Improved bone defect healing by a superagonistic GDF5 variant derived from a patient with multiple synostoses syndrome

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Abstract
Multiple synostoses syndrome 2 (SYNS2) is a rare genetic disease characterized by multiple fusions of the joints of the extremities, like phalangeal joints, carpal and tarsal joints or the knee and elbows. SYNS2 is caused by point mutations in the Growth and Differentiation Factor 5 (GDF5), which plays an essential role during skeletal development and regeneration. We selected one of the SYNS2-causing GDF5 mutations, p.N445T, which is known to destabilize the interaction with the Bone Morphogenetic Protein (BMP) antagonist NOGGIN (NOG), in order to generate the superagonistic GDF5 variant GDF5N445T. In this study, we tested its capacity to support regeneration in a rat critical-sized defect model in vivo. Microarray and histological analyses indicate that GDF5N445T-treated defects show faster and more efficient healing compared to GDF5 wild type (GDF5wt)-treated defects. Microarray-based gene expression and quantitative PCR analyses from callus tissue point to a specific acceleration of the early phases of bone healing, comprising the inflammation and chondrogenesis phase. These results support the concept that disease-deduced growth factor variants are promising lead structures for novel therapeutics with improved clinical activities.

Introduction
Bone growth and restoration comprise a well-orchestrated interplay of various factors and mediators responsible for cellular recruitment, proliferation and differentiation. Secreted growth factors, in particular Bone Morphogenetic Proteins (BMPs) and Growth and Differentiation Factors (GDFs), play a key role as they exert their effects on mesenchymal stem cells by promoting their differentiation into the chondro- or osteogenic lineage [1,2].

GDF5 is known as BMP14 or Cartilage-Derived Morphogenetic Protein 1 (CDMP1). It is a central modulator of early cartilage formation and plays an important role in the development of bones, joints, tendons and ligaments in the axial and appendicular skeleton [3–6]. As a therapeutic agent, the regeneration-supporting activities of wild type GDF5 (GDF5wt) have been investigated in various preclinical studies and clinical trials including craniofacial, peri-implant [7,8] and calvarial [9,10] bone formation, spine [11–13] and long bone surgery [14,15] as well as cartilage [16], tendon and ligament [17–19] formation and repair [20].

GDF5 is also implicated in rare diseases associated with bone overgrowth, like the multiple synostoses syndrome (SYNS2, #610017). SYNS2 is characterized by fusion of carpal and tarsal bones as well as proximal symphalangism in fingers and toes [21–24]. Previous work analyzing the signaling pathomechanism behind the SYNS2-associated GDF5 mutations p.W414R [25], p.S475N [22] and p.N445T [23] revealed that the mutations abrogate the negative feedback loop controlling GDF5 activity by extracellular antagonists like NOGGIN (NOG). Most of the aforementioned GDF5 mutations interfere with NOG binding and affect GDF5 receptor specificity due to their position within the overlapping receptor and NOG binding domain. For example, GDF5W414R is incapable of BMP receptor type 1A (BMPR1A) binding and also its binding to BMPR1B is diminished [25]. Similarly, GDF5S475N shows only
weak binding to BMPR2 [22]. In contrast, GDF5<sup>N445T</sup> displays unaltered receptor binding characteristics as demonstrated in a previous in vitro study [23]. The profound NOG insensitivity of GDF5<sup>N445T</sup> increases its biological activity compared to the wild type counterpart as shown in a chondrogenic differentiation assay [23].

The motivation behind this study was an attempt to learn from rare diseases for designing biomolecules with improved biological activities and therapeutic properties. We hypothesized that the naturally occurring superagonistic GDF5<sup>N445T</sup> molecule accelerates and improves the healing process when compared with its wild type counterpart in a critical-sized bone defect model.

Materials & methods

Recombinant proteins

Recombinant human GDF5<sup>WT</sup> and its variant GDF5<sup>N445T</sup> were provided by Biopharm GmbH and dissolved in 10 mM HCl.

Animal surgery

A group of 72 female Sprague–Dawley rats (12-week-old, weight 250–300 g; Charles River Deutschland GmbH, Germany) underwent a 5 mm critical-sized femoral segmental defect followed by stabilization with a custom-made unilateral external fixator as described previously [26,27]. The rats were administered an intraperitoneal injection of a solution containing ketamine hydrochloride (60 mg/kg, ketamin 50 mg, Actavis®, Island) and medetomidine (0.3 mg/kg, Domitor®, Pfizer, Karlsruhe). The antibiotic clindamycin-2-dihydrogenphosphate (45 mg/kg, Ratiopharm, Ulm) was also administered subcutaneously. An incision was made across the lateral aspect of the thigh, through the fascia, and the femur after pre-drilling with a 0.9 mm drill bit resulting in a 5 mm mechanical testing indicated that the osteotomy gap occupied by bone (yellow), cartilage (blue to green), and unmineralized cartilage) from non-mineralized tissue. Outcome measures included mineralized callus volume (bone volume, BV; mm<sup>3</sup>/mm<sup>3</sup>), total callus volume (TV, mm<sup>3</sup>), mineralized callus volume fraction (BV/TV, mm<sup>3</sup>/mm<sup>3</sup>) and tissue mineral content (TMC, mg HA), determined by microCT analysis 6 weeks post-operation. Rats were randomly subdivided into two experimental groups and one control group. For the two experimental groups, the sponges were soaked in resuspension buffer containing 10 mM HCl, and placed into the defect. The volume of the defect was calculated using µCT software. The rats were allowed to resume normal activity and given unrestricted access to food and water. For analysis of the early healing phase, 48 rats (n = 4 rats/group & time point) were sacrificed at days 3, 5, 7 and 14 post-operation and the callus tissue between the two inner pins was harvested for RNA extraction and gene expression analyses. A further 24 rats (n = 8 rats/group & time point) were analyzed by in vivo micro-computed tomography (microCT) at 2, 4 and 6 weeks post-operation, to assess the overall regenerative capacity of the growth factor-treated defects and the control group. Most of the animals (90.3%) tolerated the experimental procedure well and had an uncomplicated recovery through the end of the study; however, the following complications were observed: anesthesia-associated death (n = 5), pin infection (n = 1), pin breakage (n = 1). The anesthesia-associated deaths occurred in 3 rats directly following osteotomy surgery, while the other 2 rats died under anesthesia during the first in vivo microCT scan. After exclusion of these 7 rats, the remaining 17 rats, which had no complications, completed the in vivo microCT assessment (n = 5–6 rats/group & time point). These rats were sacrificed after 6 weeks of healing and histomorphometric analysis was performed (n = 4–6 rats/group).

Micro-computed tomography

Bone defect healing (n = 5–6/group) was assessed by in vivo microCT (vivaCT 40, Scanco Medical, 55 kVp, 145 µA, 150 ms integration time) at 2, 4 and 6 weeks post-operation. Analysis was performed on the basis of published procedures [28–30] using the specific parameters described below. We used a semi-automated segmentation of cross-sectional tomograms to derive the volume of interest (VOI), defined by the periosteal callus as the outer boundary and the endosteal callus as the inner boundary, excluding the original (parent) cortical bone. The VOI included the 5 mm defect region and 0.5 mm in the proximal and distal directions from the borders of the original osteotomy. A global threshold of 50% of the mineral density of the intact limb, equivalent to 351 mg HA/cm<sup>3</sup> was used to distinguish mineralized tissue (bone and calcified cartilage) from non-mineralized tissue. Outcome measures included mineralized callus volume (bone volume, BV, mm<sup>3</sup>), total callus volume (TV, mm<sup>3</sup>), mineralized callus volume fraction (BV/TV, mm<sup>3</sup>/mm<sup>3</sup>) and tissue mineral content (TMC, mg HA), defined as BV multiplied by the total mineral density (TMD). TMD was calculated using only the voxels whose intensity exceeded the threshold. In addition, the number of animals achieving bony defect bridging in the defect area was quantified. For microCT data, normal distribution was tested using a Shapiro–Wilks test and the within-subject effect of healing time (2, 4, and 6 weeks post-operation) and treatment (control, GDF5<sup>WT</sup>, GDF5<sup>N445T</sup>) were assessed using a repeated measures ANOVA. Sub-analyses were also performed using paired or unpaired t-tests, as appropriate. All values are presented as mean ± SD and statistical significance was set at p < 0.05. Analyses were performed using SAS 9.3 software.

Qualitative and quantitative histology

After in vivo microCT analysis 6 weeks post-operation, rats were sacrificed and femora were harvested for histological and histomorphometric analyses (n = 4–6/group). Femora were fixed in formaldehyde for 48 h and subsequently decalcified in EDTA for approximately 4 weeks at 37 °C. Fixed and decalcified tissues were dehydrated in graded ethanol up to 100%, transferred to xylene, and embedded in paraffin. Four micron thick longitudinal sections were prepared on a custom microtome (Leica RM 2125, Germany) and placed on glass slides. Quantitative histomorphometry was performed to analyze tissue differentiation for a single fixed region of interest (ROI), using semi-automated software (KSpolo 3.0 software, Zeiss Microlmaging GmbH, Germany). The composition of the callus tissue was quantified after staining with Movat Pentachrome by measuring the area within the osteotomy gap occupied by bone (yellow), cartilage (blue to green),...
and fibrous connective tissue formation (pink to purple), with the fibrous connective tissue also including bone marrow elements. Tissue areas (mm²) were measured and tissue fractions (%) calculated based on the ROI. For histomorphometry data, normal distribution was tested using a Shapiro–Wilk test and a one-way ANOVA was performed to assess the effect of treatment (control, GDF5wt, GDF5N445T). Sub-analyses were also performed using unpaired t-tests. All values are presented as mean ± SD and statistical significance was set at p < 0.05. Analyses were performed using SAS 9.3 software.

RNA isolation

Rat callus tissue between the two inner pins was harvested at days 3, 5, 7 and 14 post-operation (n = 4 rats/group & time point) and immediately flash frozen in liquid nitrogen. Rat tissue was subsequently pulverized in pre-cooled Teflon grinding jars (Retsch GmbH, Germany). Total RNA was isolated using Trifast reagent (Peqlab, Germany) and RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer’s instructions. RNA concentrations were determined spectrophotometrically and RNA integrity was verified by denaturing RNA gel electrophoresis. The isolated total RNA was stored at –80°C.

Quantitative PCR (Q-PCR)

Using TaqMan® Reverse Transcription Reagents (Applied Biosystems, CA), cDNA was synthesized from 1 μg of RNA. Q-PCR was performed in MicroAmp optical 384-well plates (Applied Biosystems, CA) on an ABI Prism 7900 Sequence Detection System (Applied Biosystems, C). As detection reagent, the SensiFast™ SYBR HI-ROX Kit (Bioline, Germany) was used. Primers for target genes were designed to span exon–exon junctions (Table S1). Samples were run in triplicates and results were normalized to 18S rRNA using the ΔΔCt method. Graphs display the fold change of transcript concentrations in each experimental group in comparison to the control at the earliest time point.

Q-PCR analyses of the individual rat RNAs showed comparable gene expression patterns at days 3, 7 and 14 (Fig. S1). For gene expression analyses by Q-PCR and microarray hybridizations, individual RNA samples of each experimental group (control, GDF5wt, GDF5N445T) at days 3, 5, 7 and 14 were pooled. According to the quality of the isolated RNA, three samples per group were selected and pooled for these comparisons.

Using the D’Agostino–Pearson omnibus normality test we confirmed that Q-PCR results of individual animals were normally distributed. Dependent on the data structure, we applied either a one-way ANOVA with Sidak post-hoc test or a two-tailed Student’s t-test for statistical analyses. Analyses were performed using GraphPad Prism 6 software.

Microarray analysis

The pooled RNA samples of each experimental group (control, GDF5wt, and GDF5N445T at time points days 3, 5, 7 and 14) were analyzed at the Laboratory of Functional Genomics Charité (LGFC) using the Affymetrix GeneChip expression analysis system (GeneChip Rat Gene 1.0 ST Array; Affymetrix, CA). Microarray data are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under the accession number E-MTAB-2249.

Data pre-processing was performed using the ‘aroma affymetrix’ package (version 2.9.4) which is implemented in the R software (http://www.r-project.org) [31]. In order to get gene-level summaries for each gene, default settings were used, i.e. RNA background correction, quantile normalization, and probe-level summarization (plm). Gene-level summarization on log2-scale was annotated to gene symbols using the ‘rangen10stranscriptcluster.db’ package (version 7.0.1). The ‘arrayQualityMetrics’ package (version 3.14.0) was used for quality metrics of raw data and the obtained expression set [32]. Statistical analysis was conducted based on variance over a time course of 4 measurements (day 3, day 5, day 7, and day 14). Variances were calculated for each experimental group, i.e. control, GDF5wt, and GDF5N445T. From each group, the top 1.5% of genes with the highest variance were selected. Taken together, this resulted in a total of 517 high-variance genes. For clustering analysis of the different samples according to similarity in pattern of gene expression, expression values of each of the 517 high-variance genes were scaled to a mean of zero and a standard deviation of one. For the heat plot, scaling was performed separately for each experimental group, i.e. control, GDF5wt, and GDF5N445T. The normalized values of 4 time points per group were then combined into vectors of 12 values per gene and hierarchical clustering based on these vectors was performed to group genes according to similarity in pattern of gene expression. In the line plots, summary values for the different clusters were plotted as mean ± SD after scaling all values per gene, thus allowing estimation of differences of expression levels dependent on different treatments. The lines connect the 4 time points analyzed in each group. Model-based gene set Gene Ontology (GO) analysis [33] was performed for all 517 high-variance genes, whereby GO terms with a marginal probability of at least 50% were considered as significant. Additionally, the Parent–Child-Union method of the Ontologizer [34,35] was used to analyze each cluster separately, and GO terms with Benjamini–Hochberg corrected p ≤ 0.05 were considered as significant.

Results

Enhanced bone defect healing by GDF5N445T compared to GDF5wt

The growth factor variant GDF5N445T was locally applied into a rat critical-sized femoral segmental defect model. The healing potential of GDF5N445T was assessed by in vivo microCT at weeks 2, 4, and 6 and histomorphometry at 6 weeks post-operation (Fig. 1).

In vivo microCT analysis revealed moderate mineralized tissue formation around the cortical ends of the defect site at week 2 for both the GDF5N445T– and the GDF5wt–treated defects. Periosteal, intracortical and endosteal bony bridging with marrow elements and early restoration of the medullary canal were observed in the GDF5N445T group at weeks 4 (5/6 animals) and 6 (6/6 animals) with cartilage islands and minimal remnants of mineralized cartilage in the defect region, respectively. Within the GDF5wt group endosteal bridging was achieved by 2/6 animals at week 4 and by 4/6 animals at week 6 with one animal already achieving intracortical bridging at week 6 post-osteotomy. GDF5wt–treated defects showed cartilage tissue and endochondral bone formation at the endosteal defect site with thick fibrous connective tissue fibers separating muscle tissue from the gap (Fig. 1).

Mineralized callus volume (BV) measured by microCT increased from the 4th to the 6th week post-osteotomy in both the GDF5wt– and the GDF5N445T–treated animals. In both groups, the mineralized callus tissue was primarily concentrated within the endosteal region rather than in the periosteal region, thus resulting in diminished bone marrow space over time. The GDF5N445T group showed significantly greater mineralized callus volume (BV, p < 0.01), total callus volume (TV, p < 0.01), and total tissue mineral content (TMC, p < 0.01) as compared to the GDF5wt–treated defects (Table 1). Furthermore, histomorphometry at week 6 demonstrated a significantly greater bone area for GDF5N445T (BAR, p = 0.01) as compared to GDF5wt–treated defects (Table 2).

The defects of the control group all resulted in a non-union at 6 weeks post-osteotomy. The empty defects were characterized by fibrous connective tissue and/or prolapsed muscle tissue as well as rounded cortical bone ends and newly formed bone between the cortices of each fragment, with sealing of the medullary canal from the osteotomy.

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Transcriptional feedback response after GDF5 treatment

To identify a potential negative feedback loop in direct response to the growth factor treatment, we analyzed the transcriptomes after GDF5 treatment on Gdf5 itself and a number of known extracellular modulators of GDF5 signaling at the earliest time point analyzed, i.e., day 3 after osteotomy (Figs. 2 and S2).

In response to the GDF5 treatment, expression of Gdf5 is not altered 3 days post-stimulation. The expression of most of the BMP antagonists (Fig. S2) remained also unaffected by the treatments with the notable exceptions of Nog and Chrdl2 (Fig. 2). The up-regulation of Nog likely diminishes GDF5wt action without affecting GDF5N445T activity, as GDF5N445T is NOG insensitive. In contrast, we neither observed inhibition of GDF5wt nor by GDF5N445T by CHRDLL2 (Fig. S3). Therefore, the altered NOG-interaction may contribute to the faster healing process observed for GDF5N445T.

GDF5N445T application causes distinct effects on the gene expression pattern

In order to explore and compare the early molecular changes in response to the local growth factor applications, we performed global gene expression profiling. A total of 517 genes representing the top 1.5% of high-variance genes derived from each experimental group were included into further analyses. Unsupervised hierarchical clustering was used to visualize the overall similarity of gene expression patterns in all samples. Distinct gene expression patterns can be identified already 3 or 5 days after GDF5wt and GDF5N445T application, respectively. The largest differences between the treatment groups are present 3 days after growth factor application. Notably, the gene expression patterns diverge at days 7 and 14, when the GDF5N445T-treated samples branch away from control and GDF5wt-treated samples, indicating qualitatively different treatment effects of the growth factors (Fig. S4). GO analysis of all 517 high-variance genes revealed ‘ossification’ as the GO term with the highest marginal followed by GO terms such as ‘cell contraction’ and ‘cell chemotaxis’ which are highly relevant processes in skeletal biology and fracture healing. To further explore gene expression in the time course of the healing process, genes were clustered according to their similarity of their expression pattern and visualized by a

Table 1

<table>
<thead>
<tr>
<th>Parameter/Group</th>
<th>Control n = 5</th>
<th>GDF5wt n = 6</th>
<th>GDF5N445T n = 6</th>
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<tr>
<td>BV/TV [mm³]b</td>
<td>0.6 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td>0.6 ± 0.1</td>
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<tr>
<td>2 weeks</td>
<td></td>
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<tr>
<td>4 weeks</td>
<td>0.6 ± 0.1</td>
<td>0.7 ± 0.1</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>6 weeks</td>
<td>0.7 ± 0.1</td>
<td>0.8 ± 0.1</td>
<td>0.8 ± 0.0</td>
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<tr>
<td>BV [mm³]b</td>
<td>1.0 ± 0.3</td>
<td>1.36 ± 0.3</td>
<td>1.63 ± 0.1</td>
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<tr>
<td>2 weeks</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 weeks</td>
<td>1.32 ± 3.1</td>
<td>3.30 ± 12.2</td>
<td>7.65 ± 25.3</td>
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<td>6 weeks</td>
<td>2.27 ± 10.5</td>
<td>5.38 ± 18.5</td>
<td>10.93 ± 25.5</td>
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<td>TV [mm³]b</td>
<td>1.61 ± 5.3</td>
<td>21.5 ± 4.8</td>
<td>27.1 ± 11.1</td>
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<tr>
<td>2 weeks</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 weeks</td>
<td>20.7 ± 4.8</td>
<td>47.8 ± 14.4</td>
<td>99.7 ± 25.6</td>
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<td>6 weeks</td>
<td>31.5 ± 15.8</td>
<td>67.4 ± 19.8</td>
<td>131.1 ± 32.1</td>
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<td>TMC [mg HA/cm³]b</td>
<td>6.7 ± 2.8</td>
<td>7.8 ± 1.5</td>
<td>9.2 ± 3.3</td>
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<td>2 weeks</td>
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<tr>
<td>4 weeks</td>
<td>8.5 ± 1.6</td>
<td>21.1 ± 1.4</td>
<td>47.1 ± 16.4</td>
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<td>6 weeks</td>
<td>15.7 ± 6.5</td>
<td>36.9 ± 13.4</td>
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<td>TMD [mg HA/cm³]b</td>
<td>626.7 ± 54.0</td>
<td>574.0 ± 16.3</td>
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<tr>
<td>2 weeks</td>
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<tr>
<td>4 weeks</td>
<td>646.9 ± 38.4</td>
<td>636.4 ± 18.6</td>
<td>612.5 ± 13.2</td>
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<tr>
<td>6 weeks</td>
<td>699.6 ± 36.5</td>
<td>683.7 ± 32.7</td>
<td>685.8 ± 22.9</td>
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Table 2

<table>
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<tr>
<th>Region/Group</th>
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<th>GDF5wt n = 6</th>
<th>GDF5N445T n = 5</th>
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</thead>
<tbody>
<tr>
<td>Total area [mm²]</td>
<td>24.0 ± 0.3</td>
<td>11.5 ± 0.3</td>
<td>0.3 ± 0.0</td>
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<td>Bone area [mm³]</td>
<td>12.3 ± 0.6</td>
<td>10.4 ± 0.6</td>
<td>0.4 ± 0.0</td>
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<td>Cartilage area [mm³]</td>
<td>3.2 ± 0.1</td>
<td>2.1 ± 0.1</td>
<td>0.2 ± 0.0</td>
</tr>
<tr>
<td>Fibrous connective tissue area [mm²]</td>
<td>15.4 ± 0.6</td>
<td>15.5 ± 0.6</td>
<td>0.5 ± 0.0</td>
</tr>
</tbody>
</table>
Figure 2. Expression changes of Gdf5 and its antagonists in response to GDF5 treatment. RNA pools of each experimental group at day 3 post-operation were used for microarray analysis. Gdf5 expression was not significantly altered, whereas Nog and Chrdl2 were strongly up-regulated in response to the growth factor treatment. Sfrs3 or 18S rRNA was used for normalization. Statistical analysis was performed using one-way ANOVA and a Sidak post-hoc test. Data is given as mean ± standard deviation of 3 replicates with p ≤ 0.001*** comparing either GDF5wt or GDF5N445T with the control.

Discussion

In this study, we report a side-by-side comparison of GDF5wt- and GDF5N445T-treated bone defects in comparison to control defects. The data indicate improved bony callus formation and regeneration upon GDF5N445T treatment in comparison to control or GDF5wt treatment. Previous work has shown that the N445T point mutation destabilizes the interaction with the extracellular antagonist NOG, resulting in a markedly increased GDF5 signaling output, which led in vitro to a strong chondrogenic effect on primary mesenchymal limb bud cells [23]. In light of these results, we were encouraged to evaluate the regenerative capacity of GDF5N445T in vivo using a well-established rat critical-sized defect model [27] that has already been successfully used for the evaluation of bone repair stimulation strategies [26,36]. In accordance with previous studies, we observed that osteotomy sites treated with GDF5wt heal via classical endochondral ossification [36]. Our data indicate that also GDF5N445T-stimulated regeneration follows the same general pathway. However, the molecular events appear to occur faster after local application of the GDF5N445T variant as compared to GDF5wt, in line with the accelerated bone defect healing. Molecular characterization of the healing phase indicates an early local up-regulation of BMP antagonists, such as Nog and Chrdl2 in GDF5wt- and GDF5N445T-treated rats. Whereas it is well established that NOG directly regulates GDF5 activity, there is only one study addressing the interaction between CHRD2 and GDF5 [37]. Although we could not confirm that CHRD2 inhibits GDF5 activity using an ALP assay, the signaling activity of GDF5N445T might have been retained for a longer period of...
Fig. 3. Gene cluster analysis highlights biological processes during bone defect healing. (A) Heatmap of all 517 high-variance genes at days 3, 5, 7, and 14 that were separated according to the experimental groups (control, GDF5wt and GDF5N445T). Blue indicates relatively low and red relatively high expression. Seven clusters were separated by visual inspection (1–7). (B) Expression dynamics are visualized by plotting the normalized expression levels of all genes within a cluster as a line with mean and SD at the four time points of analysis. Lines are interrupted according to treatment groups. (C) Each single cluster was analyzed using the Gene Ontologizer with Parent–Child-Union and Benjamini–Hochberg multiple testing correction. The top Gene Ontology (GO) terms are listed, whereby the bold font indicates significant GO terms (adj. \( p \leq 0.05 \)) and italic font highest ranked, but non-significant GO terms (adj. \( p \geq 0.05 \)).
time due to its NOG insensitivity. The global expression analysis of the early healing phase indicates a distinct temporal difference in inflammatory gene expression between the GDF5wt- and GDF5N445T-treated samples. Inflammation is the initial event during bone healing, in which a hematoma is formed due to accumulating blood originating from the damaged bone ends [38]. Recent studies highlight that the immune cell function is regulated during bone healing and that the modulation of immune cell function is a promising approach to improve regeneration [39–41]. In contrast to GDF5wt, we observed that the pro-inflammatory marker gene Il1b and the inflammatory chemokine Ccl3 were more rapidly declining after the GDF5N445T stimulus suggesting that these fracture sites have entered an advanced level within the healing cascade. Furthermore, the decline in inflammatory gene expression was strongly linked with an early rise in cartilage-specific matrix genes. Especially the expression of type II collagen, like Col2a1 by chondrogenic cells is an indicator for endochondral ossification accompanying cartilaginous matrix production and preceding soft callus formation [42,43].

As cell volume increases, we found the ECM molecule Acan to be strongly up-regulated, likely needed to control the osmotic pressure [44]. Ascending expression levels as response to the terminating inflammatory signal in GDF5N445T-treated rats were observed in case of Sox9, one of the central transcription factors regulating chondrocyte differentiation [45], and Comp, another key ECM component of functional cartilage [46]. An attenuated gene expression of Sox9 and Acan in GDF5N445T-treated rats was observed between days 7 and 14 post-operation.

Fig. 4. Dynamic expression of inflammatory genes during the healing process. Relative expression levels of inflammatory cytokines were quantified by Q-PCR in RNA pools of the experimental groups used for microarray analysis. Two representative inflammatory marker genes were chosen for Q-PCR analysis, Interleukin-1 Beta (Il1b) and Chemokine (C-C motif) ligand 3 (Ccl3). As endogenous control, Sfrs3 was used and expression values were normalized to control group at day 3. Statistical analysis was performed using a two-tailed Student’s t-test. Data is given as mean ± SD of 3 replicates with p ≤ 0.05*, p ≤ 0.001*** comparing GDF5N445T- with GDF5wt-treated samples.

Fig. 5. Dynamic regulation of marker genes for endochondral ossification. RNA pools of each experimental group used for microarray analysis were reversely transcribed and the representative marker genes for endochondral ossification, SRY-Box 9 (Sox9), Aggrecan (Acan), Cartilage oligomeric protein (Comp) and Collagen type 2 alpha 1 (Col2a1), were analyzed by Q-PCR. As endogenous control, Sfrs3 was used and expression values were normalized to the expression level of the control group at day 3. Statistical analysis was performed using a two-tailed Student’s t-test. Data is given as mean ± SD of 3 replicates with p ≤ 0.05*, p ≤ 0.01** and , p ≤ 0.001*** comparing GDF5N445T with GDF5wt.
suggesting that cell differentiation is triggered earlier into the osteogenic lineage as compared to GDF5wt-stimulated osteotomy sites.

The principle of rendering growth factors more potent through mutations has been previously addressed, e.g., by introducing two mutations into GDF5 yielding the bioengineered GDF5M453V, M456V (BB-1) molecule [47,48]. BB-1 shows an altered receptor binding characteristic converting GDF5 into a BMP2-like molecule. Other attempts included the introduction of the BMP2 heparin binding sites N-terminally of GDF5 (B2GDF5); however, the in vivo healing outcome using this variant was indistinguishable from GDF5wt treatment [49]. A slightly better result was obtained using the monomeric variant of GDF5, GDF5C456A, where the healing capacity was improved as compared to B2GDF5 [50].

An advantage of GDF5N445T might be the feature that only a single amino acid within the NOG binding interface is replaced retaining an unaffected signal transduction pathway and thus causing comparable effects on the gene expression pattern as compared to GDF5wt. As GDF5N445T is a naturally occurring mutation, it has already proven its markedly enhanced signaling capacity leading to bony fusions in the patients. Moreover, there are no severe side-effects reported in SYNS2 patients such as heterotopic ossification or increased cancer risk. In relation to its therapeutic potential, it appears as if critical steps during the bone defect repair become accelerated and strengthened by GDF5N445T as compared to its wild type counterpart, likely due to the avoidance of NOG-mediated inhibition. Such a bypassing of the negative feedback regulation was also reported by Song and colleagues [51] by transferring a key residue of BMP6 responsible for the NOG resistance into the corresponding position of BMP2 and BMP7. It can be assumed that the strategy employed in our study can also be adapted to other growth factors for rendering them less sensitive to endogenous feedback control and downregulation by increased antagonist expression.

As it is known that growth factors have different receptor binding preferences and therefore also different regenerative capacities, a tool box of highly potent growth factors can be generated for various therapeutic applications by transferring the same principle to other growth factors to render them more potent by introducing a homologous mutation within the NOG binding site.

Conclusion

Our study supports the concept of improving growth factor signaling and specificity for therapeutic use by modifying key residues involved in antagonist interaction. Applying this strategy to other growth factors will further enlarge the toolbox available to clinicians for personalized therapeutic use. We are convinced that a great potential for improving the signaling capacity of biomolecules lies hidden in the naturally occurring mutations identified in rare diseases. This strategy has several advantages, as the critical residues for a given biological process are identified during molecular diagnosis, the major effects are highlighted by the symptoms, and some knowledge on the biological safety and side-effects of the variants can be deduced from a thorough analysis of potential co-morbidities observed in the patients.

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Conflict of interest statement

Frank Plöger is an employee of the company Biopharm GmbH as indicated in the affiliation. Biopharm GmbH is a 100% privately owned company. The co-authors Frank Plöger and Stefan Mundlos have filed a patent on the GDF5 variant N445T (PCT/EP2006/001966). All other authors have declared that no competing interests exist.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bone.2014.12.017.

References


Glossary

Acan: Aggrecan
BMU: bone marrow
BMP: Bone Morphogenetic Protein
BMPRIA: BMP receptor type 1A
BMPRII: BMP receptor type 1B
BMPR2: BMP receptor type 2
BV: bone volume
C2b: chondromere (C-C motif) ligand 3
CDMP1: cartilage-derived
Chrd2: Chordin-like 2
Co: connective tissue
Col2a1: collagen, type II, alpha 1
Comp: cartilage oligomeric matrix protein
CT: computer tomography
GDF: Growth and Differentiation Factor
GO: Gene Ontology
HA: hydroxyapatite
IL6: interleukin 1 beta
MC: muscle
NOC: Noggin
P: PAR: quantitative polymerase chain reaction
Sry: Sry (sex determining region Y) box 9
SYNS2: multiple synostoses syndrome 2
TMC: tissue mineral content
TMD: tissue mineral density
TV: total volume
VOI: volume of interest
Wb: woven bone
Wt: wild type