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Molecular signaling within growth plates of the radius and tibia after periosteal stripping: an experimental study in lambs

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Summary

Periosteal transection and elevation is a standard treatment for angular limb deformities in foals. It is used to correct axis deviations in the limbs at an early age to assure that the foals grow up with straight limbs to improve their chances to reach their full potential as future athletes. Although clinically proven, its mechanisms of action were never elucidated on a more basic scientific level. In this experimental study the molecular response to periosteal stripping was investigated within the growth plate and adjacent perichondrium. The study was based on the hypothesis that a growth restraining feedback loop related to Indian hedgehog (Ihh), parathyroid hormone related protein (PTHrP) and parathyroid hormone receptors (PTHR) was responsible for the corrective effect of periosteal stripping. Twelve 3 months old lambs underwent periosteal stripping of the distal lateral radius and tibia on one side. The contralateral side served as non-operated controls. Two animals each group were sacrificed at 2, 6, 10, 14, 18 and 21 days after surgery and the growth plates with minimal adjacent bone tissue were harvested for histological investigations. After decalcification, paraffin-embedded sections with routine hematoxylin-eosin stains were prepared to assess morphology and length of growth plates, whereas immunohistochemistry of Ihh, PTHrP, PTHR and the two cytokines fibroblast- (FGF) and transforming growth factor (TGF) was performed to study different protein expression between operated limbs and controls. The results indicate that periosteal stripping caused an up-regulation of Ihh in the early pre- and hypertrophic zone of the growth plate, followed by an increase of PTHrP mainly in the perichondrium, while an increase of PTHR was noticed in all zones, although highest in the perichondrium and hypertrophic zones. The growth factors FGF and TGF were upregulated in all zones, but FGF in response to periosteal stripping was more intensely expressed in the proliferative zone and the highest peak of TGF was found in the perichondrium. Length measurements of the various growth zones revealed significant negative correlations between the proliferative and pre-and hypertrophic zones, indicating that indeed a negative feed back loop after periosteal stripping exists coupled by the Ihh/PTHrP/PTHR cascade.The hypothesis that periosteal stripping had an effect on the Ihh/PTHrP/PTHR related feedback loop in epiphyseal growth was confirmed in this experimental study in lambs. Since these mechanisms are very basic and similar in most species, it can be safely assumed that the effects in foals are similar. In fact, the asymmetric mechanical load in animals suffering from axis deviation may even increase the enhancing effect of length correction.

Keywords: growth plate, periosteal stripping, molecular signaling, foals, axis deviation

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Molekulare Signalübertragung in der Wachstumsfuge von Radius und Ulna nach Periost Stripping: eine experimentelle Studie in Lämmern

Chirurgisches Durchtrennen und Anheben des Periosts ("Periost Stripping") ist seit den frühen 80er Jahren eine Standardbehandlung bei Fohlen mit Achsenfehlungen ihrer Gliedmaßen. Das Ziel der Behandlung ist auf der kürzeren Seite der Wachstumsfuge einen erneuten Wachstumsschub auszulösen und damit eine Korrektur der Fehlstellung zu erreichen. Auch wenn die Behandlung klinische Erfolge zeitigt, so ist der dahinterstehende Mechanismus auf einer mehr grundlegenden, wissenschaftlichen Basis nach wie vor nicht bekannt. In der vorliegenden Studie wurde die molekulare Antwort auf das Periost Stripping innerhalb der Wachstumsfuge untersucht, wobei eine wachstumsbehindernde Rückkoppelung im Rahmen der Signalübertragung von Indian hedgehog (Ihh), parathyroid hormone related protein (PTHrP) und dessen Rezeptoren (parathyroid hormone receptors (PTHR)) angenommen wurde. Bei 12 gesunden, 3-Monate alten, männlichen Lämmern wurde an der lateralen, distalen Seite des Radius und der Tibia ein Periost Stripping ausgeführt. Die nicht operierte, kontralaterale Seite diente als Kontrolle. Jeweils zwei Tiere wurden nach 2, 6, 10, 14, 18 and 21 Tagen getötet und ihre Wachstumsfugen mit darüberliegendem Perichondrium und angrenzendem Knochen histologisch untersucht. Dekalzifizierte, in Paraffin eingebettete Proben wurden mit Hematoxylineosin angefärbt, um die Morphologie und Struktur der Wachstumsfugen zu vergleichen. Immunhistochemie wurde verwendet, um die Regulierung von Ihh, PTHrP, PTHR und den Zytokinen, Fibroblast (FGF)- und Transforming Growth Factor (TGF) bei den operierten und Kontrollwachstumsfugen zu vergleichen. Die Resultate zeigten, dass Periost Stripping eine Aufregulierung von Ihh in der präund hypertrophen Zone, gefolgt von einer zeitlich verzögerten Aufregulierung von PTHrP vor allem im Periochondrium der Wachstumsfuge verursacht. Ein Anstieg von PTHR wurde in allen Zonen beobachtet, jedoch am meisten ebenfalls im Perichondrium und der hypertrophen Zone. Die Wachstumsfaktoren FGF und TGF wurden in allen Zonen aufreguliert, jedoch wurde die höchste Konzentration von FGF in der proliferativen Zone und bei TGF im Perichondrium gefunden. Längenmessungen der verschiedenen Zonen der Wachstumsfuge resultierten in einer signifikant negativen Korrelation zwischen der proliferativen und prä-/hypertrophen Zone, was bedeutet, dass tatsächlich eine negative Rückkoppelung dieser Signalmoleküle aus der Ihh/PTHrP/PTHR-Kaskade für diesen Effekt des Längenwachstums verantwortlich ist. Die Hypothese, dass Periost Stripping einen Effekt auf die Ihh/PTHrP/PTHR bezogene Kaskade eine Rückkoppelung auslöst, konnte in dieser Studie bestätigt werden. Da diese molekularen Mechanismen sehr grundlegend sind und für die meisten Spezies gelten, kann ein identischer Mechanismus für Fohlen angenommen werden. In Wirklichkeit kann die zusätzlich veränderte mechanische Signalübertragung bei Fohlen mit Achsenfehlstellungen den wachstumsbeschleunigenden Effekt noch verstärken.

Schlüsselwörter: Wachstumsfuge, Periosteal Stripping, Molekulare Signalübertragung, Achsenabweichung, Fohlen

Introduction

Axis deviation and growth disturbances due to failure in the epiphyseal growth plates are common orthopedic problems in foals and children (*Auer* and *Martens* 1982, *Auer* and *Rechenberg* 2006, *Campbell* 1977, *Younger* et al. 1997) presenting a challenge for therapy (*Canadell* and *de Pablos* 1985, *Hunt* et al. 1990). The growth plates are responsible for the longitudinal growth in long bones (*van der Eerden* et al. 2003) and are interposed between the epiphyses (or secondary ossification centers) and the metaphyses, where ossification takes place. The growth plates consist of 5 different zones (*van der Eerden* et al. 2000, *van der Eerden* et al. 2003) with the reserve zone being adjacent to the epiphysis, followed by the proliferation-, transition-, hypertrophic- and finally ossification zone at the metaphysis, which leads into the ossified long bone shaft.

The growth plate is a very vulnerable, relatively soft structure. Once it has been traumatized either by accidents, radiation (*Bakker* et al. 2003, *Pateder* et al. 2001) or unknown, internal regulating incidents, it hardly ever recovers (*Davis* and *Green* 1976, *Farnum* et al. 2000). This generally leads to premature closure of the growth plate such that ossification sets in and longitudinal growth is abruptly stopped (*Bakker* et al. 2003, *Canadell* and *de Pablos* 1985, *Davis* and *Green* 1976). If damage affects the growth plate equally, the bone may keep its normal longitudinal axis and is just left shorter compared to the contralateral bone. However, if only one side of the growth plate is affected, bone growth is retarded only unilaterally and either medial or lateral axis deviation is the consequence (*Beck* et al. 2001, *Gladbach* et al. 2000, *Mielke* and *Stevens* 1996) (Fig.1). Depending on the age of the individual patient, this may lead to repeated surgical interventions, such as epiphysiodesis (*Gladbach* et al. 2000, *Ogilvie* and *King* 1990) and lengthy distraction procedures of long bones, at least in children (*Canadell* and *de Pablos* 1985, *Davis* and *Green* 1976, *Graf* and *Biemer* 1993, *Scott* et al. 1996).

Fig. 1 A foal with severe angular deformity of the front limbs is shown in the picture.

In foals, growth disturbances are usually diagnosed at a young age and usually managed by hoof manipulations, temporary epiphysiodesis, or hemicircumferential transection of the periosteum and periosteal elevation. The latter technique, also called periosteal stripping, which is performed on the concave aspect of the bone in the juxtametaphyseal region of the physis, is capable of stimulating the growth plate to resume its longitudinal bone growth, at least in many cases (*Auer* and *Martens* 1982, *Auer* 1985, *Auer* and *Rechenberg* 2006). The outcome seems to be dependent on the location and age of the patient. Thus, axis correction by periosteal stripping is recommended in foals younger than 3 months of age (*Auer* and *Rechenberg* 2006) or even less than 60 days (*Dutton* et al. 1999), although it also depends on the location and physiological closure of the growth plate.

Although this surgical technique has been widely applied in equine surgery, the surgical outcome has been debated recently (*Read* et al. 2002). Furthermore, the underlying mechanism for growth plate stimulation has never been investigated on a cellular and/or molecular level. It may be related to a phenomenon called "catch-up growth" (*Bakker* et al. 2003) that was observed in humans, after growth retarding conditions such as Cushing syndrome, hypothyroidism, anorexia nervosa, malnutrition, growth hormone deficiency or fractures of the femoral shaft (*Beck* et al. 2001, *Wessel* and *Seyfriedt* 1996). In rats and rabbits it could be experimentally induced through local dexamethasone or estrogen injections (*van der Eerden* et al. 2003). However, it is more likely that the effect of the periosteal transection and elevation is related to an interruption of cell signaling (also called "cross talk") between the elevated periosteum/perichondrium and the cells of the different zones within the growth plate.

The mechanisms of how longitudinal growth is regulated within the growth plates are complex (*van der Eerden* et al. 2003) and await further clarification regarding systemic and local influences through endocrine hormones and signal transduction by local cytokines and other molecules. Only through recent advances in the field of molecular biology was it possible to elucidate at least some of these mechanisms. It could be demonstrated that the interaction of several signaling molecules between the different zones of the growth plate plays a major role for undisturbed physeal growth. Among those molecules, indian hedgehog (ihh), parathyroid hormone related peptide (PTHrP) (*Juppner* 2000), PTH/PTHrP receptor 1 (PTHrPR) (*Chung* et al. 1998, *Chung* et al. 2001, *Huch* et al. 2003, *Juppner* 1995, *Kronenberg* et al. 1998, *Tiet* and *Alman* 2003, *van der Eerden* et al. 2000), estrogen receptors (ER) and systemic estrogen levels, fibroblast growth factor 1, 2 and 3 (FGFs) (*Huch* et al. 2003), transforming growth factor β2 (TGF-β) (*Alvarez* et al. 2002, *Serra* et al. 1997), bone morphogenetic protein 2,4, 6 and 7 (BMPs) (*Chung* et al. 2001, *van der Eerden* et al. 2000), insulin-like growth factor 1 (IGF-1) and vascular endothelial factor (VEGF) play a major role (*Pateder* et al.

2001). The expression of these molecules could be related to the cells of the specific zones and through the use of transgenic mice (knock-out mice) their functions in the process of bone growth/endo-chondral ossification could be explained. Indian hedgehog is mainly synthesized in prehypertrophic and hypertrophic chondrocytes (*Chung* et al. 1998, *Chung* et al. 2001, *Kronenberg* and *Chung* 2001) and seems to be a regulator of chondrocyte differentiation via the signaling molecule Patched 1 (Pct1) (*Alvarez* et al. 2001, *Alvarez* et al. 2002), Gli (*Semevolos* et al. 2005, *Vortkamp* et al. 1996) and Smoothened (Smo) (*Juppner* 2000, *van der Eerden* et al. 2000). PTHrP was found in cells of the proliferating zone as well as in the early hypertrophic chondrocytes and periarticular perichondrium inhibiting the pathway of chondrocyte differentiation. PTHrPR1 is mainly expressed in the cells of the late proliferating and prehypertrophic zone and is responsible for the effect of PTHrP/PTH to take place (*Juppner* 1995, *Kronenberg* and *Chung* 2001). A growth restraining feedback loop (*Chung* et al. 2001, *Kronenberg* and *Chung* 2001, *van der Eerden* et al. 2000, *van der Eerden* et al. 2003) between the two molecules Ihh and PTHrP (Fig.2) regulates longitudinal growth within the growth plate, such that Ihh is an up-stream positive regulator of PTHrP. If Ihh secretion is increased, PTHrP molecules are up-regulated and inhibit differentiation of proliferating chondrocytes into hypertrophic chondrocytes, thus promoting longitudinal growth. Since Ihh and PTHrP are not expressed in close vicinity and molecule diffusion through the extracellular matrix (EM) is limited, intermediates, such as Ptc (*Tiet* and *Alman* 2003), TGFβ2 (*Alvarez* et al. 2002, *Serra* et al. 1999), BMPs (*Chung* et al. 2001) and the growth factors FGF and TGF-β that are located in the periochondrium participate in this regulatory process (*Alvarez* et al. 2001, *Alvarez* et al. 2002, *Pateder* et al. 2000, *Serra* et al. 1999). VEGF is involved to attract vascular supply from the metaphysis. Ihh has two pathways available to regulate longitudinal growth and bone formation in the growth plate. Apart from its negative feedback loop in conjunction with PTHrP via the signaling molecule Pct, it also has an effect on already predetermined chondrocytes to further differentiate into the osteoblastic lineage (*Chung* et al. 2001). Together with BMP it regulates bone formation at the ossification site and is responsible for forming a bone collar at the metapyhseal end of the growth plate.

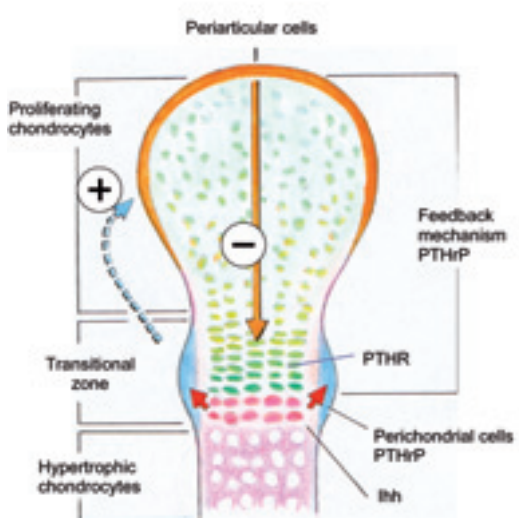

Fig. 2 The diagram shows the mechanism of signal transduction in the growth plate. Changes of Ihh concentrations in the pre- and hypertrophic zone regulates the response of PTHrP in the perichondrium and also the PTHR in most zones, however, mainly in the pre- and hypertrophic zone. FGF and TGF up-regulation enhance the proliferative and lengthening effect of the feed back loop.

Disturbances of this regulatory mechanism between Ihh/PTHrP and PTHrPR1 were demonstrated in horses when osteochondral lesions were compared to normal cartilage (*Semevolos* et al. 2002). In addition, misregulation was confirmed also in enchondroma, and osteochondroma in children (*Tiet* and *Alman* 2003). Therefore, it can be expected that this regulatory axis is deregulated also in axis deviations and premature closure of the epiphysis and after surgical periosteal transsection. The goal of the experiments conducted in this study was to investigate whether periosteal stripping affects the feed back loop between Ihh/PTHrP and PTHrPR1 in growth plates of normal, growing lambs. It was based on the hypothesis that the surgical interruption of the crosstalk between the perichondrium and the growth plate would up-regulate the expression of Ihh, PTHrP and PTHrPR, as well as TGF and FGF in the chondrocytes of the growth plate and periochondrium. To prove this hypothesis, the expression of these molecules (IHH, PTHrP, PTHrPR, FGF and TGF) was followed over a time period of 21 days using immunohistochemistry and comparing operated limbs to their contralateral, non-operated limbs as controls.

Materials and methods

Study design and animal groups: Twelve three months old, male lambs were weaned from their mothers shortly before surgery. Periosteal stripping was performed at the lateral aspect of the distal radial and tibial metaphysis using the contralateral limbs as controls. Two animals each were sacrificed at day 2, 6, 10, 14, 18 and 21 after surgery. Growth plates were sectioned in a dorsopalmar/plantar fashion immediately adjacent to the site of periosteal stripping.

Surgery

Animals were fasted for 3 hours before surgery. Sedation was achieved with Medetomidin (Graeub AG, Bern Switzerland; 10μg/kg BW) or xylazine (Streuli Pharma AG, Uznach, Switzerland; 0.1mg/kg BW) and perioperative antibiosis (Clamoxyl®, 7mg//kg i.m., Pfizer AG, Zurich, Switzerland) and analgesia (Rimadyl®, 2mg/kg BW, Pfizer AG, Zurich, Switzerland; Temgesic®, 0.03mg/kg BW, Essex Chemie AG, Lucerne, Switzerland) was initiated intravenously through a jugular catheter. Anesthesia was induced with ketamine (Vétoquinol AG, Ittigen, Switzerland; 2mg/kg BW) and diazepam (Roche, Rheinach, Switzerland; 50μg/kg BW) or propofol (Fresenius Kabi, Stans, Switzerland; 2-4mg/kg BW) and maintained through inhalation anesthesia with isoflurane (Abbott AG, Baar, Switzerland; 1-1.5%) in 100% oxygen via tracheal intubation. Intravenous fluids were applied (10ml/kg BW Ringer Lactate) through the jugular catheter and prophylactic tetanus serum (Veterinaria AG, Pfäffikon, Switzerland; 2ml per lamb) was injected subcutaneously.

The original technique as described for foals was applied in lambs (*Auer* and *Martens* 1982). Briefly, after routine aseptic preparation of the limbs and animals in lateral recumbency, the surgical approach was performed laterally in the uppermost limbs through a 2-4 cm vertical skin incision in the distal metaphysis of the radius or tibia respectively. The subcutaneous tissue was bluntly disected before the T-incision of the periosteum was made with the help of a No: 12 Bard Parker scalpel blade. The vertical incision of the periost was placed in the frontal plane joining distally the horizontal incisions, which were continued caudally and cranially in a hemicircumferential manner. The triangular periosteal flaps were elevated using a periosteal elevator and loosely repositioned into their original position before the subcutaneous tissues were closed using resorbable suture material in a simple continuous pattern followed by an identical pattern applied intracutaneously.

Postoperative care

The distal limbs were placed in a soft bandage, animals were recovered from anesthesia and placed back in their stalls, where they were kept in small groups until sacrifice. Animals were allowed to ambulate freely and were offered food and water ad lib. Postoperative antibiosis (Clamoxyl®) and analgesia (Temgesic® 0.03mg/kg twice at 4 hours interval; Rimadyl® 2mg/kg) was continued for 3 days and bandages were removed after 3-5 days.

Sample harvesting and preparation for histology

All lambs were euthanatized at the university-owned local slaughterhouse and the growth plates harvested immediately after the animals were dead. Sections of 2 mm thickness were cut in parasagittal planes of the distal radii and tibiae using a band saw (Fig. 3). The samples were subsequently fixed in 4% freshly prepared and buffered 4% formaldehyde for 2 days, followed by decalcification in 12% EDTA at room temperature and constant stirring for 4% formaldehyde for 2 days, followed by decalcification in 12% EDTA at room temperature and constant stirring for at least 28 to 42 days. When decalcification was complete, the samples were embedded in paraffin according to the routine protocol of our loaboratory. Eight sections were cut from each block, of which one was used for routine hematoxylin-esoin (HE) staining, whereas the others were used for immunohistochemical procedures using antibodies against Ihh, PTHrP, PTHrPR, FGF and TGF.

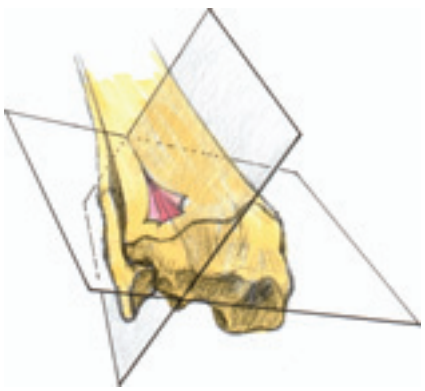

Fig. 3 The schematic drawing shows the plane of growth plate dissection for histology. The vertical plane was chosen for histology.

Immunohistochemistry

Growth plate slides (2-3 micrometer) were mounted on positively charged slides deparaffinized and rehydrated, watered in tap water for 5 min. and counterstained for 2 to 4 min. in hemalaun. Endogenous peroxidase was inactivated by treatment with 3% H_2O_2 (3% H_2O_2 with 0,2% NaN^3 (sodium azide) in water) for 10 min. and a protein block (DakoCytomation Protein Block, X0909, Switzerland) was applied for 10 min. at RT (room temperature). No pretreatment was used for following antibodies: IHH, PTHrP, FGF and TGF. For the usage of the antibody PTHrPR the slides had to be pretreated with citrate puffer (Dako Target Retrieval, S2031, pH6) for 20 min. Avidin and Biotin blockage was preformed in all slides for 20 min. each (Avidin Biotin Blocking Kit SP-2001, Vector Laboratories, USA). The antibodies were diluted as follows: rabbit anti-IHH (H88 human, sc-13088, Santa Cruz Biotechnology) 1:3, rabbit anti-PTH-rP (H137 human, Santa Cruz Biotechnology), mouse anti-PTH-R Type 1 Ab1 (clone 3D1.1 human MS-1270, LabVision Neomarkers) 1:10, rabbit anti-FGF-2 (basic human AB 1458, Chemicon) 1:1000 and mouse anti-TGFß (human MAB 1032, Chemicon) 1: 100. Incubation for all antibodies was over night at RT. As secondary antibody the LSAB+ Kit was used for 15 min. at RT for each step (K5003, HRP/AEC, Rabbit/Mouse). As chromogen AEC (Aminoethyl Carbazole Substrate Kit, Invitorgen, 00-2007) was used. Between every step the slides were washed with phosphate buffer solution (pH 8).

Evaluation

The histology slides were evaluated under light microscopy (Leica® DMR, Leica Microsystems Wetzlar GmbH, Wetzlar, Deutschland). The HE stained sections were qualitatively screened for normal, resp abnormal morphology. In addition, using histomorphometric measures (QUIPS/Qwin® Leica, Heerbrugg Switzerland), the lengths of the proliferative, prehypertrophic and hypertrophic zone was measured at both ends and divided at equal distances within the growth plates (4 measurements/growth plate, 8 measurements per group and day). Percentages for each zone were calculated in relation to the entire length of the growth plates. Length measurements were always taken parallel to the columnar arrangement of the prehypertrophic and hypertrophic zone. As for the evaluation of the various immunohistochemistry sections, two approaches were used; first, staining and second, its intensity were recorded for the proliferative, prehypertrophic and hypertrophic zone, each separately, and at both ends and in the middle of the growth plate (3 measurements/zone, 9 measurements for the entire growth plate per animal.). The perichondrium was also scored separately. The scores for the staining were set at 0= no staining, 1= positive staining, whereas the scores for the intensity of staining were set at 0=none 1=mild, 2= moderate and 3= intense staining. The sum of the three regions was used for statistical evaluation. Two reviewers that were blinded to the groups and days conducted the scoring within a period of 3 weeks.

Statistical evaluation

Scores for each zone and group were evaluated separately (6 measurements per group/day), as well as for each type of long bone (radius and tibia). Means were calculated using one way factorial analysis of variance (ANOVA) and Posthoc tests according to Bonferroni to assess individual differences between the two groups (operated vs. controls) and days (2, 6, 10, 14, 18 and 21 days). In addition, non–parametric statistical tests were applied (Kruskall Wallis) to verify p-values, but no other statistical significances were found and, therefore, only results of the parametric tests are reported in this text.

Results

Animal experiments

No complications were recorded during surgery, recovery from anesthesia and the postoperative period. Periosteal strippings could be performed without problems and all surgical procedures were performed by the same surgeon (JA).

Preparation of histology/immunhistology

Harvesting of growth plates went without problems. However, due to decalcification requirements sections had to be cut thin enough (2-3mm), which sometimes resulted in separation of the soft, cartilaginous part of the growth plate and the metaphysis. Care was taken to place them correctly and adjoining to each other within the paraffin block. Unfortunately, ruptures of parts of the growth plates could not always be avoided during sectioning, mounting and staining of the thin sections for immunohistochemistry. However, there was always more than 70% left of the growth plates to guarantee adequate scoring. Antibodies for Ihh, PTHrP, PTHrPR, FGF and TGF were validated before the experiments and cross reactions were excluded. There were always PBS controls for each antibody and group. Immunostaining with PTHrP antibodies proved difficult, since growth plates had to be pretreated with the microwave leading to substantial damage of the growth plate morphology. Therefore, not all sections could be completely evaluated on all three locations.

Qualitative assessment

The morphology of all growth plates was considered as normal without changes to the structural organization of the different zones. The perichondrium of the operated groups was much thicker compared to the controls and covered with proliferative fibrous tissue. Other than this, no differences were seen between groups and days.

Quantitative length measurements of growth plate

Measuring length was not always straight forward, since over the entire growth plate the overall thickness and also direction of the columnar arrangement varied. Nevertheless, commonly it was similar in the operated and control limbs and, therefore, the sum of all 4 measurements provided acceptable mean values of the percentage of each zone in relation to the entire growth plate (Fig. 4). No statistically significant differences were found between operated and control limbs, neither for the length of the entire growth plate, nor for the individual zones. However, there was a clear tendency to show increased overall length of the entire growth plate of the operated limbs between days 10-18 for both, the radius and the tibia. If individual zones were compared, the operated limbs had a tendency to show an incre-

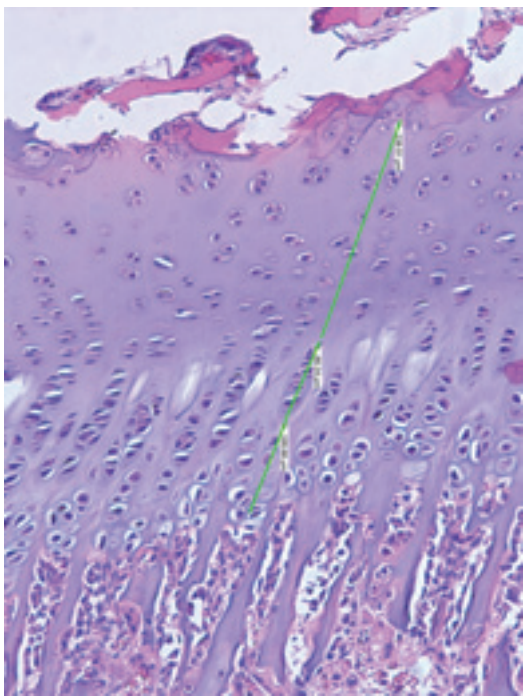

Fig. 4 The photograph demonstrates a sample for length measurements of histological sections (Quips, Leica®Heerbrugg, Switzerland; paraffin section, HE staining).

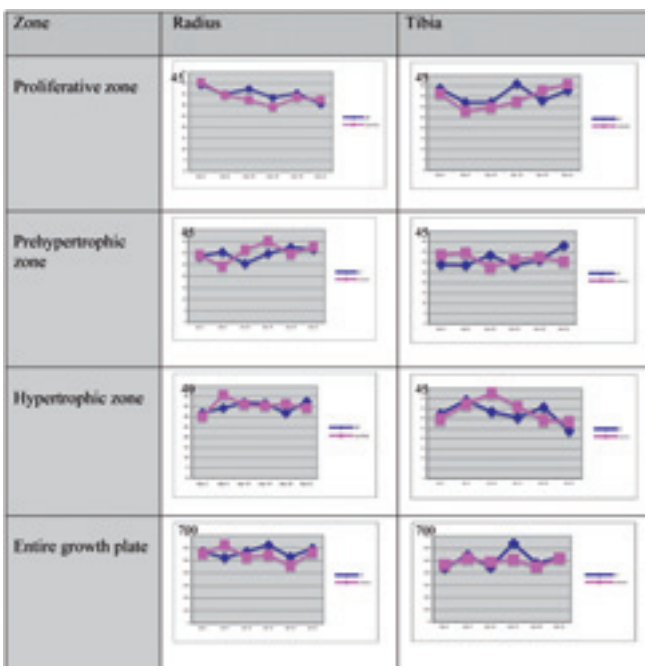

Fig. 5 The graph shows the curves of length measurements over time. Note the clear tendency for operated limbs to have an increased overall length of the growth plate, specifically of the proliferative zone.

ased length of the proliferative zone between days 2-14 in the tibia, and for days 6-18 in the radius. In the prehypertrophic zone results were variable, whereas in the hypertrophic zone there was a tendency for the operated limbs to show lower values compared to the controls for both, the radius and the tibia (Fig. 5).

Scoring of immunohistochemistry sections

As in the HE sections, it could not always be avoided that ruptures between the growth plates and the metaphyses occurred. Nevertheless, it was always possible to score three regions, although sometimes the middle region had to be shifted off the central part. Staining was easily visible and background noise was minimal in all sections. Statistically significant differences were recorded within the graphs (Fig. 6) and only for differences between groups (operated versus controls) and time points within groups. Expression of the different factors was studied over time in parasagittal planes. There was generally an undulating pattern over time for all variables scored and for operated and non-operated limbs, but always more pronounced in the operated limbs. This was true for both, the radius and the tibia.

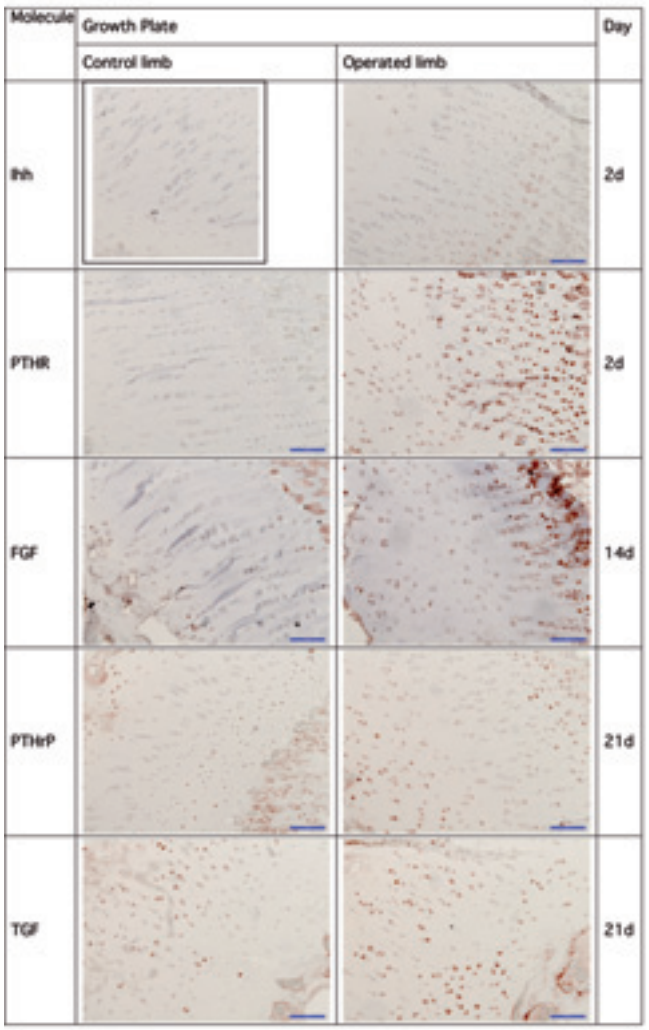

Fig. 6 Immunohistochemistry sections of operated limbs (right column) and controls (left column) show differences in intensity staining (red coloration) for all molecules. The pictures are taken on days, where the individual molecules showed their peak expression in the operated limbs. Staining for Ihh was increased mainly in the late pre- and hypertrophic zone at 2 days, whereas PTHR was more intensly stained in the hypertrophic zone and perichondrium. PTHrP was more intense in the prehypertrophic zone, while FGF and TGF were more intensely stained in the perichondrium and the proliferative zone.

Ihh

Staining was present in all zones and also the perichondrium, but more pronounced in the late prehypertrophic and hypertrophic zone. Overall, the operated sides showed higher scores mainly at days 2, 10 and 14. As for the intensity of staining (Fig. 7), scores were higher in the operated limbs mainly in the early period between day 2 and 10, and slightly less up to day 18 and even on day 21. The highest scores were found in the hypertrophic zone and perichondrium.

PTHrP

Staining was moderate in all zones of the growth plates. The highest scores for PTHrP staining were found in the perichondrium, the lowest in the prehypertrophic zone, although in the latter the scores for the operated limbs were still higher compared to the non-operated site. The distribution for the intensity of staining was similar. Overall, the operated limbs showed an increase in scores in the early (days 2) and later time periods and there, mainly from day 14-18, or even up to 21 in the prehypertrophic zone (Fig. 8).

PTHR

Staining of PTHR was present in all zones and the perichondrium and more less equally high (score 1), although the prehypertrophic zone showed slightly less staining in the radius compared to the proliferative and hypertrophic zone, especially at the later time points (day 10, 18 and 21). The highest scores of the intensity of staining were found for the hypertrophic zone in both, the radius and the tibia. Overall, the pattern for PTHR peaked in the early time periods (between days

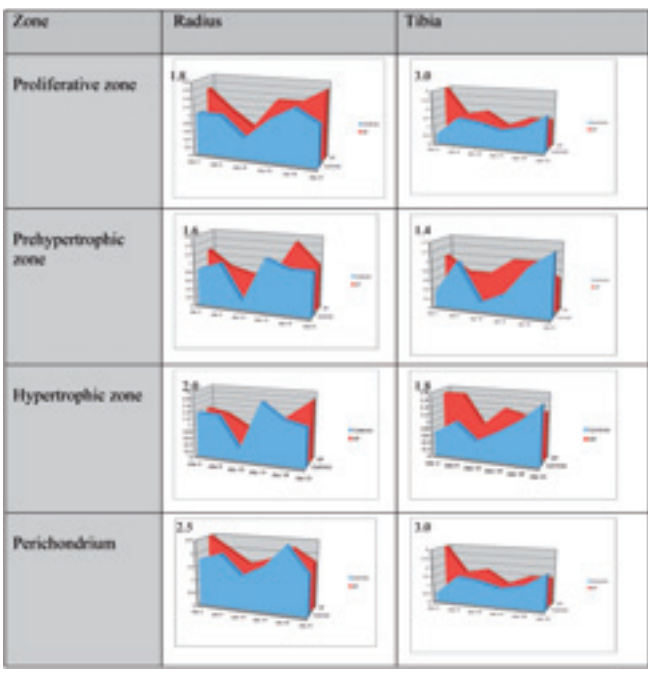

Fig. 7 The regulation of Ihh is depicted in graphs of the various zones and over time. Note, that in operated limbs the Ihh is upregulated relatively early after periosteal stripping. Also note the undulating pattern for both, operated limbs and controls (all graphs).

2-6) and later between days 14 to 18. Although initial peaks were generally higher in the growth plate of the tibia, the scores for the remaining days were pretty much similar between the two bones (Fig. 9).

FGF

All zones and the perichondrium stained well for FGF with a tendency for the operated limbs for higher scores. As for the intensity of staining, this growth factor was expressed throughout all zones of the growth plates with higher scores in the operated limbs. The highest scores were found in the proliferative zones and perichondrium in both, the radius and the tibia, although there was a slightly more undulating pattern in the tibia compared to the radius, most pronounced in the perichondrium. Scores were usually elevated in the second half of the time period, although a rapidly decreasing peak was found at day 2 in all zones and the perichondrium (Fig. 10).

TGF

As with FGF, TGF staining was present in all zones and the perichondrium. The intensity of TGF staining was high throughout the zones and the perichondrium, but also in an undulating pattern always increasing towards the later time points (days 18-21, peaks already between day 10 and 14).

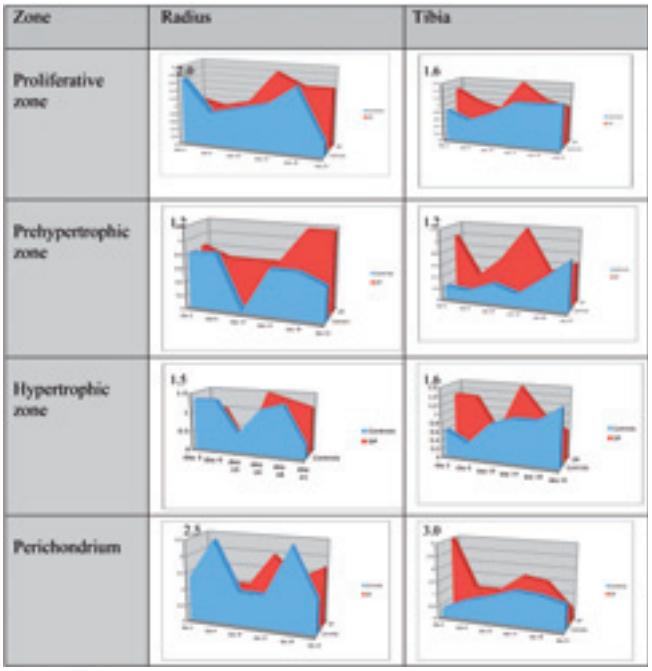

Fig. 8 PTHrP is mainly upregulated in the perichondrium and at a later time point than Ihh.

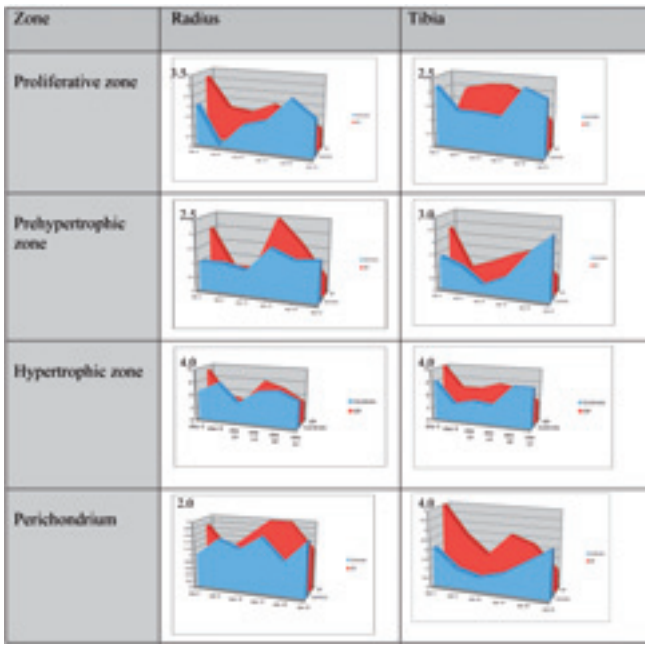

Fig. 9 The upregulation of PTHR in operated limbs is similar to the curves observed in PTHrP. The highest scores were found for the hypertrophic zones

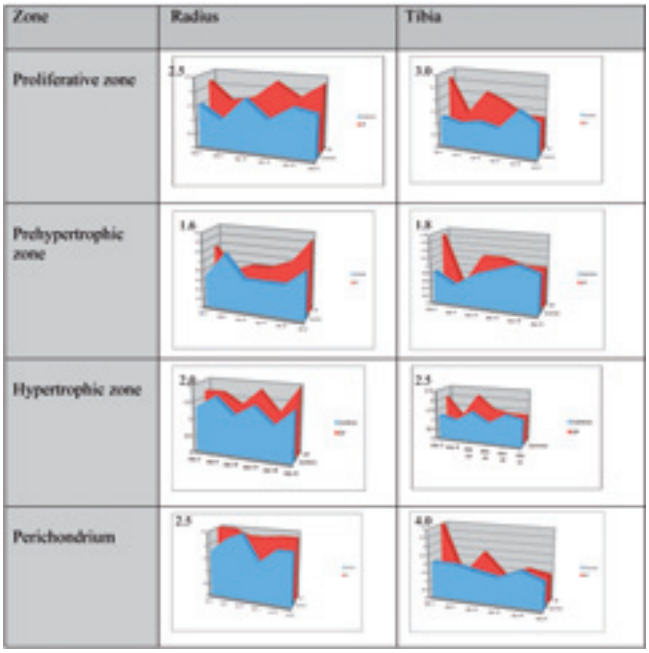

Fig. 10 FGF in response to periosteal stripping was upregulated mostly in the proliferative zone and the perichondrium.

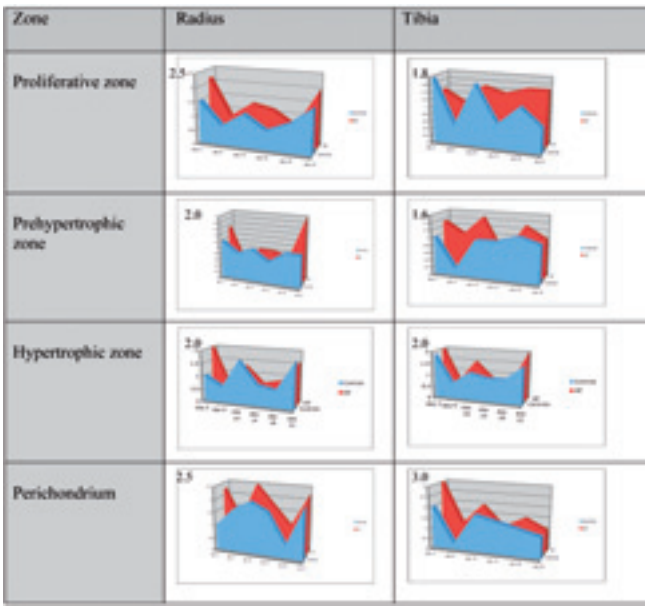

Fig. 11 The highest peaks for TGF upregulation in the operated limbs were found in the perichondrium followed by the proliferative zones.

The highest scores were found for the periochondrium followed by the proliferative zones in both, the radius and the tibia (Fig. 11).

Correlations

As for the length measurements, there were very high and negative correlations in the radius between the proliferative- and prehypertrophic- as well as hypertrophic zones ($p<0.0001$). There was no correlation between the prehypertrophic and hypertrophic zones. Correlations found for the tibia were similar ($p<0.0001$), except that negative correlations were statistically significant also between the prehypertrophic and hypertrophic zones. Correlations for protein expressions in the radius and the tibia are only reported for intensity of staining and are outlined in Tab.1a-d.

Discussion

This experimental study in growing lambs could demonstrate that periosteal stripping at an early time point triggers a growth restraining feed back loop initiated by an up-regulation of Ihh in the early pre- and hypertrophic zone of the growth plate, followed by an increase of PTHrP mainly in the perichondrium, while an increase of PTHR was noticed in all zones, although highest in the perichondrium and hypertrophic zones. The growth factors FGF and TGF were upregulated in all zones, but FGF in response to periosteal stripping was more intensely expressed in the proliferative zone and the highest peak of TGF was found in the perichondrium. Length measurements of the various growth zones revealed significant negative correlations between the proliferative zones and pre-and hypertrophic zones, indicating that indeed a negative feed back loop after periosteal stripping exists coupled by the Ihh/PTHrP/PTHR cascade. The results suggest that early intervention, when growth plates are still open and responsive to various stimuli, may be very beneficial in clinical cases.

Lambs served well as animal models to study protein expression in the growth plates after periosteal transection and elevation. The surgeries were efficient, easily standardized and growth plates of the radius and tibia were still active at the chosen age. None of them was closed, neither in control nor operated limbs. Nevertheless, there were some limitations such as animal numbers (2 lambs per time point) and thus, number of growth plates retrieved. It would have been preferable to increase the numbers of growth plates for each time point and performing the study at a younger age of the animals. This was not possible for several reasons of which

Table 1a Correlations for intensity scores (IZ) and zones in the tibia

Variables 1	Variable 2	Correlation coefficient	p-Value
IZ1-Ihh	IZ2-Ihh	0.788	0.002
	IZ3-Ihh	0.798	0.002
	IZ1-PTHrP	0.701	0.011
	IZ2-PTHrP	0.719	0.008
	IP-PTHrP	0.593	0.042
	IP-PTHR	0.609	0.036
	IZ2-FGF	0.584	0.046
IZ2-Ihh	IZ3-Ihh	0.881	0.000
	IP-Ihh	0.835	0.001
	IZ1-PTHrP	0.730	0.007
	IZ2-PTHrP	0.742	0.006
	IZ3-PTHrP	0.582	0.047
	IP-PTHrP	0.749	0.005
	IZ2-PTHR	0.689	0.013
	IP-PTHR	0.756	0.004
	IP-FGF	0.677	0.016
IZ3-Ihh	IP-Ihh	0.601	0.039
	IZ2-PTHrP	0.679	0.015
	IP-PTHrP	0.645	0.023
IP-Ihh	IZ2-PTHrP	0.760	0.004
	IP-PTHrP	0.896	0.000
	IZ2-PTHR	0.667	0.018
	IP-PTHR	0.927	0.000
	IP-TGF	0.805	0.002
	IZ2-FGF	0.625	0.030
	IP-FGF	0.822	0.001
IZ1-PTHrP	IZ3-PTHrP	0.614	0.033
	IZ1-FGF	0.678	0.015
	IZ3-FGF	0.643	0.024
IZ2-PTHrP	IZ3-PTHrP	0.636	0.026
	IP-PTHrP	0.891	0.000
	IZ2-PTHR	0.698	0.012
	IZ2-PTHR	0.757	0.004
	IP-TGF	0.672	0.017
	IZ2-FGF	0.741	0.006
	IP-FGF	0.831	0.001
IZ3-PTHrP	IZ2-PTHR	0.590	0.043
IP-PTHrP	IZ2-PTHR	0.681	0.015
	IZ2-TGF	0.604	0.038
	IP-TGF	0.771	0.003
	IZ2-FGF	0.748	0.005
	IZ3-FGF	0.723	0.008
	IP-FGF	0.896	0.000
IZ1-PTHR	IZ2-PTHR	0.676	0.016
IZ2-PTHR	IP-TGF	0.692	0.013
	IP-FGF	0.763	0.004
IP-PTHR	IZ2-TGF	0.660	0.020
	IP-TGF	0.901	0.000
	IZ2-FGF	0.704	0.011
	IZ3-FGF	0.644	0.024
	IP-FGF	0.892	0.000
IZ1-TGF	IZ2-TGF	0.713	0.009
	IZ3-TGF	0.852	0.000
	IZ2-FGF	0.673	0.016
IZ2-TGF	IZ3-TGF	0.788	0.002
	IP-TGF	0.782	0.003
	IZ2-FGF	0.685	0.014
	IZ3-FGF	0.591	0.043
	IP-FGF	0.696	0.012
IZ3-TGF	IZ2-FGF	0.729	0.007
IP-TGF	IZ2-FGF	0.612	0.034
	IZ3-FGF	0.577	0.050
	IP-FGF	0.806	0.002
IZ1-FGF	IZ2-FGF	0.731	0.007
	IZ3-FGF	0.933	0.000
	IP-FGF	0.586	0.045
IZ2-FGF	IZ3-FGF	0.782	0.004
	IP-FGF	0.815	0.001
IZ3-FGF	IP-FGF	0.755	0.005

Table1b Correlations for intensity scores (IZ) and zones in the radius

Variables 1	Variable 2	Correlation coefficient	p-Value
IZ1-Ihh	IZ2-Ihh	0.786	0.002
	IZ1-PTHrP	0.701	0.011
	IP-PTHR	0.609	0.036
	IZ1-TGF	0.595	0.041
IZ2-Ihh	IZ1-PTHrP	0.730	0.007
	IZ2-PTHrP	0.743	0.007
	IZ3-PTHrP	0.763	0.004
	IP-PTHrP	0.580	0.048
	IZ1-PTHR	0.743	0.006
	IP-PTHR	0.781	0.003
	IP-TGF	0.579	0.049
	IZ2-FGF	0.670	0.017
	IZ3-FGF	0.643	0.024
IP-Ihh	IZ2-PTHrP	0.652	0.022
	IP-PTHrP	0.899	0.000
	IZ-2-PTHR	0.693	0.013
IZ1-PTHrP	IZ2-PTHrP	0.800	0.002
	IZ3-PTHrP	0.803	0.002
	IP-PTHrP	0.624	0.030
	IZ1-PTHR	0.668	0.017
	IZ2-PTHR	0.747	0.005
IZ2-PTHrP	IZ3-PTHrP	0.784	0.003
	IP-PTHrP	0.778	0.003
	IZ1-PTHR	0.837	0.001
	IZ2-PTHR	0.798	0.002
IZ3-PTHrP	IZ1-PTHR	0.720	0.008
IP-PTHrP	IZ1-PTHR	0.665	0.018
	IZ2-PTHR	0.698	0.012
IZ2-PTHR	IZ3-PTHR	0.760	0.004
	IP-PTHR	0.702	0.011
IZ3-PTHR	IP-PTHR	0.737	0.006
IP-TGF	IZ2-FGF	0.851	0.000
	IZ3-FGF	0.719	0.008
	IP-FGF	0.943	0.000
IZ1-FGF	IZ3-FGF	0.645	0.023
	IP-FGF	0.608	0.036
IZ2-FGF	IZ3-FGF	0.604	0.038
	IP-FGF	0.928	0.000
IZ3-FGF	IP-FGF	0.715	0.009

the most important were costs, animal welfare and also amount of work to be performed. However, each histology section could be scored at 3 locations and at 4 locations length measurements could be performed, resulting in 6 scores and 8 measurements per time point and for the scores in three different zones of the growth plates. In addition, both (semi-) quantifications were followed over time, which allowed verification at different time points. Increasing animal numbers may have resulted in less variations and more statistically significant differences; however, results over time demonstrated tendencies that were clear enough to support our original hypothesis that periosteal stripping elicits a Ihh/PTHrP/PTHR negative feed back loop in growth plates. In addition, meanwhile in a second series in our laboratory, where 6 lambs per group were compared only at one time point at 4 weeks after surgery we could confirm our results sufficiently to draw safe conclusions (results published elsewhere: *Liesegang* et al. 2010).

Table 1c Correlations for length measurements of zones in the radius

Variables 1	Variable 2	Correlation coefficient	p-Value
proliferative	prehypertrophic	-.656	0.000
	hypertrophic	-.679	0.000
prehypertrophic	hypertrophic	-.108	0.348

Table 1d Correlations for length measurements of zones in the tibia

Variables 1	Variable 2	Correlation coefficient	p-Value
proliferative	prehypertrophic	-.631	0.000
	hypertrophic	-.616	0.000
prehypertrophic	hypertrophic	-.233	0.050

With the preparation of histological slides a few challenges had to be met. In our preliminary study with control tissues to validate all antibodies used for lambs (MH), we could demonstrate that cryosections showed slightly better results. However, if bone is involved, cryosections are not feasible, at least in larger animals. Although only little bone was included in extension from the growth plates into the metaphyses and epiphyses, this was sufficient to destroy the morphology of all growth plates rendering every section unfeasible for immunohistochemistry. Decalcifiaction and embedding in paraffin was a "must" and could not be avoided. In order to speed up the process of decalcification, thin slices had to be cut in the fresh bone, which detached some parts of the growth plates in their weakest connection, often in the center of the sections. Nevertheless, there were always enough good parts of the growth plates left for scoring and getting an adequate number of each growth plate and time point.

Immunohistochemical staining for each antibody were always performed in one batch and at the same time and by the same highly experienced technician (KZ). Therefore, we consider the undulating pattern of protein expression as real and not related to technical problems. This assumption is supported by the fact that undulation was pretty constant throughout the different protein expressions and correlations between them were very high. In the literature, undulating patterns of these protein expressions are not reported to the knowledge of the authors, neither in development (*Pathi* et al. 1999, *Vortkamp* 2001), nor after surgical interventions, such as periosteal stripping. Furthermore, most of the studies were conducted in laboratory rodents (*Alvarez* et al. 2001, *Karp* et al. 2000, *Vortkamp* et al. 1996) and no in vivo study was found in larger animals such as sheep or even horses. Changes of Ihh and PTHrP expression were reported in horses affected with OCD (*Semevolos* et al. 2005), but not after periosteal stripping. To the authors' knowledge, this is the first study reporting differences in protein expressions in growth plates of growing larger animals and after periosteal stripping.

Undulation of protein expression occurred also in the non-operated sites and quite frequently parallel to those of the operated limbs. The exact reason for the development of this pattern could not be demonstrated in this study and probably will be impossible to prove also in studies dedicated to this

problem. However, if a negative feed back loop is responsible for "kicking off" longitudinal growth in long bones, an undulating pattern may be well explained. The undulating pattern in protein expression could also be logically explained in normal or undisturbed growth plates. If Ihh is the triggering molecule to initiate or inhibit longitudinal growth, it makes sense that the translational protein expression and effective lengthening of the growth plate involved in the growth restraining feed back loop are awaited before new signals are transmitted again. The results of our controls and operated limbs suggest that this biological pattern involves under physiological conditions a 10 day cycle.

If individual protein expressions in our investigation were studied over time and compared to the locations and sequence postulated in embryonal development by *Vortkamp* et al (1996), our results matched largely their findings. These authors described the sequence of the signal cascade in the embryonic development of growth plates in mice, where Ihh up-regulation in the pre- and hypertrophic zones induced the expression of the second signal, the PTHrP, mainly in the perichondrium. *Weisser* et al (2002) studied this expression pattern in bovine chondrocytes of growth plates and showed additionally that the expression of MMPs varied accordingly. PTH1-34 and PTHrP1-40 inhibited MMP13 expression in the hypertrophic zone indicating that mineralization in this area was delayed as expected in the growth restraining feedback loop. Similar to our results they found that PTHR was not only expressed in the prehypertrophic-, but mainly in the hypertrophic zone. These findings were (like ours) different from mice (*Vortkamp* et al. 1996) and may be specific for ruminants.
The actual length measurements of the different zones, again, were not significantly different between control and operated limbs. However, the statistically strong negative correlation between lengths of the various zones of the growth plates supported our hypothesis that periosteal stripping initiates the signaling cascade, where Ihh and PTHrP/PTHR expression is responsible for length control. The reason why actual length measurements of the various zones showed no statistically significant differences may also be related to the fact that our lambs had normal growth plates and no axis deformation before experimental periosteal stripping was performed. It is probably safe to assume that in foals with angular limb deformity the surgically elicited molecular response may be much more pronounced due to the false and uneven mechanical load across the physis (*Auer* 2006). The molecular response may also be longer sustained in these cases. Most likely this assumption will never be proven, since animal experiments with naturally affected foals will be hard to conduct, not only for costs but also for ethical reasons.

Experimental surgical studies in lambs (*Meynaud-Collard* et al. 2009) and foals (*Read* et al. 2002) failed to demonstrate the beneficial aspect of periosteal transsection and elevation compared to controls. Therefore, both research groups concluded that periosteal stripping has no benefit for correcting axis deviations, which makes their findings contrary to clinical experience and also to our results of the current study. Both studies used an experimental design, where growth plates were surgically manipulated initially, before corrective measures were taken several weeks thereafter. No differences were found between controls and treated limbs. The failure to show differences may well be related to the experimental design in both studies. In awareness of the subtle molecular regulatory mechanisms of the growth plates, it may be safely assumed that the initial surgical intervention to create the axis deviation and the removal of the growth-restraining devices already triggered a molecular response and signal cascade such, that the second, corrective measure did not make another difference. Our current study, however, suggests that periosteal stripping indeed may elicit a corrective response in limbs with axis deviations.

Future studies of our group are directed towards comparing different treatment modalities for periosteal stimulation in foals including using biotechnology products that can entertain a prolonged action for physeal correction.

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