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Evaluation of fibrin based gene-activated matrices for BMP2/7 plasmid co-delivery in a rat non-union model

ABSTRACT

PURPOSE: Treatment of large segmental bone defects still is a challenge in clinical routine. Application of Gene-activated matrices (GAMs) based on fibrin, BMP 2/7 plasmids and non-viral transfection reagents (cationic polymers) could be an innovative treatment strategy to overcome this problem.

The aim of this study was to determine the therapeutic efficacy of fibrin GAMs with or without additional transfection reagents for bone morphogenic protein (BMP)2 and BMP7 plasmid co-delivery in a rat femur non-union model.

METHODS: In this experimental study a critical size femoral defect was created in 27 rats. At 4 weeks after the surgery animals were separated into 4 groups and underwent a second operation. Fibrin clots containing BMP2 and BMP7 plasmids with and without cationic polymer were implanted into the femoral defect. Fibrin clots containing recombinant human (rh) BMP2 served as positive and clots without supplement as negative controls.

RESULTS: At 8 weeks animals which received GAMs containing the cationic polymer and BMP 2/7 plasmids showed decreased bone volume compared to animals treated with GAMs and BMP2/7 only. Application of BMP2 and BMP7 plasmids in fibrin GAMs without cationic polymer lead to variable results. Animals which received rhBMP 2 protein showed increased bone volume and osseous unions were achieved in 2 out of 6 animals.

CONCLUSIONS: Cationic polymers decrease therapeutic efficiency of fibrin GAM based BMP2/7 plasmid co-delivery in bone regeneration. Non-viral gene transfer of BMP2/7 plasmids needs alternative promoters (e.g. by sonoporation, electroporation) to promise beneficial clinical effects.

INTRODUCTION

Treatment of large segmental bone defects still is a challenge in clinical routine. In the last years the U.S. Drug and Food Administration approved the clinical use of recombinant human (rh) BMP2 and BMP7 for special indications and provided an innovative tool in the treatment of substantial bone defects[1]. Despite encouraging results in clinical routine therapy costs still are high and the local use of highly increased BMP concentrations raises safety concerns[2]. An alternative strategy is the delivery of BMP transgenes instead of recombinant growth factors in order to generate a local sustained release of host-cell produced, highly bioactive growth factors[3-6]. Recent experimental data showed a synergistic effect of BMP2 and BMP7 therapeutic gene co-delivery[7-9]. This promises to increase therapeutic efficiency in upcoming clinical studies. Such Gene therapeutic approaches could increase the efficiency of BMPs and reduce costs as well as potential side effects. Viral delivery of DNA vectors to the target tissue was shown to be highly effective but could result in a fatal immune response of the host[10,11]. In contrast non-viral gene transfer using plasmids is regarded as a relatively safe but inefficient strategy[12,13].

Therefore, several strategies have been developed to overcome barriers to non-viral gene delivery and to enhance transfection efficacies[14,15]. The use of cationic polymers or lipoplexes and other reagents for non-viral gene delivery is a well established method which can significantly increase transfection efficacies *in vitro* and *in vivo[16-18]*. In recent years

several studies succeeded to increase passive gene transfer by lipofection or cationic polymer based gene delivery in animal models: Critical size mandibular defects in rats were successfully treated by liposome-mediated gene transfer of BMP2 in an *ex vivo* setting[19]. *In vivo* experiments showed increased bone formation and osseointegration when using liposomal BMP2 vectors in calvaria defects in pigs[20]. Using fibrin based gene-activated matrices (GAMs) is another strategy which showed promising results in promoting passive gene transfer[21]. A previous *in vivo* study assessed wound healing in an epigastric flap rat model. Animals treated with VEGF plasmids and GAMs consisting of fibrin and lipofectamine showed increased vascularisation and flap survival compared to the control groups[22]. Up to now there is no study focusing on the effects of transfection-reagent enhanced *in vivo* co-delivery of BMP2 and BMP7 plasmids in GAMs in a segmental bone defect model.

Therefore, the aim of this study was to determine the therapeutic efficacy of fibrin GAMs with or without additional poly(hydroxyalkylene)imine (PEI) cationic transfection reagents for bone morphogenic protein (BMP)2 and BMP7 plasmid co-delivery in a rat femur non-union model.

MATERIALS AND METHODS

BMP2 and BMP7 plasmids

Preparation and *in vitro* testing of the BMP2 and BMP7 plasmids are described elsewhere[23]. The plasmids were amplified in *E.coli* TOP10 (Life Technologies Ltd, Paisely, UK, ♯C404003) and isolated as endotoxin-free maxipreps using the EndoFree Plasmid Maxi or Giga kits (Quiagen GmbH, Hilden, Germany, ♯12362). (for plasmid maps, see Fig.1).

Experimental protocol

All experiments performed in this study were approved by the Animal Protocol Review Board of the City Government of Vienna. Animals were maintained and treated in accordance with the requirements of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (DHHS Publikation NIH 86-23, 1985). We used a critical size defect model in rat femurs which was previously validated including biomechanical testing and micro CT analysis[24]. We studied male Spraque-Dawley rats at an age of 20 to 24 weeks weighing at least 350 g. The animals were randomized and separated into four groups (n=6-9). All animals underwent surgery to create a standardized critical sized defect (3.8 mm) of the right femur. A silicone spacer was placed into the defect to avoid formation of vascularized scar tissue. At four weeks all animals underwent a second surgery. The silicone spacer was removed. The defect was filled with fibrin clots (V=160 µl), which served as gene activated matrix (GAM) for all groups. Beneficial effects using fibrin as GAM for gene delivery was extensively studied and previously published[21,22].

The fibrin sealant (Tisseel®, Baxter AG, Vienna, Austria) was prepared according to manufacturer's recommendations. 31.25 µl of fibrinogen, 31.25 µl of thrombine (c=400IU/mL) and supplements (recombinant human BMP2, BMP2/7 Plasmids, cationic polymer) were mixed in an Eppendorf tubecap under sterile conditions. Saline was added up to a volume of 160 µl. The fibrin clot was allowed to solidify at 37°C for 5 minutes before implantation.

In the first group (**FIBRIN**, n=6) animals received a fibrin clot without supplement. In the second group (**rhBMP2**, n=6) animals received a fibrin clot containing 10 µg recombinant human BMP2 (InductOS®, Pfizer, Vienna, Austria). In the third group (**BMP2/7**, n=9) animals received a fibrin clot containing 20 µg BMP2 and 20 µg BMP7 plasmid vectors. In the fourth group (**PEI_BMP2/7**, n=6) animals received a fibrin clot containing 20 µg BMP2

plasmids, 20 µg BMP7 plasmids and 40 µl poly (hydroxyalkylene) imine (Turbofect®, Thermo Scientific Inc., Vilnius, Lithuania[25]). At 8 weeks all animals were sacrificed and union rate, bone volume and bone density was determined.

Surgical Procedure

Animals were anaesthetized by intramuscular injection of ketamine hydrochloride (110 mg /kg body weight; Ketamidor, Richter Pharma, Austria) and xylazine hydrochloride (12 mg /kg body weight; Rompun, Bayer Healthcare, Germany). Each rat was placed on a thermostatic heating plate in a lateral decubitus position. A lateral approach was used to expose the femoral shaft. A 2 mm 5-hole AO titanium plate was fixed onto the anterolateral surface of the femur using 4 AO cortical screws (Synthes, Swizerland). A 3.8 mm segmental bone defect was created by double osteotomy in the the femur´s midshaft using a reciprocating saw (CORE Reciprocating Saw, Stryker Instruments, USA) under guidance of a template. To simulate a delayed union the femoral gap was filled with a 3.8 mm silicone spacer, which was firmly sutured onto the plate. The wound was closed in two layers and animals were allowed free movement. Analgesia was postoperatively provided by subcutaneous administration of meloxicam (0.15 mg /kg body weight; Metacam, Boehringer Ingelheim, Germany) for four days.

At four weeks all animals underwent a second intervention. The segmental bone defect was exposed through a small skin incision under anaesthesia. The silicone spacer was removed and the proximal and distal femoral bony edges were debrided using a burr (CORE Micro Drill, Stryker Instruments, USA) under constant irrigation with physiologic saline solution. After creating bleeding bony surfaces fibrin clots with or without supplements were applied according the experimental protocol. The wound was closed by layers.

At 8 weeks anaesthetized animals were sacrificed. The right femurs were explanted for further measurements. The surrounding soft tissue was removed and the specimens were fixed in 4%

neutral buffered formalin for further radiological and histological assessments.

Imaging

For assessment of union rate and bone volume, the femura were explanted and the titanium plate and screws were replaced by a synthetic radiolucent device. The bone was scanned using a microtomographic system (μCT 20; Scanco Medical, Zurich, Switzerland) between 2mm proximal and distal of the defect. A 3D image was reconstructed using 100 horizontal micro CT slide images 30 μm thick giving a resolution of 20 μm. Bone tissue was isolated using an automatic threshold procedure. The callus cross-sectional area was assessed in an interfragmentary horizontal slice.

In a first step all rat femurs were classified. Specimens exhibiting a visible bony bridge which increased 30% of the total cross sectional defect area were defined as unions. Bony bridges below 30% were defined as narrow unions. Specimens without new bone formation in the defect area were classified as oligotrophic or atrophic non unions according to Weber and Cech[26]. In a second step we performed a quantitative analysis of newly formed bone within the defect area. Bone volume was expressed in mm^3 per defect.

Histology

After completing the radiological measurements, rat bone specimens were fixed in 4% neutral buffered formalin for 48h and rinsed in tap water overnight. After that, specimens were submitted to a quick decalcification with an agent containing 14% hydrochloric acid (New decalc®, Medite, Burgdorf, Germany) and subsequently rinsed with running tap water overnight.

Decalcified bone samples were dehydrated and embedded in paraffin, and 4 μm sections were

cut on a rotary microtome. The paraffin sections were deparaffinized in xylene, rehydrated in graded alcohols, and stained with hematoxylin & eosin (H&E).

Statistics

All data (except the percentage of bone union) are presented as median ± interquartile range. Statistical analysis was performed using a standard software package (Graph Pad Prism, Graph Pad Software Inc., CA, USA). Normal distribution of data was tested by the Kolmogorov-Smirnov test. Groups were compared using one-way ANOVA. Post test was performed using the Tukey´s Multiple Comparison test. P-values less than 0.05 were considered statistically significant.

RESULTS

Union Rate

Union rate was assessed 8 weeks after creation of the femoral critical size defect and 4 weeks after implantation of the fibrin clots containing plasmids with/without cationic polymer. Unions were classified according the system of Weber and Cech[26].

Osseous unions were found in two out of six animals in the rhBMP2 group. Further two animals in this group showed narrow unions. Oligotrophic non unions were found in one out of six animals in the FIBRIN group, three out of nine animals in the BMP2/7 group and two out of six animals in the rhBMP2 group (see Fig.2).

Bone volume

Bone volume within the critical size defect was determined in all 27 treated rat femurs (see Fig. 3). Animals in the PEI_BMP2/7 group showed significantly decreased bone volume compared to the rhBMP2 ($p < 0.01$) and the BMP2/7 ($p < 0.05$) group. Animals in the rhBMP2 group showed significantly increased bone volume compared to the FIBRIN ($p < 0.05$) and the PEI_BMP2/7 group ($p < 0.01$). There was no significant difference comparing bone volume in the rhBMP2 and the BMP2/7 group.

Histological findings

Rat femur sections were subjected to H&E staining to analyze tissue characteristics and bone quality. Representative histological specimens of each group are presented (see Fig. 4A-D). Samples in the FIBRIN group showed tight fibrous tissue filling up the gap. Signs of incipient osteogenesis were found on one defect margin adjacent to the former plate location. In contrast samples in the rhBMP2 group showed extended bone formation within the defect. Lamellar bone in cortical regions and woven bone in the medullary region predominated the histological sections. Samples in the BMP2/7 group showed distinctive callus formation featuring extended osteogenic areas as well as cartilaginous tissues within the defect. Sufficient bony bridging was not achieved in any of the specimens. Samples in the PEI_BMP2/7 group missed any signs of bone formation. The defect was filled up with loose fibrous tissue. Osteogenic or chondrogenic precursor cells were absent.

DISCUSSION

In the present study we assessed the effects of PEI based cationic polymers and fibrin GAMs on passive gene transfer of BMP2 and BMP7 plasmids in a femoral non-union rat model.

The main finding of the study presented herein was a detrimental effect of the cationic transfection reagent on union rate and bone formation *in vivo*. Coadministration of cationic polymer for transfection in fibrin GAMs failed to enhanced the therapeutic effect in the experiments. In the PEI_BMP2/7 group 6 out of 6 femurs showed atrophic non unions (see Fig. 2). Bone volume in this group was significantly decreased compared to animals treated with BMP2 and BMP7 plasmids without cationic polymer (see Fig. 3). Histological sections showed absence of chondrogenic or osteogenic cells (see Fig. 4D). On one hand this finding is remarkable as there are previous studies showing beneficial effects of additional reagents such as liposomes[19,20] or polyethyleneimine based cationic polymers on BMP gene delivery and bone regeneration[20,19,27,28]. Administration of a BMP2 plasmid in combination with lipoplexes increased bone formation and osseointegration in a rat and pig[20,19] model. On the other hand it is well established that liposomal reagents as well as cationic reagents exhibit cytotoxic effects in a dose dependent manner. In cell cultures administration of lipoplexes caused cell shrinking, decreased mitose rates and vacuolisation of the cytoplasm. Beyond that there is evidence of interaction with the immune mediated cytokine cascade. *In vivo* administration of lipoplexes led to local and systemic inflammation. Blood cell agglutination was caused due to the positive charge of the lipids and resulted in local microinfarction and vascular inflammatory response[17,18]. *In vitro* tests overcome this problem by using tumorous cell lines, which are held under optimal conditions. Cell loss due to the cytotoxic effects of lipoplexes or cationic polymers can be accepted in certain cases, especially *in vitro* where high cell numbers are employed for transfection. In contrast our two-step surgery model of an atrophic non union provides a limited number of osteogenic precursor cells and a situation of compromised vascularisation. It is possible that the negative effects of the addition of the cationic polymer in this study are a consequence of the cytotoxic effects of PEI on the limited amount of host progenitor cells required for transgene expression and regeneration in this clinically relevant animal model.

Another important finding of this study is the insignificant therapeutic effect of passive gene transfer when administering exclusive BMP2 and BMP7 plasmids. 3 out of 9 animals in the BMP2/7 group showed oligotrophic non unions. Histological sections showed extensive areas of incipient osteogenesis without sufficient new bone formation (see Fig. 5B). There was however a tendency of increased bone volume in the BMP2/7 group compared to the negative control (Fibrin group). Nevertheless, inconsistent results due to single outliers failed to show statistically significant differences. This insignificant therapeutic effect was found in numerous studies, which demonstrated non-viral gene transfer as a safe but relative inefficient strategy to increase bone growth[10,12,13]. Results of this study highlight the importance of promoting non-viral gene transfer by alternative methods. Currently sonoporation or electroporation seem to be the most promising strategies in terms of safety, efficacy and minimal invasiveness[9,29-31]. Further studies are needed to clarify if those strategies are capable to increase bone growth in a clinical relevant segmental bone defect model.

As a positive control animals in the rhBMP2 group were treated by application of recombinant growth factor. Without any surprises animals showed increased bone volume compared to the Fibrin and PEI_BMP2/7 group. As a consequence osseous unions as well as narrow unions occurred in 2 out of 6 animals respectively. The results are supported by a previous study[32].

In conclusion, the addition of PEI-based cationic polymers to fibrin GAMs failed to promote non-viral gene transfer of BMP2 and BMP7 plasmids in this study. Application of BMP2 and BMP7 plasmids in fibrin GAMs without cationic polymer lead to variable results in bone volume without sufficient new bone formation to bridge the femoral critical size defect. It indicates a potential advantage of delivering BMP2 and 7 transgenes in fibrin gels for tissue regeneration if enhanced by methods such as the recently developed matrix-assisted

sonoporation[33] or methods without application of biomaterials relying only on physical stimuli for gene delivery such as sonoporation or electroporation[9,31].

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CONFLICT OF INTEREST

Competing financial interests of all authors have been disclosed**.** No conflict of interest exists.

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FIGURE CAPTIONS

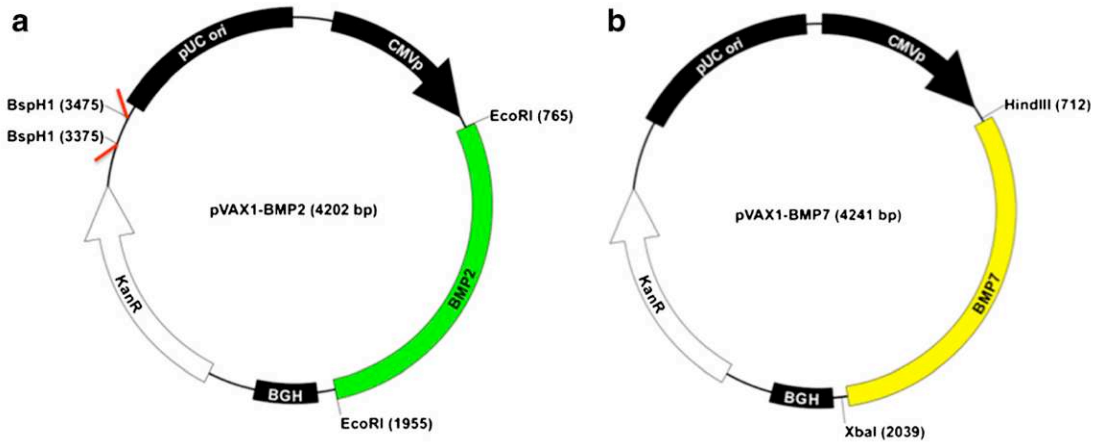

Fig. 1: Constitutive BMP2 and BMP7 expression plasmids: pVAX1-BMP2 and pVAX1-BMP7.

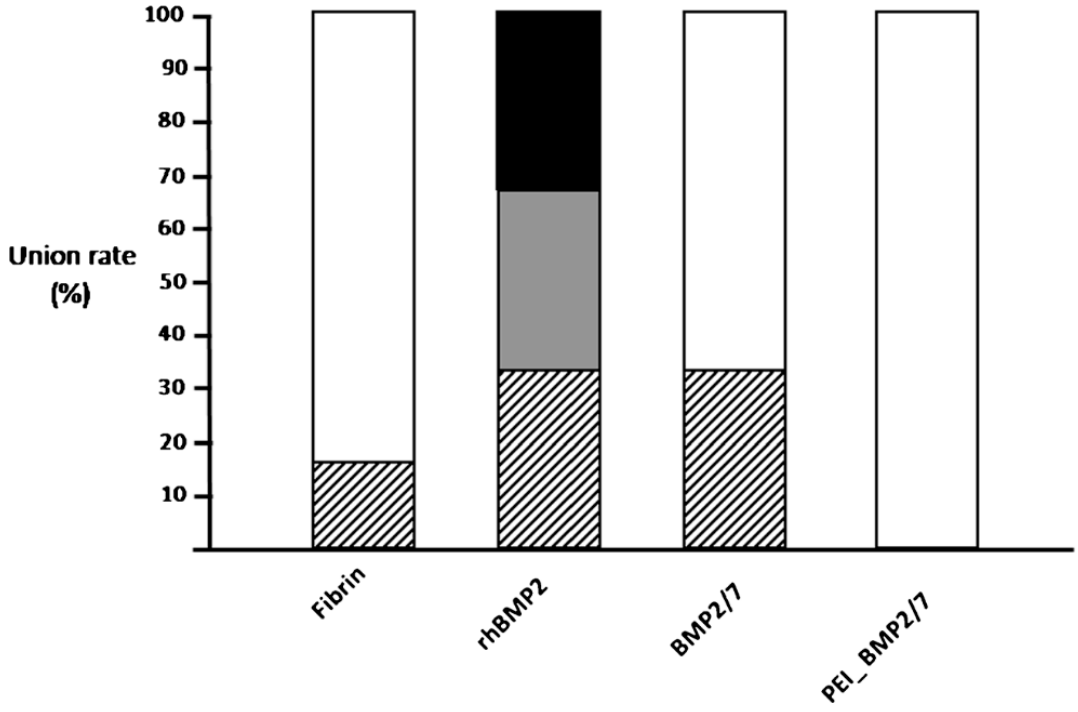

Fig. 2: Union rate at 4 weeks following treatment of a 3.8mm critical size femoral defect (%). Black and grey areas represent unions and narrow unions respectively. Black/white stripped and white areas represent oligotrophic and atrophic non unions respectively.

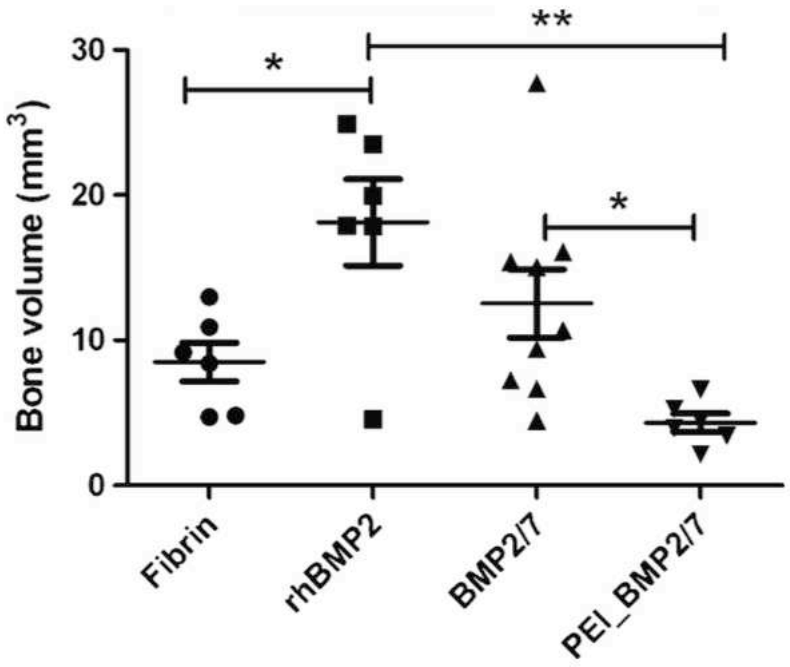

Fig. 3: Bone volume at 4 weeks following treatment of a 3.8 mm critical size femoral defect (mm^3). * indicates $p<0.05$, ** indicates $p<0.01$. Data are presented as median ± interquartile range.

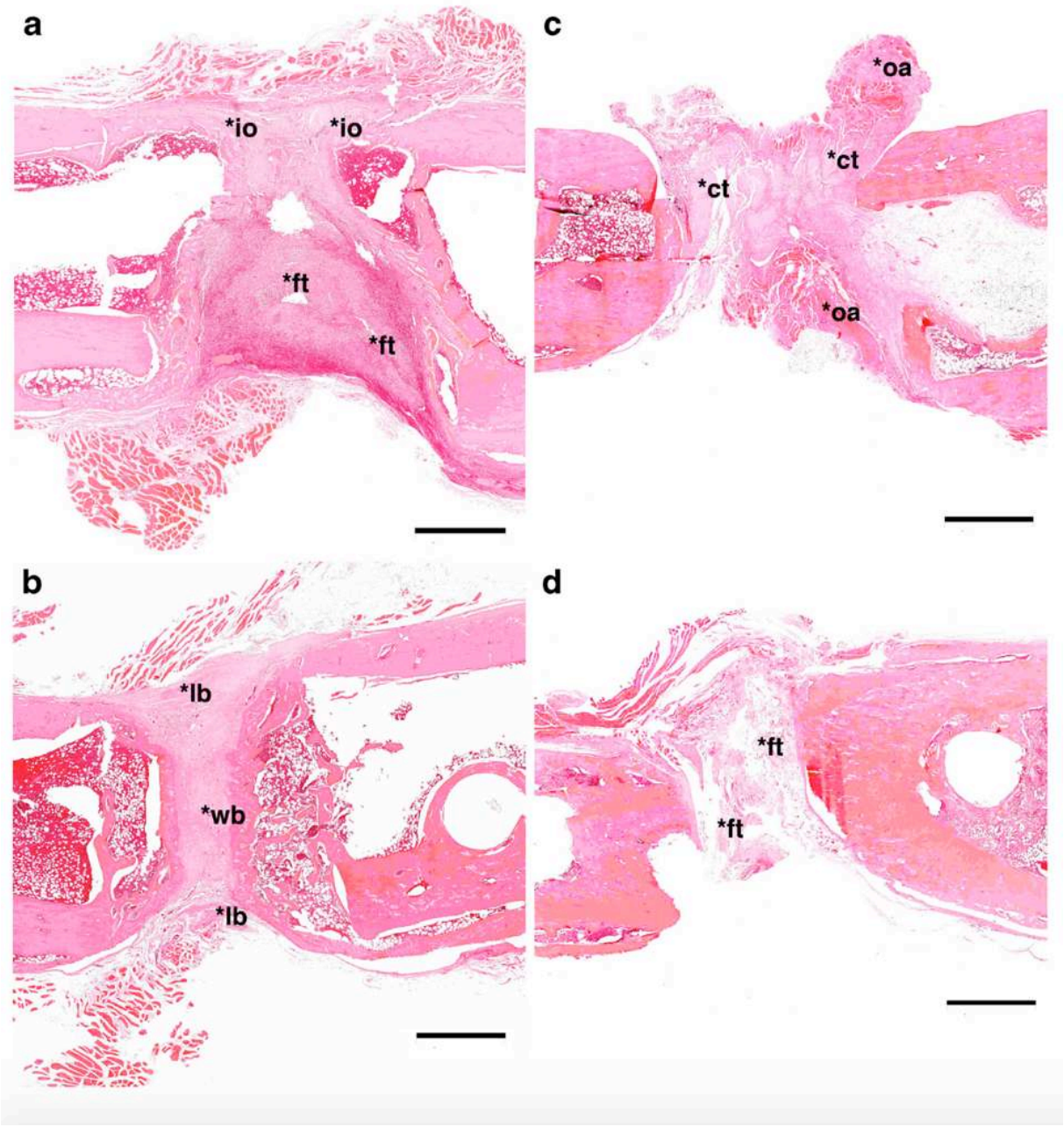

Fig. 4: Histological sections at 4 weeks following treatment of a 3.8 mm critical size femoral defect (H&E staining, magnification x10). **A:** Samples in the FIBRIN group show tight fibrous tissue filling up the gap. Signs of incipient osteogenesis are found on one defect margin adjacent to the former plate location. **B:** In contrast samples in the rhBMP2 group show extended bone formation within the defect. Lamellar bone in cortical regions and woven bone in the medullary region predominate the histological sections. **C:** Samples in the BMP2/7 group show distinctive callus formation featuring extended osteogenic areas as well as cartilaginous tissues within the defect. Sufficient bony bridging is not achieved in any of

the specimens. **D:** Samples in the PEI_BMP2/7 group miss any signs of bone formation. The defect is filled up with loose fibrous tissue. Osteogenic or chondrogenic precursor cells are absent.