significant shrinkage. Hence, three collagen scaffolds were inserted in a stepwise manner and loaded with 100 µl. The fourth scaffold served as a seal.
Right panel: The maximum loading capacity of the PCL scaffold was 150 µl, which resulted in equal distribution throughout the scaffold. The top and bottom of the loaded PCL scaffolds are shown. Two PCL scaffolds (Ø 10 mm, h = 5 mm) were inserted in each defect.

available, and the model had been established at our institution, which eliminates most learning curve issues (Compston et al. 1986; Jensen et al.).

The sample size was guided by our previous experiments in which this model was used and by literature reports (Compston et al. 1986; Stockmann et al. 2012; Jensen et al.). The same power, significance level, and expected effect size as in Paper 1 were applied. Our previous data showed a large standard deviation in the primary outcome measure, bone volume fraction (BV/TV), which denotes the bone volume relative to the volume of the bone defect. As the thickness of the frontal and parietal bone increases from anterior to posterior, we assumed that the regeneration potential would also vary and consequently that the large standard deviation was site-dependent. To diminish the standard deviation, Paper 3 was designed to make three pairwise comparisons across the midline. To enable the investigation of the site-dependency in a subsequent analysis extending beyond this thesis, each treatment group was chosen to be in each position three times. Consequently, the sample size of the six groups was equal (n = 18). The localization of the three pairs was determined by block randomization, and EPO treatment was either randomized to the left or the right side of the calvaria. These measures ensured that the results were not biased by a possible site-dependent regenerative potential at the three locations (Baas 2008).

The limitations of this model include a difference in development compared with most other bones. In embryogenesis, the calvaria develops through intramembranous ossification rather than endochondral ossification. However, endochondral healing was observed in a murine calvarial defect model (Sun et al. 2012). Another limitation is the absence of weight bearing, which may result in an alternation of the healing response in other clinically relevant settings. Furthermore, the morphology and almost avascular nature of the parietal and frontal bone, which become apparent when drilling holes without bleeding, is likely to change the bone healing mechanism and could potentially limit access to circulating progenitor cells.

The stepwise scaffold insertion of two PCL scaffolds (Ø 10 mm, height 5 mm), each loaded with 150 µl, and four collagen scaffolds (Ø 10 mm), whereof three were loaded with 100 µl and the last functioned as a seal, was determined by the maximum loading capacity of the scaffolds and the volume of the loaded scaffolds (Figure 3). Importantly, the hydrophilicity of the PCL scaffolds was increased with sodium hydroxide treatment.

Observation time

Holstein et al. described an accelerated bone healing with a declining effect size over time (Holstein et al. 2007). Relatively short observation times, e.g. 6 weeks in Paper 1 and 5 weeks in Paper 3, therefore were chosen to maximize the chance of finding statistically significant differences between groups. Here, we applied the same rationale as that described by Baas (2008). In this time window, in which the slope of the healing/bone formation rate is steepest, the likelihood of detecting differences between groups peaks (Figure 4). At the chosen observation time, healing was expected to be at a stage at which differences in bone formation and neovascularization between the groups would be identifiable if present.

Furthermore, it is clinically relevant to assess the time to radiological and clinical healing as well as the final result. This

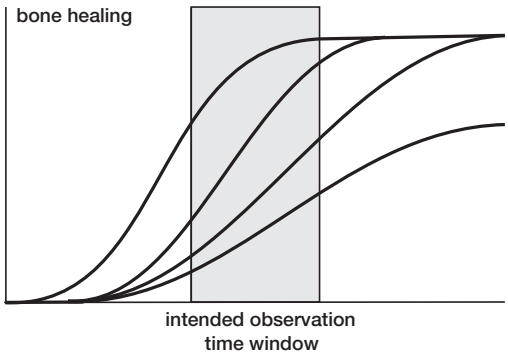


Figure 4. The observation time of Papers 1–3 was intended to be within the illustrated time window.

could, for example, involve long-term assessment of fracture union and bone quality after healing had ended. Because of the initial reports by Holstein et al., we chose to use early time points to evaluate the potential acceleration of bone healing owing to the use of EPO. In the clinic, an increased initial healing rate could potentially shorten the recovery period.

Regarding Paper 1, it should be noted that the fusion rate does not increase any further after 6 weeks of observation in the rabbit posterolateral spinal fusion model according to Boden et al. (Boden et al. 1995). The meta-analysis confirmed that most researchers followed this recommendation, but an even shorter observation time of 4-5 weeks might be sufficient to determine the fusion rate in this model (Riordan et al. 2013). However, this statement is controversial and hence a matter of debate (Rölfing and Bünger 2013b).

Regarding Paper 2, the observation time was chosen based on previously published reports using a similar set-up (Kim et al. 2012). Moreover, the cultured cells detached from the plates at later time points than 14 days in pathway inhibitor experiments, which limited the intended observation time of 3 weeks.

Regarding Paper 3, the follow-up time was chosen to match the application of a commercially available collagen scaffold, which is absorbed within approximately 3 weeks via phagocytosis and enzymatic degradation according to its product information (Sangustop, Aesculap AG, Tuttlingen, Germany). The observation time of 5 weeks was also chosen based on the results of a previous longitudinal study in which this animal model was used (Stockmann et al. 2012).

Outcome evaluations

Given the different types of pre-clinical studies performed, numerous different outcome evaluations were applied. The primary outcome evaluations were the estimation of bone volume with CT scans after rabbit posterolateral fusion in Paper 1, the determination of the mineralization rate with Arsenazo III and AZR assays read on an ELISA reader in Paper 2, and the estimation of BV/TV with high-resolution quantitative CT and histomorphometry in Paper 3.

Figure 5. Illustration of the defined region of interest, fusion mass (white), and its delineation towards the adjacent vertebral body (grey).

Estimation of bone volume with CT scans

Choi et al. described the assessment of the volume of the fusion mass after spondylodesis using autograft (Choi et al. 2007). Post-mortem, neither the radiation dose nor movement artifacts are limiting factors and it is therefore possible to apply the optimal technical configuration of the CT scanner to achieve the best possible spatial resolution. The maximal spatial resolution is defined by the minimum slice thickness of the CT scanner. Using a 64 Slice Dual Source SOMATOM Definition CT scanner (Siemens AG Medical Solutions, Erlangen, Germany), the best technically spatial resolution was 0.6 mm. Because the animals neither breathe nor move, scans could be performed with 50% overlap to further increase the accuracy. The acquisition parameters were 150 Eff.mAs, 100 kV, 0.6 mm slice thickness, 0.3 mm increment, and 0.5 mm pitch. Image reconstruction of the acquired raw data is equally important. To preserve the high resolution and to achieve an isotropic three-dimensional spatial resolution of 0.6 mm, the field of view was set to 307 mm, and a B40f medium filter was applied. On these axial CT images, the region of interest was defined as previously described (Choi et al. 2007). In brief, a blinded observer encompassed the fusion mass while having the coronal and sagittal planes on the screen at the same time. This procedure was important as defining the medial border of the fusion mass can be challenging due to the adjacent vertebral body. If the adjacent vertebral body interfered with the fusion mass (Figure 5), transversal images above or below were taken into consideration when defining the medial border. When all sections were encompassed, the fusion volume was computed from the two-dimensional areas of interest.

The internal validity of the study could potentially have been larger, if the inter- and intra-observer variability had been reported after performing multiple independent, blinded analyses.

An alternative semi-automatic approach was considered when planning the evaluation method. Automatic bone volume rendering, in which the observer only defines the cranial, caudal, and lateral borders and a threshold of Hounsfield units

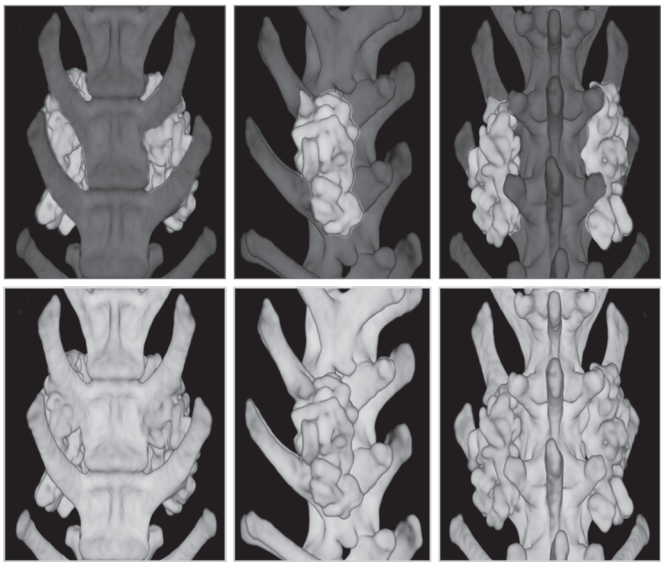


Figure 6. 3D reconstructions of vertebrae L4–L6.
Upper panels: The method applied in Paper 1, which estimates the volume of the fusion mass only, is shown. The adjacent vertebral bodies (grey) are delineated and not measured.
Lower panels: An alternative semiautomatic method estimating the combined volume of the fusion mass and vertebrae (L4) + L5 + L6 + (L7) is illustrated.

could have diminished the subjective element of defining the borders of the fusion mass. For instance, it would have been possible to define a volume of interest from the upper endplate of the vertebral body L4 to the lower endplate of L6 and laterally bounded by the tip of the transverse processes. This could have been followed by computing the bone volume with a predefined threshold (Figure 6 lower panels). However, if this method had been applied in Paper 1, the contribution of the bone volume of vertebra bodies L4–L7 would have biased the result. This assumption is based upon the hypothesis that the bone volume of L4–L7 is highly correlated with the weight of the animal, which was 3.792 ± 0.324 kg. Hence, this standard deviation of about 8.5% would have had substantial influence on the measured combined bone volume of (L4) + L5 + L6 + (L7) + fusion mass (Figure 6 lower panels). This is especially important, given the expected effect size of 20% difference in bone volume (see sample size calculation).

In Paper 3, the volume of interest was clearly delimitated from the adjacent bone, and this methodology was applied in high-resolution quantitative CT analysis because it enjoys the advantage of being more objective.

A limitation of Paper 3 was the resolution of the CT scanner (XtremeCT, Scanco Medical AG, Brüttisellen, Switzerland). Unfortunately, the μCT scanner (μCT 40, Scanco) used in Paper 1 was no longer available. Analyses were therefore performed with the best possible, but lower voxel size of 82 μm3 using an XtremeCT scanner. According to the Nyquist criteria, the trabecular thickness and space between trabeculae should at least be twice the voxel size to be accurately measured. Fortunately, our previous μCT data have shown that, first, a trabecular structure is present after calvarial healing and that, second, the trabecular thickness ranged from 150 to 210 μm. Thus, we were able to avoid that low resolution scans relative to the structure of interest may underestimate the bone mineral density due to partial-volume effects and overestimate object thickness (Bouxsein et al. 2010).

Histomorphometry and its correlation with 3D imaging

Histomorphometry is considered the gold standard for determining tissue morphology. Stereology allows the investigator to draw conclusions about the three-dimensional microscopic structures based upon the data from two-dimensional analysis of histological sections. The techniques and requirements regarding tissue processing including randomization, sampling, cutting, and probing have been described in detail (Vesterby et al. 1987; Gundersen et al. 1988). The application of these stereological principles includes thorough sampling and it is hence extremely time-consuming. Researchers therefore often sample only a limited fraction of the volume of interest. If the volume of interest is cylindrical (Paper 3), it is often the central section of the circle that is investigated; an approach that introduces a central selection bias (Schlegel et al. 2006; Baas 2008). Fortunately, this bias is negligible under most circumstances (Baas 2008).

Guidelines regarding the planning, conduction, and reporting of histomorphometric and quantitative CT analyses were followed, and the adherence to stereological principles increased the internal validity of the results (Bouxsein et al. 2010; Dempster et al. 2013). Inter- and intra-observer variation should be stated if possible, because such variation is reported to be substantial even with identical methodology (Compston et al. 1986). Depending on the analyzed outcome measure, the coefficient of variance may range from 9% to 69% for inter-observer variance and from 3% to 22% for intra-observer variance (Compston et al. 1986). These data highly threaten the internal validity and hence the external validity and generalizability of histomorphometric data in general. Nonetheless, it has been shown that correlation of the bone structural measurements between μCT and histomorphometry is very good, and even excellent when estimating bone volume to tissue volume (Müller et al. 1998; Chappard et al. 2005; Thomsen et al. 2005). The latter was the primary outcome measurement in Paper 3. Consequently, quantitative CT can be employed to obtain reliable morphometric results in less time than two-dimensional histomorphometry. In the author's opinion, the biggest advantage of quantitative CT compared with histological analysis is that the entire volume of interest is analyzed rather than multiple two-dimensional samples, and that the analysis is semi-automatic and therefore less subjective. Recent advances in μCT protocols also enabled the assessment of the secondary outcome measure in Paper 1, vascularization (Fei et al. 2010). However, that protocol has not been implemented at our institution so far.

Importantly, the term "bone" is ambiguous and may convey one of three different meanings. The first meaning, defined by the American Society of Bone and Mineral Research, is: bone matrix including mineralized and not yet mineralized matrix (osteoid). The second is: mineralized bone matrix excluding osteoid; and the third meaning is bone as a tissue including bone matrix, bone marrow, and soft tissue; in the interest of terminological uniformity, the third meaning should be referred to as bone tissue (Dempster et al. 2013). A major disadvantage of radiological analysis is that it can only visualize mineralized bone, but not osteoid. For the quantification of osteoid as an indicator of ongoing bone formation, histomorphometric evaluation is required. Notably, bone remodeling occurs throughout life, and bone formation and osteoid will therefore always be present to some degree. Furthermore, light microscopy enables the observer to discriminate between mineralized bone as either old (lamellar) and newly formed (woven) bone because of its morphology, and it allows the observer to quantify fibrous tissue, blood vessels, and other outcome measures of interest.

Mineralization assays

Mineralization is an essential step in bone formation. In vitro, a large array of methods is available for the determination of calcium deposition, including Von Kossa staining, Arsenazo III assay, AZR staining and AZR assay (Gregory et al. 2004). Both the Von Kossa staining and the Arsenazo III assay have certain limitations. Only qualitative or semi-quantitative evaluation under the microscope is possible with the Von Kossa technique. The Arsenazo III assay estimates calcium deposition, however its disadvantage is that it is a quantitative method only. Consequently, other means to visualize and measure mineralization are preferred at our institution. AZR was and is still the standard method endorsed at our institution, because it is versatile and allows the both quantitative evaluation of mineralization with an ELISA reader and inspection under the microscope. The basic principle of AZR is that it precipitates in the presence of calcium. Previously, the employed AZR protocol included the quantitative de-staining with cetylpyridinium chloride (Stiehler et al. 2008). While conducting the experiments of Paper 2, the material safety classification of cetylpyridinium chloride was unfortunately altered and its use was prohibited at our institution. Therefore, before the altered version of the AZR protocol using 5% SDS in 0.5 M HCl for quantitative de-staining was tested and validated, an Arsenazo III assay was used in parts of Paper 2 as previously reported (Stiehler et al. 2009).

The limitations of the staining methods include the need to wash the cells many times to remove excessive staining. At late time points in Paper 2, this repeated rinsing step resulted in detachment of the cells from the culture plate, which made them unavailable for analysis.

Other means to verify the osteogenic differentiation of cells, such as DNA, RNA, and protein analyses at earlier time points are frequently described and often required for publication in high-ranking, peer-reviewed journals. However, measuring the primary endpoint of mineralization after 10, 14, and 21 days fulfills the call to minimize the number of statistical tests, and it moreover saves time and financial expenses as compared to using the additional methods. Nevertheless, the secondary outcome evaluation of alkaline phosphatase assay (ALP) was employed to investigate early time points in Paper 2.

Ethical considerations

Under the heading "the removal of inhumanity", Russel and Burch published a concept known as the "three R's" of laboratory animal science in 1959: "replacement" meaning the substitution of animals of insentient material if possible; "reduction" meaning limiting the number of animals sacrificed; and "refinement" meaning the improvement of animal welfare (Russell et al. 1992). These values are today the cornerstones of humane animal research.

In order to facilitate implementation of these general aims in pre-clinical studies using laboratory animals and to improve scientific publication standards, the ARRIVE guidelines and the Gold Standard Publication Checklist (GSPC) were published (Kilkenny et al. 2010; Hooijmans et al. 2011). While the authors of the latter guidelines may provide a more detailed checklist, accreditation and pre-clinical use of these guidelines favor the ARRIVE guidelines with currently 231 vs. 47 citations. Either of these guidelines should be followed when conducting pre-clinical studies in animals.

The continuous need for improvement of existing animal models in orthopedics on the road to clinical translation has been described (An and Friedman 1999). To ensure that ethical considerations are taken into account in animal models, minute planning and execution are mandatory. This includes the proper selection of animal species, procedure, observation time, and sample size calculations to fulfill the requirement of "reduction", which implies using the least number of animals to answer the scientific questions with reasonable probability.

In the present thesis, these principles were followed. In brief, we performed sample size calculations, defined humane endpoints and applied the stipulated guidelines. Furthermore, all experiments complied with local laws and were approved by The National Authority, no. 2006/561-1178 and 2012-15-2934-00362.

Statistical considerations

Sample size calculation

As a direct consequence of the discussed ethical considerations, a sample size calculation should be performed before pre-clinical animal studies are conducted. A sample size calculation justifies the size of the study populations and demonstrates that

the study is capable of answering the scientific questions posed (Kirkwood and Sterne 2003). Its key goal is to avoid waste of time and resources and it should thus be in the very interest of any researcher besides being a requirement of many funding agencies, journals, and universities. The factors that determine the number of study objects needed are listed below.

The power of a study is the probability to detect a true positive effect. The power thus describes a study's capability to reject a false null hypothesis. The power of a study is also called the sensitivity of a study meaning the chance of finding that two study populations statistically significantly differ when in fact they are not identical. The risk of false negative results (type II error) is given by the equation 1 – power. In this thesis, a power of 80% was chosen. Given that the effect size and standard deviations were as predicted during sample size calculations, the chance of success was only 80%. Increasing the power or likelihood of success would have had large consequences for the sample size.

The significance level, meaning the strength of the evidence required to reject the null hypothesis, is most often 5% ($p \leq 0.05$) in biomedical research, including the present thesis. The significance level is also the alpha value, which denotes the maximal risk of false positive results, e.g. rejecting a true null hypothesis (type I error). If we had chosen a smaller P-value, the required sample size would have been larger.

The most difficult part of conducting meaningful sample size calculations is to predict the minimal clinically relevant difference, e.g. the effect size of the intervention in the population. The smaller the true effect size, the larger the sample size needed.

The standard deviation is inversely related to the sample size. Both the biological variability between study objects and the technical variability, e.g. the precision of the methodology applied, determine the standard deviation. Both the size of the true effect and the standard deviation should be based upon the primary outcome measure of the study. If comparable studies and evaluation methodology have previously been published, such literature is the best resource to estimate approximate values of the effect size and the expected standard deviation.

Another important factor to consider is the study design. For instance, a paired study design measuring the same variable within a subject at different time points is statistically much stronger than an unpaired design analyzing the variable in two different subjects. In fact, the sample size needs to be doubled if an unpaired design is chosen (Johansen 2002; Baas 2008).

The calculated sample size is the number of objects available for analysis. This number needs to be sufficiently large to account for the dropout or exclusion of study objects throughout the study. Formulae for sample size calculations can be found in medical statistics textbooks (Johansen 2002; Kirkwood and Sterne 2003). The right formula must be applied based on the outcome measure (mean, rate, proportion, odds ratio, etc.) (Campbell et al. 1995).

Multiple testing

Multiple testing, relevant when testing several treatment groups or time points, increases the risk of false positive results (type I errors) and inflates the significance level. This disadvantage can be accounted for with alpha adjustments. In this context, the Bonferroni correction is commonly used; here the P-value is divided by the number of statistical tests performed before statistical significance is reported. However, statistically stronger means of correction have also been published. The preferred solution of the author is to state the actual uncorrected P-values, to disclose this approach in the materials and methods section, and consequently let the reader interpret the significance of the results. In general, the least possible number of statistical tests should be applied.

Summary of Papers

Paper 1: Erythropoietin augments bone formation in a rabbit posterolateral spinal fusion model

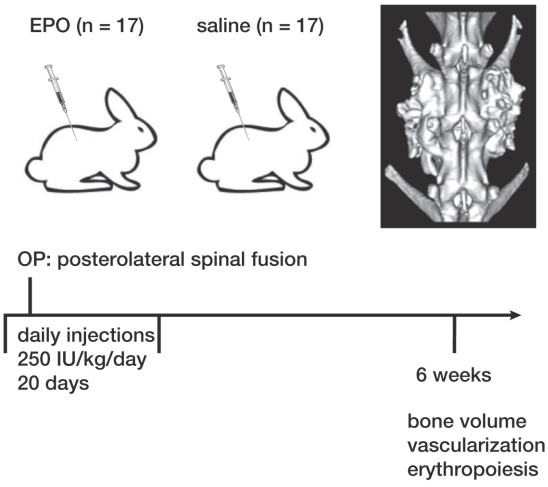


Figure 7. Study design of Paper 1.

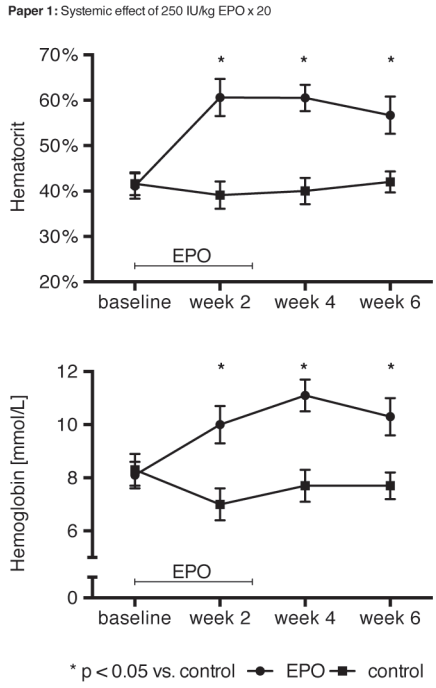


Figure 8. Hematocrit and hemoglobin levels after 20 subcutaneous injections of 250 IU/kg EPO.

The aim of Paper 1 was to test the efficacy of low-dose, continuous EPO as an autograft enhancer (Rölfing et al. 2012). Based upon the results achieved by Holstein et al., we hypothesized that EPO facilitates angiogenesis and that this leads to improved bone formation and consequently an increase in the spinal fusion rate. In this unpaired study, 34 adult male New-Zealand white rabbits underwent a standardized posterolateral fusion procedure (Boden et al. 1995). The animals received subcutaneous injections of either EPO (250 IU/kg epoetin beta, n=17) or saline (n=17) for 20 days starting 2 days prior to operation. The primary outcome measure was bone volume assessed with CT evaluation ad modum Choi after 6 weeks (Choi et al. 2007). Vascularization was assessed with histomorphometry of actin-stained vessels by an independent blinded laboratory technician. The fusion rate, which is a clinically relevant outcome measure, was determined both with CT, X-ray, and manual palpation by two blinded, independent observers: a senior radiologist and a spine surgeon. Furthermore, the hematopoietic effect was investigated with blood samples drawn 2, 4, and 6 weeks postoperatively. Bone morphometry within the fusion mass was analyzed using µCT.

The main finding of this study was that EPO increased bone volume to 3.85 cm^3 (95% CI 3.66–4.05) compared with 3.26 cm^3 (95% CI 2.97–3.55) in the control group ($p < 0.01$). Bone morphometric µCT analysis did not show significant differences in bone micro-architecture within the fusion mass. Notably, a trend was observed towards an increased fusion rate, which is of utmost clinical importance. An indirect way of osteogenic action of EPO was found, as vascularization was doubled in the EPO group ($p < 0.01$). Other potential ways of action were not investigated in this study. The systemic effect of EPO was documented. EPO could only be detected during rhEPO treatment, because the employed rhEPO ELISA kit did not detect rabbit EPO. However, the biological response of rhEPO was documented by hematocrit levels reaching 60.6 ± 4.1% after 2 weeks (Figure 8).

The need to alter the applied continuous, low-dose treatment regime was evident if clinical translation should be possible. In conclusion, EPO increased the bone volume and can hence be classified as an autograft enhancing factor. Further in vitro and in vivo studies were warranted.

Paper 2: The osteogenic effect of erythropoietin on human mesenchymal stromal cells is dose-dependent and involves non-hematopoietic receptors and multiple intracellular signaling pathways

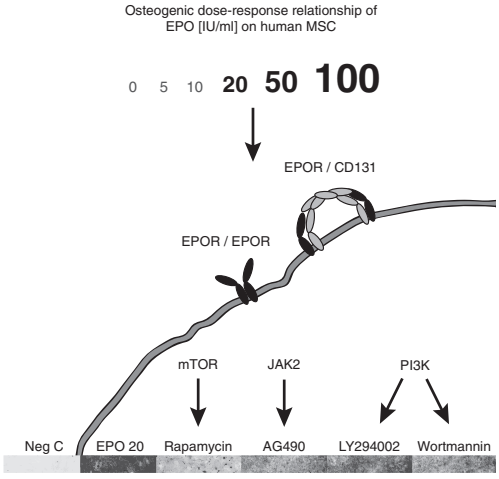


Figure 9. Graphical summary of Paper 2.

The main purpose of Paper 2 was to determine the minimum therapeutically effective dose also known as the lowest effective dose on hMSCs and to describe the dose-response relationship over a wide range of EPO concentrations (0, 5, 10, 20, 50, 100 IU/ml). Furthermore, the presence and co-expression of the cell surface receptors EPOR and CD131 and the involvement of intracellular signaling pathways were investigated (Rölfing et al. 2013). Commercially available hMSCs from two donors were either cultured in proliferation medium (negative control), osteogenic medium (positive control), or osteogenic EPO medium for up to 21 days. Mineralization assays were used as the main outcome evaluation to determine the lowest effective dose and the involvement of the three intracellular signaling pathways in pathway blocking experiments. The investigated pathways were (1) mTOR signaling blocked with rapamycin, (2) JAK2 abrogated with AG490, and (3) PI3K reversibly impeded with LY294002 and irreversibly impeded with Wortmannin. Quantitative flow cytometry and qualitative confocal microscopy for EPOR and CD131 were utilized in receptor experiments.

The highlights of this study were that it represents the first demonstration of a proportional dose-response relationship for the osteogenic effect of continuously applied EPO on hMSCs. The lowest effective dose was 20 IU/ml. Both EPOR and CD131 were expressed on the cell surface; however, only 22.3–58.1% of hMSCs expressed EPOR. Qualitative confocal microscopy suggested that this percentage in fact was higher, and the flow cytometry data were likely to be biased by technical challenges. This finding in conjunction with the absent effect at low doses indicated that the osteogenic functioning was exercised through the proposed non-hematopoietic EPOR/CD131 receptor (Brines and Cerami 2008). In agreement with a review about the pleiotropic effects of EPO in other tissues, we observed that multiple intracellular signaling pathways were responsible for yielding the maximal osteogenic effect on hMSCs (Figure 10) (Brines and Cerami 2008).

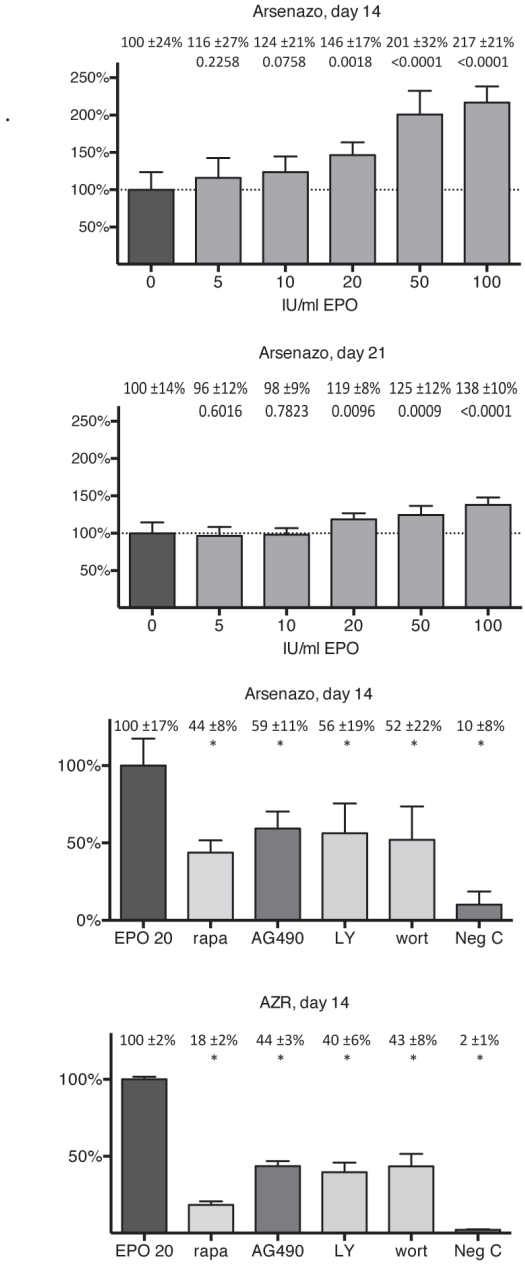


Figure 10. The osteogenic effect of EPO in vitro.
Upper two panels: Dose-response relationship on hMSCs and determination of the lowest effective dose with mineralization assays Arsenazo after 14 and 21 days.
Lower two panels: Investigation of the involved intracellular pathways, mTOR blocked with rapamycin (rapa), JAK2 with AG490, and PI3K with LY294002 (LY) and Wortmannin (wort). Proliferation medium served as negative control (Neg C). Arsenazo and AZR after 14 days are shown. * $p < 0.0001$

Paper 3: A single topical dose of erythropoietin applied on a collagen carrier enhances calvarial bone healing in pigs

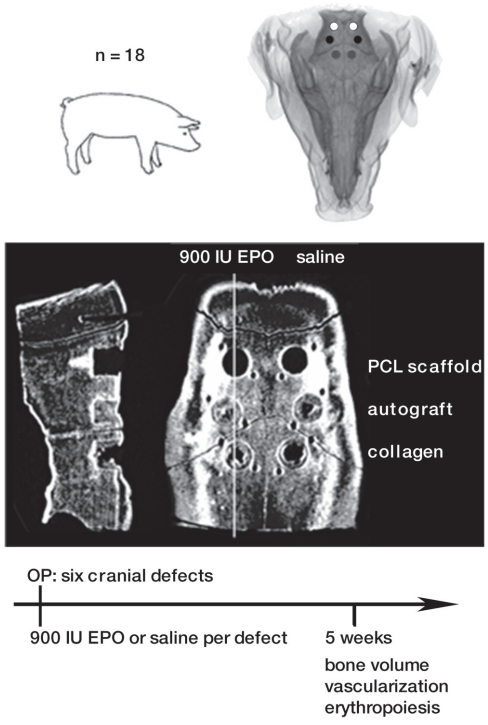


Figure 11. Study design of Paper 3.

In order to enable clinical translation of the promising data from cell studies and small-animal studies, it was necessary to test the efficacy of EPO in a large-animal model and to use a treatment regime that does not increase hematological quantities to unacceptably high levels (Rölfing et al.). Aiming for clinical progress and feasibility, the purpose of Paper 3 was to evaluate the efficacy of a single, low-dose EPO to stimulate bone healing in swine.

The hypothesis was that 900 IU/ml site-specifically applied EPO increases bony ingrowth in a porcine calvarial defect model. The combined dose of EPO per animal was 18.5 IU/kg. A within-subject study design allowed the pairwise comparison of in total six cranial defects (Ø 10 mm, height 10 mm) in adolescent pigs. The three comparisons were 1) autograft ± EPO, 2) collagen scaffold ± EPO, and 3) PCL scaffold ± EPO (Figure 12). The primary outcome measure was the bone volume fraction (BV/TV) assessed with quantitative CT (82 μm^3 voxel size) after 5 weeks of observation. Secondary outcome measures included histomorphometry and blood sample analyses to document a potential systemic effect.

The main finding of this paper was that a single topically administered low dose of EPO increased bone healing in the collagen group. However, the median effect size was moderate. The gold standard, autograft, exhibited an excellent healing capacity and was able to regenerate the bone defect almost

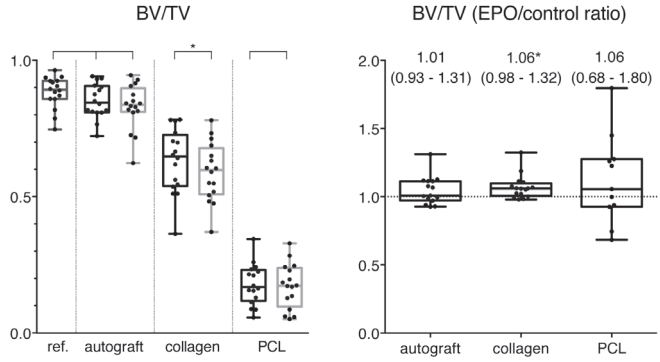


Figure 12. BV/TV results assessed with quantitative CT (82 µm3 voxel size). Healthy bone comprised a reference (ref.). Median (min-max) are given. * p = 0.001.

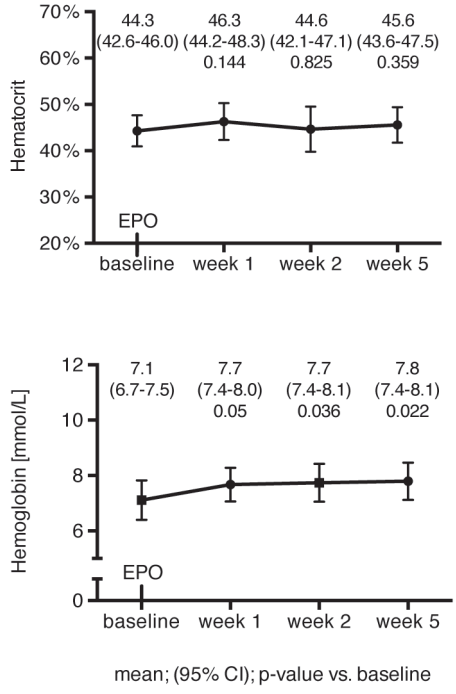


Figure 13. The systemic effect of a single topical EPO administration of 18.5 IU/kg on hematocrit and hemoglobin.

completely. The PCL scaffold inhibited bone regeneration and performed worse than the negative control group, a saline-soaked collagen carrier.

Notably, vascularization did not statistically significantly differ between EPO-treated and placebo-treated defects.

The systemic effects of a single topical dose of 18.5 IU/kg EPO are depicted in Figure 13. None of the observed differences were considered statistically significant after correction for multiple comparisons.

In summary, EPO increased bone healing when applied on a collagen carrier. Despite the moderate effect size, the applied dose is considered to be safe and could potentially be applied in the clinic.

Discussion of the results

Confirming and contrasting literature reports

The key findings of this PhD thesis were that continuous, systemically administered low-dose EPO increased bone volume in a rabbit spinal fusion model. Furthermore, a significant increase in vascularization was found as presented in Paper 1, but not in Paper 3. EPO stimulated the osteogenic differentiation of hMSCs in a dose dependent manner through direct, receptor-mediated signaling likely via EPOR and CD131 and triggered JAK2, PI3K, and mTOR intracellular pathways. Finally, topical, single low-dose EPO augmented the bone volume if applied on a collagen carrier, but failed to improve the healing capacity of PCL in a porcine calvarial defect model.

An extensive exploration of how the results of Papers 1–3 compare with the published literature is given in the individual papers, to which the reader is kindly referred. In the following the internal and external validity of the results of this thesis will be discussed.

Internal validity

The internal validity of a study is the extent to which the observed effect of a treatment can be attributed to the treatment rather than to chance or a systematic distortion of the results, i.e. a bias. In other words, the term internal validity describes the reliability and reproducibility of a given study. The internal validity of a study must be excellent in order to allow conclusions to be drawn and generalizations from the achieved results to me made. The generalizability, also known as the external validity of the presented results, consequently depends upon an adequate study design and a sound study conduct. According to van der Worp et al., the internal validity is threatened by four types of bias (van der Worp et al. 2010).

Selection bias can arise from biased allocation to treatment groups and can be prevented by randomization and concealed allocation. Randomization was performed in Paper 1 and 3. However, concealed allocation was only feasible in Paper 1. Comparable baseline characteristics between groups show effective randomization in Papers 1 and 3. In Paper 2, for any given outcome evaluation both treated and control cells were seeded from the same flask onto the same 96-well plate. No selection bias was therefore supervened in Paper 2.

Performance bias can occur if there was a systematic difference in care between the treatment groups, other than the studied intervention. This potential source of bias was accounted for by placebo treatment in Paper 1 and 3. However, the results may have differed if, for instance, control animals were not injected with saline and therefore handled and cared for in a different manner than the EPO-treated animals in Paper 1. In this model, postoperative handling is known to impair bone healing (Feiertag et al. 1996; Riordan et al. 2013). The risk of performance bias was reduced by blinding the surgeons in Paper 1 and by exchanging all media at the same time points in Paper 2.

Detection bias can systematically distort the results if the observer assessing the outcome has knowledge of the treatment assignment. The author of this thesis analyzed the main outcome evaluations, the bone volume in Paper 1 and the bone volume fraction (BV/TV) in Paper 3. In Paper 1, independent persons performed randomization and postoperative handling. Consequently, both the surgeons and the observers were blinded. This measure ensured unbiased analysis. However, randomization, operation, and BV/TV analysis were performed by the same person in Paper 3. This potential source of bias was minimized by consecutive numbering of the 102 defects, which concealed the treatment information until statistical analysis was performed. Hence, the collection of the data, including the BV/TV calculations, was conducted blinded.

Attrition bias can be introduced by an unequal occurrence and handling of deviations from protocol and loss to follow-up between treatment groups. In Paper 1, 14 of 17 animals per group were available for analysis. Loss to follow-up was caused by perioperative death due to anesthetic complications and bleeding in three EPO-treated animals and two saline-treated controls, and one deep infection in the control group. Because of this and because of the blinding, attrition bias was non-existent in Paper 1. Paper 3 utilized a within-subject study design, and the loss to follow-up of a single animal therefore did not introduce this form of bias. Moreover, none of the available data were excluded before statistical analysis. In Paper 2, only outliers beyond the 99% quintile were excluded from the analysis of the primary outcome evaluation. This was primarily the case in very high-end measurements in the EPO group. This exclusion could potentially introduce a systematic distortion of the results; however, it would minimize rather than enlarge the difference between the groups. The correctness of excluding these outliers was confirmed by the fact that repetition of the experimental setup resulted in a similar effect size.

Besides bias, the internal validity relies also on the appropriateness of the method used to measure the outcome. For strengths and limitations of the primary outcome evaluations of Papers 1–3 please refer to *Methodological considerations.*

In conclusion, the described measures diminished potential sources of bias. In this light, the results and answer of the three hypotheses of the individual papers are internally valid.

External validity

External validity can be defined as the extent to which the findings of an experiment can be generalized to the human condition (van der Worp et al. 2010). The goal of most pre-clinical research is either to gain a deeper knowledge of the mechanisms governing an observed phenomenon or to pave the way to clinical translation of new treatment modalities. These were also the aims of the present thesis. Notably, the generalizability of the pre-clinical, experimental data with regards to the human condition is of utmost importance.

Designing an ideal study that enjoys both a high internal and external validity is challenging. In many experimental studies, the need to control variation caused by genetics or heterogeneity limits the external validity (Festing and Altman 2002). Hence, the potential disparity between the results of pre-clinical studies and clinical trials is partly rooted in the design of the pre-clinical studies the aim of which is often to maximize the chance of establishing statistical significance (van der Worp et al. 2010). Common causes of reduced external validity were summarized by van der Worp et al. (2010). The causes that also affect the generalizability of the findings of the present thesis include:

- First, we only studied degenerative disease in young healthy, female animals and therefore cannot necessarily extrapolate these findings to older, male animals – or, indeed, man;
- Second, the animals formed a rather homogeneous group, whereas patients comprise a rather heterogeneous group;
- Third, the induced disease/injuries may differ between animals and man;
- Fourth, the treatment timing and the doses applied in animal studies may not be clinically safe in man; and
- Fifth, there may be disparity in outcome measures in animal studies and clinical trials.

Any generalizability of the performed pre-clinical experiments into clinical practice should therefore be performed with caution. Below, these aspects will be discussed for Paper 1–3.

In Paper 1, the observed increase in bone volume in the presence of systemically applied EPO took place in the setting of a posterolateral spinal fusion procedure in healthy adult rabbits. In humans, this procedure is most commonly performed in elderly patients suffering from degenerative spinal disorders. Moreover, the main limiting factor for translation of the findings of Paper 1 into the clinical setting is the supra-physiological dose of EPO that leads to an extremely high hematocrit, which invites a concomitant risk of adverse events.

In Paper 2, hMSCs of two healthy donors were studied in two-dimensional monoculture. Besides the possibility that the actual biological variation in cell responses may be more pronounced than that observed in Paper 2, one must be aware that EPO elicits its osteogenic action not only through hMSCs. EPO also stimulates multiple additional cell-based mechanisms, i.e. osteoblasts as well as hematopoietic and vascular progenitor cells (Shiozawa et al. 2010; Guo et al. 2012; McGee et al. 2012; Rölfing et al. 2012). As describe above, the applied continuous dose is the biggest challenge for in vivo trials. On the other hand, in the setting of ex vivo tissue engineering that aims at designing three-dimensional bony constructs before implementation into the patient's body, the highest tested dose of 100 IU/ml is likely to be applicable without modification. Hence, the external validity of that finding of Paper 2 is expected to be high.

Paper 3 was designed to overcome the dosing issue of Papers 1 and 2 by investigating a single, topical dose in a large-animal model. If successful, this approach could be used in subsequent clinical trials. As expected, EPO did not have a significant systemic effect and the dosing is therefore considered to be safe. However, the effect size of the osteogenic potential decreased compared with the findings of Paper 1 and 2. Under suboptimal healing conditions, i.e. in the collagen carrier group, EPO statistically significantly increased the median bone volume fraction.

As pointed out above, the most important and challenging step in the clinical translation to determine is the optimal dosage. According to a recently published paper, topical EPO treatment reduced the time to clinical and radiographic union after tibiofibular fracture from 21.50 ± 3.18 to 19.35 ± 2.66 weeks (Bakhshi et al. 2013). Notably, only two out of 30 patients in the EPO group suffered from non-union while this was the case in six out of 30 control patients. No adverse events were recorded. After personal communication with the corresponding author of that study the dosing could be vaguely defined as three vials of 4000 IU EPO (unknown type and manufacturer) in slim patients and up to five vials in obese patients (Rölfing and Bünger 2013a). Injections at the fracture site were administered 1-2 weeks after the injury was sustained. Regrettably, this double-blinded randomized control trial did not meet CONSORT guidelines on the performing and reporting such studies (Schulz et al. 2010). However, in agreement with that paper, we believe that the site-specific EPO treatment, administered as a single dose or a few repetitive doses, is likely to be the most promising manner in which the osteogenic potential of EPO may be directly exploited in vivo. However, one should bear in mind that a higher dosage and continuous EPO treatment may be applicable in tissue engineering.

Association versus causality

The assessment whether EPO causally contributed to the observed effects presupposes that Papers 1–3 were internally

valid. As discussed above, the internal validity of the primary outcome measures of the individual papers was deemed to be high. Hence, EPO caused the observed increase in bone volume in Papers 1 and 3, and mineralization in Paper 2.

However, it may be questioned if the secondary outcome measures, for instance the augmented vascularization in Paper 1, were responsible for the observed osteogenic effect. The following section will therefore discuss the secondary outcome measures and their causality versus association with the observed effect.

In Paper 1, vascularization was increased in the EPO group compared with the control group. Vascular ingrowth from the decorticated bone and the surrounding muscle were described in the studied spinal fusion model (Toribatake et al. 1998; Bawa et al. 2006). According to Toribatake et al., a high degree of vascularization of the fusion mass is associated with union, while a low degree of vascularization is associated with non-union. Although this association does not prove causality, several papers indicate a causal relationship between a good blood supply and improved bone healing. For instance, limiting the main blood supply of the fusion mass by not decorticating the adjacent vertebral body resulted in non-union (Boden et al. 1995). However, the causality of the observed association of increased vascularization with increased bone volume could not be proven with the applied methodology. In contrast to our findings in Paper 1, no statistically significant difference in blood vessel densities was found in Paper 3. It is therefore hardly likely that the observed moderate increase in bone volume fraction was caused by neovascularization. However, in light of the findings of Papers 1 and 3 and the biology of healing, it seems plausible that neovascularization is at least partly responsible for the osteogenic effect of EPO.

In Paper 2, the presence of EPOR and CD131 on the cell surface of hMSCs was detected by means of flow cytometry and confocal imaging. However, the detection and quantification of a staining-methodology is only valid if the method is both sensitive and specific. Regarding EPOR, the specificity of multiple antibodies has been questioned (Elliott et al. 2006; Sinclair et al. 2010; Singbrant et al. 2011). The careful selection of antibodies and the validated staining protocol did increase the likelihood of truly measuring the presence of EPOR and CD131 rather than the unspecific presence of the stain. The methodology of Paper 2, allows us to conclude that EPO increased mineralization of hMSCs. Because only hMSCs were present, we may also draw the conclusion that these cells must express a receptor to which EPO can bind. The fact that low concentrations of EPO (i.e. 5 and 10 IU/ml) failed to statistically significantly enhance mineralization supports the speculation that the low-affinity EPOR/CD131 instead of the high-affinity EPOR/EPOR receptor facilitated the osteogenic response of EPO. However, the paper cannot indisputably determine if the observed effect of EPO on hMSCs was mediated by the homodimeric EPOR, the heterodimeric EPOR/CD131, or a potential third receptor. In order to further investigate the involvement of these receptors, future research in this field should include receptor-blocking experiments. Estimating mineralization after continuous blocking of the homodimeric EPOR or the heterodimeric EPOR/CD131 could determine if both, either or none of the receptors are involved in mediating the increase in mineralization after EPO stimulation.

In contrast to the receptor experiments, intracellular pathways were investigated with blocking experiments. The observed decrease in mineralization after blocking of JAK2, mTOR, and PI3K could consequently determine causality. Hence, we may draw the conclusion that all three pathways contribute to the osteogenic differentiation of hMSCs and mineralization. If other potential means to investigate intracellular pathways were applied without performing blocking experiments, for instance the determination which signaling molecules were phosphorylated, the paper would only have been able to document association not causality.

The effect of EPO on bone healing

Papers 1 and 3 documented that EPO can stimulate bone healing. This is true for two different species, rabbit and swine, and two different dosing strategies, continuous systemic and single topical dosing. However, both papers exclusively investigated early time points. Likewise, Paper 2 had a short observation time of maximum three weeks. The question therefore remains:

"Does EPO increase bone healing in the long term?"

The short observation times were used in this thesis to evaluate the outcome at the steepest point of the theoretical healing curves in order to maximize the likelihood of finding statistical differences between the treatment groups (Figure 14) (Baas 2008). In the clinic, the final outcome is at least as important as the initial healing response. This thesis did not investigate the long-term outcome and hence cannot provide an adequate answer to this important question.

However, the following theoretical scenarios can be derived from Figure 14. After 100% completion of the healing process, EPO-treated and placebo-treated bone may have regenerated to the same extent. Assuming that the findings of this thesis are valid and translate to an improved recovery time, the healing process could either have started earlier or progressed at a higher pace (Figure 14 left panel). Another scenario is, that EPO improves the outcome in the long term if the intrinsic healing is compromised (Figure 14 right panel).

Papers 1–3 support the hypothesis of an improved early bone healing process. In agreement with the findings of this thesis, the majority of the published pre-clinical studies document an advantageous effect of EPO on early bone healing (Holstein et al. 2007; Garcia et al. 2011; Holstein et al. 2011). Taken together with the first data from a clinical trial, these findings indicate an improved healing process and

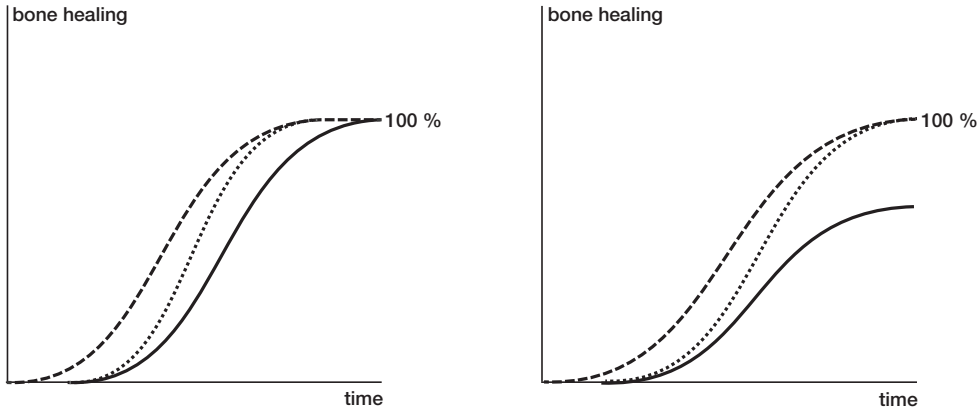


Figure 14. Theoretical scenarios about the effect of EPO from the beginning to the completion of the bone healing process. The left panel illustrates an improved early healing response and shortened recovery time. The right panel shows a potentially improved outcome after bone healing has ended. The healing curve without EPO is presented in black. Dashed lines depict theoretical healing curves with EPO.

shortened recovery time after EPO treatment (Bakhshi et al. 2013). However, if this effect declines over time, as observed in vitro in Paper 2 and in vivo by Holstein et al., there may be no difference in the final outcome between EPO-treated and placebo-treated bone healing (Holstein et al. 2007). In contrast, the fact that Bakhshi et al. report that only two out of 30 EPO patients and six out of 30 control patients suffered from non-union may imply a beneficial outcome in the long term (Bakhshi et al. 2013).

In conclusion, investigations that can answer the compelling questions about the recovery time and the long-term outcome in the clinical setting are warranted.

Conclusion and perspectives

The general aim of this PhD project was to investigate the efficacy of EPO in promoting bone regeneration in pre-clinical experiments. Furthermore, the determination of the cellular ways of action, the establishment of a dose-response relationship, and the application of a clinically safe dose in a large-animal study should pave the way for clinical translation.

Answering the three hypotheses of the present PhD project, the main findings of this thesis are:

Paper 1: Continuous, systemically administered low-dose EPO increased bone volume in the applied rabbit spinal fusion model.

Paper 2: EPO stimulated the osteogenic differentiation of hMSCs in a dose-dependent manner through direct, receptor-mediated signaling likely via EPOR and CD131, and it triggered JAK2, PI3K, and mTOR intracellular pathways.

Paper 3: Site-specific, single, low-dose EPO augmented the bone volume when applied on a collagen carrier, but failed to improve the healing capacity of PCL in a porcine calvarial defect model. Autograft exhibited an excellent healing capacity both with and without EPO.

EPO is able to enhance bone formation and to increase neovascularization, which is likely to contribute to osteogenesis. Moreover, a direct way of action via stimulation of hMSCs was documented.

Besides these advantages, the limitation of repetitive systemic EPO administration was highlighted in Paper 1. Although no thromboembolisms or adverse effects were observed in the wake of EPO treatment, the hematocrit rose to a critically high level, which makes the clinical implementation of this treatment regime impossible.

For the first time, the lowest effective dose was established in vitro. This is a prerequisite for commencing clinical trials as adverse effects including thromboembolism were described after high-dose EPO treatment. Notably, EPO may be utilized to overcome the major limitation of large-scaled, three-dimensional cell-based tissue regeneration, which is that it fosters vascularization and bony ingrowth in cell-loaded large 3D scaffolds. According to the findings of this thesis, the proportional dose-response relationship can be exploited to its full extent in the ex vivo setting, using the highest and most effective concentration of 100 IU/ml prior to implantation of cell/scaffold constructs. Hence, the primary findings of Paper 2 constitute an important step on the road to clinical translation and the paper offers information for a broad audience of scientists, who can be inspired to utilize EPO both ex vivo and in vivo. Paper 2 also demonstrated that hMSCs express the non-hematopoietic cell surface receptor EPOR/CD131 and that numerous intracellular pathways elicit the osteogenic potency of EPO. This also extends the present work beyond the past literature and thus becomes of interest for basic scientists.

Paper 3 was an endeavor to directly utilize EPO, and it was the first study to examine the osteogenic efficiency of EPO in a large-animal model. A clinically relevant, single low-dose of EPO was used. The direct topical EPO administration could potentially be employed in the clinic, for instance in the treatment of fractures that do not heal (non-unions) or that have a poor prognosis a priori, such as open fractures with limited vascular supply due to soft tissue damage.

Finally, the effect of EPOR/CD131-specific molecules on bone could be investigated. These molecules lack the ability to induce erythropoiesis because they cannot bind to the homodimeric EPOR on hematopoietic cells, but they do, however, possess non-hematopoietic potencies through their affinity to EPOR/CD131. Future research should therefore further clarify whether only EPOR/CD131 and not the homodimeric EPOR contributes to the pleiotropic effects on bone. If this holds true, utilization of EPOR/CD131-specific molecules can overcome the safety concerns and still offer the full advantageous effects of EPO on bone healing. Future research may also explore the intracellular mechanisms and the cellular interplay. However, the translation of the promising data from cell studies and animal experiments into clinical practice remains a pertinent challenge.

References

Acevedo AM, Rodríguez ML, Becerra HA, Cardona AF, Martí Carvajal AJ. Erythropoiesis–stimulating agents for myelodysplastic syndromes. The Cochrane Library. Wiley Online Library; 2012.

An YH, Friedman RJ. Animal models in orthopaedic research. CRC Press; 1999.

Anagnostou A, Lee ES, Kessimian N, Levinson R, Steiner M. Erythropoietin has a mitogenic and positive chemotactic effect on endothelial cells. Proc. Natl. Acad. Sci. U S A 1990; 87(15): 5978–82.

Arcasoy MO. Non-erythroid effects of erythropoietin. Haematologica 2010; 95(11): 1803–5.

Baas J. Adjuvant therapies of bone graft around non-cemented experimental orthopaedic implants. Acta Orthop Suppl 2008; 79(s330): 2–43.

Bakhshi H, Kazemian G, Emami M, Nemati A, Yarandi HK, Safdari F. Local erythropoietin injection in tibiofibular fracture healing. Trauma Monthly 2013; 17(4): 386–8. Available from: http://doi.org/10.5812/traumamon.7099

Bawa M, Schimizzi AL, Leek B, Bono CM, Massie JB, Macias B, et al. Paraspinal muscle vasculature contributes to posterolateral spinal fusion. Spine. 2006; 31(8): 891–6.

Besarab A, Bolton WK, Browne JK, Egrie JC, Nissenson AR, Okamoto DM, et al. The effects of normal as compared with low hematocrit values in patients with cardiac disease who are receiving hemodialysis and epoetin. N Engl J Med 1998; 339(9): 584–90.

Boden SD, Schimandle JH, Hutton WC. An experimental lumbar intertransverse process spinal fusion model. Radiographic, histologic, and biomechani-cal healing characteristics. Spine 1995; 20(4): 412–20.

Bohr S, Patel SJ, Shen K, Vitalo AG, Brines M, Cerami A, et al. Alternative erythropoietin-mediated signaling prevents secondary microvascular throm-bosis and inflammation within cutaneous burns. Proc. Natl. Acad. Sci. U S A 2013; 110(9): 3513–8.

Bouxsein ML, Boyd SK, Christiansen BA, Guldberg RE, Jepsen KJ, Müller R. Guidelines for assessment of bone microstructure in rodents using micro-computed tomography. J Bone Miner Res. 2010; 25(7): 1468–86.

Bozlar M, Kalaci A, Aslan B, Baktiroglu L, Yanat AN, Tasci A. Effects of erythropoietin on fracture healing in rats. Saudi Medical Journal 2006; 27(8): 1267–9. Available from: http://www.smj.org.sa/PDFFiles/Aug06/05BriefCom1267-1275.pdf

Brines M, Cerami A. Erythropoietin-mediated tissue protection: reducing collateral damage from the primary injury response. J Intern Med 2008a; 264(5): 405–32.

Brines M, Grasso G, Fiordaliso F, Sfacteria A, Ghezzi P, Fratelli M, et al. Erythropoietin mediates tissue protection through an erythropoietin and common beta-subunit heteroreceptor. Proc Natl Acad Sci USA. 2004; 101(41): 14907–12.

Brines M, Patel NSA, Villa P, Brines C, Mennini T, De Paola M, et al. Non-erythropoietic, tissue-protective peptides derived from the tertiary structure of erythropoietin. Proc. Natl. Acad. Sci. U S A 2008b; 105(31): 10925–30.

Burger D, Xenocostas A, Feng QP. Molecular basis of cardioprotection by erythropoietin. Curr Mol Pharmacol. 2009; 2(1): 56–69.

Campbell MJ, Julious SA, Altman DG. Estimating sample sizes for binary, ordered categorical, and continuous outcomes in two group comparisons. BMJ 1995; 311(7013): 1145–8.

Cervellini I, Sacre S, Ghezzi P, Mengozzi M. Erythropoietin does not affect TNF and IL-6 production directly. J Biol Regul Homeost Agents 2013; 27(1): 189–96.

Chappard D, Retailleau-Gaborit N, Legrand E, Baslé MF, Audran M. Comparison insight bone measurements by histomorphometry and microCT. J Bone Miner Res 2005; 20(7): 1177–84.

Chateauvieux S, Grigorakaki C, Morceau F, Dicato M, Diederich M. Erythropoietin, erythropoiesis and beyond. Biochem Pharmacol 2011; 82(10): 1291–303.

Chen L, Cheng F-J, Tang J-M. [The effect of recombinant human erythropoietin on the migration of bone marrow derived mesenchymal stem cells in vitro]. Zhonghua Xue Ye Xue Za Zhi. 2008; 29(12): 811–4.

Choi Y, Oldenburg FP, Sage L, Johnstone B, Yoo JU. A bridging demineralized bone implant facilitates posterolateral lumbar fusion in New Zealand white rabbits. Spine 2007; 32(1): 36–41.

Compston JE, Vedi S, Stellon AJ. Inter-observer and intra-observer variation in bone histomorphometry. Calcif Tissue Int 1986; 38(2): 67–70.

Dempster DW, Compston JE, Drezner MK, Glorieux FH, Kanis JA, Malluche H, et al. Standardized nomenclature, symbols, and units for bone histomorphometry: a 2012 update of the report of the ASBMR Histomorphometry Nomenclature Committee. J Bone Miner Res 2013; 28(1): 2–17.

Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 2006; 8(4): 315–7.

Ehrenreich H, Weissenborn K, Prange H, Schneider D, Weimar C, Wartenberg K, et al. Recombinant human erythropoietin in the treatment of acute ischemic stroke. Stroke 2009; 40(12): e647–56.

Elliott S, Busse L, Bass MB, Lu H, Sarosi I, Sinclair AM, et al. Anti-Epo receptor antibodies do not predict Epo receptor expression. Blood 2006; 107(5): 1892–5.

Fei J, Jia F, Peyrin F, Françoise P, Malaval L, Vico L, et al. Imaging and quantitative assessment of long bone vascularization in the adult rat using microcomputed tomography. Anat Rec (Hoboken) 2010; 293(2): 215–24.

Feiertag MA, Boden SD, Schimandle JH, Norman JT. A rabbit model for nonunion of lumbar intertransverse process spine arthrodesis. Spine 1996; 21(1): 27–31.

Festing MFW, Altman DG. Guidelines for the design and statistical analysis of experiments using laboratory animals. ILAR J 2002; 43(4): 244–58.

Garcia P, Speidel V, Scheuer C, Laschke MW, Holstein JH, Histing T, et al. Low dose erythropoietin stimulates bone healing in mice. J Orthop Res 2011; 29(2): 165–72.

Ghezzi P, Bernaudin M, Bernaudinb M, Bianchi R, Bianchic R, Blomgren K, et al. Erythropoietin: not just about erythropoiesis. Lancet 2010; 375(9732): 2142.

Gregory CA, Gunn WG, Peister A, Prockop DJ. An Alizarin red-based assay of mineralization by adherent cells in culture: comparison with cetylpyridini-um chloride extraction. Anal Biochem 2004; 329(1): 77–84.

Gundersen HJ, Bendtsen TF, Korbo L, Marcussen N, Møller A, Nielsen K, et al. Some new, simple and efficient stereological methods and their use in pathological research and diagnosis. APMIS 1988; 96(5): 379–94.

Guo L, Luo T, Fang Y, Yang L, Wang L, Liu J, et al. Effects of erythropoietin on osteoblast proliferation and function. Clin Exp Med 2012 Nov 8. [Epub ahead of print]. Available from: http://doi.org/10.1007/s10238-012-0220-7

Hankenson KD, Dishowitz M, Gray C, Schenker M. Angiogenesis in bone regeneration. Injury 2011; 42(6): 556–61.

Heeschen C, Aicher A, Lehmann R, Fichtlscherer S, Vasa M, Urbich C, et al. Erythropoietin is a potent physiologic stimulus for endothelial progenitor cell mobilization. Blood 2003; 102(4): 1340–6.

Hellebostad M, Hågå P, Cotes PM. Serum immunoreactive erythropoietin in healthy normal children. Br J Haematol 1988; 70(2): 247–50.

Holstein JH, Menger MD, Scheuer C, Meier C, Culemann U, Wirbel RJ, et al. Erythropoietin (EPO): EPO-receptor signaling improves early endochondral ossification and mechanical strength in fracture healing. Life Sci 2007; 80(10): 893–900.

Holstein JH, Orth M, Scheuer C, Tami A, Becker SC, Garcia P, et al. Erythropoietin stimulates bone formation, cell proliferation, and angiogenesis in a femoral segmental defect model in mice. Bone 2011; 49(5): 1037–45.

Hooijmans C, de Vries R, Leenaars M, Ritskes-Hoitinga M. The Gold Standard Publication Checklist (GSPC) for improved design, reporting and scientific quality of animal studies GSPC versus ARRIVE guidelines. Lab Anim 2011; 45(1): 61.

Hönig JF, Merten HA. The Göttingen mini pig as experimental animal in the human medical osteologic fundamental research. Zeitschrift für Zahnärztliche Implantologie 1993; 2: 237–43.

Isner JM, Asahara T. Angiogenesis and vasculogenesis as therapeutic strategies for postnatal neovascularization. J Clin Invest 1999; 103(9): 1231–6.

Jacobs K, Shoemaker C, Rudersdorf R, Neill SD, Kaufman RJ, Mufson A, et al. Isolation and characterization of genomic and cDNA clones of human erythropoietin. Nature 1985; 313(6005): 806–10.

Jaquet K, Krause K, Tawakol-Khodai M, Geidel S, Kuck K-H. Erythropoietin and VEGF exhibit equal angiogenic potential. Microvasc Res 2002; 64(2): 326–33.

Jelkmann W. Erythropoietin: structure, control of production, and function. Physiol Rev 1992; 72(2): 449–89.

Jelkmann W. The enigma of the metabolic fate of circulating erythropoietin (Epo) in view of the pharmacokinetics of the recombinant drugs rhEpo and NESP. Eur J Haematol 2002; 69(5-6): 265–74.

Jelkmann W. Erythropoietin after a century of research: younger than ever. Eur J Haematol 2007; 78(3): 183–205.

Jelkmann W. Efficacy of recombinant erythropoietins: is there unity of international units? Nephrol Dial Transplant 2009; 24(5): 1366–8.

Jenkins JK. Testimony > Erythropoiesis-Stimulating Agents (ESA) [Internet]. www.fda.gov. 2007 [cited 2013 Jul 17]. pp. 1–7. : Available from: http://www.fda.gov/NewsEvents/Testimony/ucm110908.htm

Jensen J, Rölfing JHD, Le DQS, Kristiansen A, Hokland L, Bendtsen M, et al. Surface-modified functionalized PCL scaffolds for bone repair: in vitro and in vivo experiments. J Biomed Mater Res A. 2013. [Epub ahead of print]. Available from http://doi.org/10.1002/jbm.a.34970

Johansen K. Basal sundhedsvidenskabelig statistik. Munksgaard Danmark, Copenhagen 2002.

Joshi D, Tsui J, Ho TK, Selvakumar S, Abraham DJ, Baker DM. Review of the role of erythropoietin in critical leg ischemia. Angiology 2010; 61(6): 541–50.

Jung Y, Song J, Shiozawa Y, Wang J, Wang Z, Williams B, et al. Hematopoietic stem cells regulate mesenchymal stromal cell induction into osteoblasts thereby participating in the formation of the stem cell niche. Stem Cells 2008; 26(8): 2042–51.

Keating A. Mesenchymal stromal cells: new directions. Cell Stem Cell 2012; 10(6): 709–16.

Khan SN, Lane JM. Spinal fusion surgery: animal models for tissue-engineered bone constructs. Biomaterials 2004; 25(9): 1475–85.

Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal re-search. PLoS Biol 2010; 8(6): e1000412. Available from http://doi.org/10.1371/journal.pbio.1000412

Kim J, Jung Y, Sun H, Joseph J, Mishra A, Shiozawa Y, et al. Erythropoietin mediated bone formation is regulated by mTOR signaling. J Cell Biochem 2012; 113(1): 220–8.

Kirkwood BR, Sterne J. Medical Statistics. Wiley-Blackwell 2003.

Koh S-H, Noh MY, Cho GW, Kim KS, Kim SH. Erythropoietin increases the motility of human bone marrow-multipotent stromal cells (hBM-MSCs) and enhances the production of neurotrophic factors from hBM-MSCs. Stem Cells Dev 2009; 18(3): 411–21.

Koury MJ, Bondurant MC. The molecular mechanism of erythropoietin action. Eur J Biochem 1992; 210(3): 649–63.

Krzyzanski W, Wyska E. Pharmacokinetics and pharmacodynamics of erythropoietin receptor in healthy volunteers. Naunyn-Schmied Arch Pharmacol. Springer-Verlag; 2007; 377(4-6): 637–45.

Leist M, Ghezzi P, Grasso G, Bianchi R, Villa P, Fratelli M, et al. Derivatives of erythropoietin that are tissue protective but not erythropoietic. Science 2004; 305(5681): 239–42.

Macdougall IC, Roberts DE, Coles GA, Williams JD. Clinical pharmacokinetics of epoetin (recombinant human erythropoietin). Clin Pharmacokinet 1991; 20(2): 99–113.

Maiese K, Chong ZZ, Shang YC, Wang S. Erythropoietin: new directions for the nervous system. Int J Mol Sci 2012; 13(9): 11102–29.

McGee SJ, Havens AM, Shiozawa Y, Jung Y, Taichman RS. Effects of erythropoietin on the bone microenvironment. Growth Factors 2012; 30(1): 22–8.

Mehdizadeh H, Sumo S, Bayrak ES, Brey EM, Cinar A. Three-dimensional modeling of angiogenesis in porous biomaterial scaffolds. Biomaterials 2013; 34(12): 2875–87.

Mihmanli A, Dolanmaz D, Avunduk MC, Erdemli E. Effects of recombinant human erythropoietin on mandibular distraction osteogenesis. J Oral Maxillo-fac Surg 2009; 67(11): 2337–43.

Müller R, Van Campenhout H, Van Damme B, Van Der Perre G, Dequeker J, Hildebrand T, et al. Morphometric analysis of human bone biopsies: a quan-titative structural comparison of histological sections and micro-computed tomography. Bone 1998; 23(1): 59–66.

Nair AM, Tsai Y-T, Shah KM, Shen J, Weng H, Zhou J, et al. The effect of erythropoietin on autologous stem cell-mediated bone regeneration. Biomaterials 2013; 34(30): 7364–71.

Olsson-Gisleskog P, Jacqmin P, Perez-Ruixo JJ. Population pharmacokinetics meta-analysis of recombinant human erythropoietin in healthy subjects. Clin Pharmacokinet 2007; 46(2): 159–73.

Prunier F, Pfister O, Hadri L, Liang L, Del Monte F, Liao R, et al. Delayed erythropoietin therapy reduces post-MI cardiac remodeling only at a dose that mobilizes endothelial progenitor cells. Am J Physiol Heart Circ Physiol 2007; 292(1): H522–9.

Rankin EB, Wu C, Khatri R, Wilson TLS, Andersen R, Araldi E, et al. The HIF signaling pathway in osteoblasts directly modulates erythropoiesis through the production of EPO. Cell 2012; 149(1): 63–74.

Ribatti D, Presta M, Vacca A, Ria R, Giuliani R, Dell'Era P, et al. Human erythropoietin induces a pro-angiogenic phenotype in cultured endothelial cells and stimulates neovascularization in vivo. Blood 1999; 93(8): 2627–36.

Riordan AM, Rangarajan R, Balts JW, Hsu WK, Anderson PA. Reliability of the rabbit postero-lateral spinal fusion model: a meta-analysis. J Orthop Res 2013; 31(8): 1261–9.

Rölfing JHD, Baatrup A, Stiehler M, Jensen J, Lysdahl H, Bünger C. The osteogenic effect of erythropoietin on human mesenchymal stromal cells is dose-dependent and involves non-hematopoietic receptors and multiple intracellular signaling pathways. Stem Cell Rev. 2013 Sep 20. [Epub ahead of print]. Available from http://doi.org/10.1007/s12015-013-9476-x

Rölfing JHD, Bendtsen M, Jensen J, Stiehler M, Foldager CB, Hellfritzsch MB, et al. Erythropoietin augments bone formation in a rabbit posterolateral spinal fusion model. J Orthop Res 2012 Jul; 30(7): 1083–8.

Rölfing JHD, Bünger C. Queries regarding local erythropoietin injection in tibiofibular fracture healing. Trauma Monthly 2013a; 18(2): 101–2. Available from http://doi.org/10.5812/traumamon.12192

Rölfing JHD, Bünger C. Recommendations regarding the rabbit posterolateral spinal fusion model. J Orthop Res 2013b; 31: 1860. Available from http://doi.org/10.1002/jor.22465

Rölfing JHD, Jensen J, Jensen JN, Greve A-S, Lysdahl H, Chen M, et al. A single topical dose of erythropoietin applied on a collagen carrier enhances calvarial bone healing in pigs. Acta Orthop. In press.

Russell WMS, Burch RL, Hume CW. The principles of humane experimenal technique [Internet]. Hyperion Books; 1992. Available from http://altweb.jhsph.edu/pubs/books/humane_exp/het-toc

Schlegel KA, Lang FJ, Donath K, Kulow JT, Wiltfang J. The monocortical critical size bone defect as an alternative experimental model in testing bone substitute materials. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2006; 102(1): 7–13.

Schulz KF, Altman DG, Moher D, CONSORT Group. CONSORT 2010 statement: updated guidelines for reporting parallel group randomised trials. PLoS Med 2010; 7(3): e1000251.

Schweiberer L, Büchner F. Bone transplantation in animals and man. In: Altman H, Büchner F, Cottier H, Grundmann E, Holle G, Letterer E, editors. Handbuch der allgemeinen Pathologie. 6 ed. Springer, Berlin, Heidelberg, New York 1977; 617–54.

Shiozawa Y, Jung Y, Ziegler AM, Pedersen EA, Wang J, Wang Z, et al. Erythropoietin couples hematopoiesis with bone formation. PLoS ONE 2010;5(5):e10853.

Sinclair AM, Coxon A, McCaffery I, Kaufman S, Paweletz K, Liu L, et al. Functional erythropoietin receptor is undetectable in endothelial, cardiac, neu-ronal, and renal cells. Blood 2010; 115(21): 4264–72.

Singbrant S, Russell MR, Jovic T, Liddicoat B, Izon DJ, Purton LE, et al. Erythropoietin couples erythropoiesis, B-lymphopoiesis, and bone homeostasis within the bone marrow microenvironment. Blood 2011; 117(21): 5631–42.

Singh AK, Szczech L, Tang KL, Barnhart H, Sapp S, Wolfson M, et al. Correction of anemia with epoetin alfa in chronic kidney disease. N Engl J Med 2006; 355(20): 2085–98.

Stiehler M, Bünger C, Baatrup A, Lind M, Kassem M, Mygind T. Effect of dynamic 3-D culture on proliferation, distribution, and osteogenic differentia-tion of human mesenchymal stem cells. J Biomed Mater Res A 2009; 89(1): 96–107.

Stiehler M, Lind M, Mygind T, Baatrup A, Dolatshahi-Pirouz A, Li H, et al. Morphology, proliferation, and osteogenic differentiation of mesenchymal stem cells cultured on titanium, tantalum, and chromium surfaces. J Biomed Mater Res A 2008; 86(2): 448–58.

Stockmann P, Park J, Wilmowsky von C, Nkenke E, Felszeghy E, Dehner J-F, et al. Guided bone regeneration in pig calvarial bone defects using autologous mesenchymal stem/progenitor cells - a comparison of different tissue sources. J Craniomaxillofac Surg 2012; 40(4): 310–20.

Storring PL, Gaines Das RE. The International Standard for Recombinant DNA-derived Erythropoietin: collaborative study of four recombinant DNA-derived erythropoietins and two highly purified human urinary erythropoietins. J Endocrinol 1992; 134(3): 459–84.

Sun H, Jung Y, Shiozawa Y, Taichman RS, Krebsbach PH. Erythropoietin modulates the structure of bone morphogenetic protein 2-engineered cranial bone. Tissue Eng Part A 2012; 18(19-20): 2095–105.

Sølling C. PhD thesis: Organ protective and immunomodulatory effects of erythropoietin. Faculty of Health Sciences, Aarhus University, Aarhus 2011.

Thomsen JS, Laib A, Koller B, Prohaska S, Mosekilde L, Gowin W. Stereological measures of trabecular bone structure: comparison of 3D micro com-puted tomography with 2D histological sections in human proximal tibial bone biopsies. J Microsc 2005; 218(Pt 2): 171–9.

Tonia T, Mettler A, Robert N, Schwarzer G, Seidenfeld J, Weingart O, et al. Erythropoietin or darbepoetin for patients with cancer. Cochrane Database Syst Rev. Wiley Online Library; 2012; 12: CD003407.

Toribatake Y, Hutton WC, Tomita K, Boden SD. Vascularization of the fusion mass in a posterolateral intertransverse process fusion. Spine 1998; 23(10): 1149–54.

van der Worp HB, Howells DW, Sena ES, Porritt MJ, Rewell S, O'Collins V, et al. Can animal models of disease reliably inform human studies? PLoS Med 2010; 7(3): e1000245.

Vesterby A, Kragstrup J, Gundersen HJ, Melsen F. Unbiased stereologic estimation of surface density in bone using vertical sections. Bone 1987; 8(1): 13–7.

Wen D, Boissel JP, Tracy TE, Gruninger RH, Mulcahy LS, Czelusniak J, et al. Erythropoietin structure-function relationships: high degree of sequence homology among mammals. Blood 1993; 82(5): 1507–16.

Wu YW, Bauer LA, Ballard RA, Ferriero DM, Glidden DV, Mayock DE, et al. Erythropoietin for neuroprotection in neonatal encephalopathy: safety and pharmacokinetics. Pediatrics 2012; 130(4): 683–91.

Yu Q, Chen L, You Y, Zou C, Zhang Y, Liu Q, et al. Erythropoietin combined with granulocyte colony-stimulating factor enhances MMP-2 expression in mesenchymal stem cells and promotes cell migration. Mol Med Rep 2011; 4(1): 31–6.