Detection of PTHrP and Ihh in fetal epiphyseal growth plate zones

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&
Radboud University Nijmegen, Dept. Biology

Wille A.
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Summary

Long bones grow via a process called endochondral ossification. In this process chondrocytes synthesize cartilage matrix that during growth is calcified in a carefully controlled manner. This occurs in the growth plate which consists of a number of different differentiated cell zones that expresses specific growth regulating proteins.

Endochondral ossification is influenced by different external factors. It is known that mechanical loading plays an important role in remodelling of tissues. PTHrP and Ihh are considered to be one of the important regulating protein mechanisms during bone growth. The expression of these proteins is depended upon the differentiation stage of cells in the different growth plate zones. According to present studies, mechanical loading would have a decreasing effect on the expression of these proteins.

During the last few years computer models were developed concerning the growth of bone and its biochemical regulation mechanisms. To be able to validate these computer models experimental data of growth protein expression and there effects is required. Therefore, techniques have to be created to isolate the different growth plate zone cells, detect proteins PTHrP/Ihh and detect concentration differences in these proteins after mechanical loading exposure. This study aimed to develop these different techniques.

Viable epiphyseal growth plate cells were isolated from porcine fetuses. The epiphyseal growth plate cells were separated on their different differentiations using discontinues Percoll gradient. Different research groups and additional experiments like Immunohistochemical staining with Alkaline Phosphatase showed that the epiphyseal growth plate cells were separated on their different differentiation stages with success. It also showed that a minimum of $\sim 6.0 \times 10^7$ cells were needed to have easy separation of the different growth plate zones.

Immunochemical staining, using antibodies against porcine PTHrP and Ihh, showed different molecular weight bands intracellular after gel electrophoreses. Molecular weight comparison showed that in all likeliness PTHrP and Ihh were detected. However, additional experiments, e.g. protein sequencing, have to be run to get 100% proof. Only intra cellular PTHrP and Ihh detection was accomplished.

For mechanical loading experiments in which decrease of PTHrP and Ihh production is assumed, an immunochemical staining experiment was run that showed that 4x dilution of recommended amount of cells would still show protein bands.

Because of the many different protein bands for PTHrP and Ihh it was not possible to obtain any reliable data from Immunohistochemical staining on cryo slides. More specific antibodies for PTHrP and Ihh have to be found and tested.

Overall all the results pointed out that the techniques used to isolate the epiphyseal growth plate cells and separate them on their different differentiation
stages was successful. The detection of PTHrP and Ihh in these isolated epiphyseal growth plate cell zones was possible.
Foreword

I chose this placement at the Eindhoven University of Technology, Department of Biomedical Engineering because of my past studies and future aims. As a microbiologist and IT professional I could not choose between these different branches. 2 years ago, I decided to try and combine the two by starting a new study at Radbout University Nijmegen. For this study two placements of 9 months each are to be accomplished. Trying to combine the two professions I decided to accept the invitation of this placement. It showed to be a very interesting and knowledgeable experience.

I would like to thank René van Donkelaar for offering this placement and hope that my work will be a benefit to his ongoing research. I also want to thank the following persons for their support and help during my study; Reinout Hesselink, Jasper Foolen, Birgit Faber, Linda Meijer, Machiel Resink and Edel Kelleher. Wishing you all success with your current research and future possibilities.

Alain Wille
2006
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1. Introduction

The primordial of the appendicular skeleton are the limb buds, which consist of meso-dermal structures covered by ectoderm. The embryonic limb buds follow a condensation of the mesenchymal cells which in turn differentiate into cartilage cells (chondrocytes). The chondrocytes produce a cartilaginous model or matrix that is used in architecture the production of bone in a later stage. Around the cartilage an outer layer of cells, the perichondrium, is formed. The cartilage grows by interstitial and appositional growth, and a vascular system develops to invade the perichondrium. This is followed by a collar of bone laid down around the airshaft of the bone. The ossification of the midshaft is a result of the inner perichondrial cells differentiating into osteoblasts. During which the osteoblasts together with capillaries invade the centre of the shaft to form the so called primary (or diaphyseal) ossification centre and dispositions spongy bone. The embryonic bone increases in width by appositional growth, the spongy bone core gradually becomes resorbed to form a marrow cavity.
After some time, usually after birth, a secondary ossification (epiphyseal) centre is formed in long bones. The difference between the primary ossification centre and the secondary ossification centre is that the spongy bone is being maintained and no marrow cavity is formed. The area between epiphysis and diaphysis is the epiphyseal growth plate. The growth of the cells in the growth plate continues in columns and the cartilage matures at the diaphysial side followed by calcification. This process continues until late adolescent age when the growth plate completely calcifies. [3-6]

This process of endochondral ossification is regulated through different factors. As in most biological processes, if these factors are disturbed, in this case growth retardation can follow. An example of this kind of growth retardation is seen in Jansen’s metaphyseal chondrodysplasia, a dwarfing condition with severe abnormalities of the growth plate.[7]

1.1 Epiphyseal growth plate

The epiphyseal growth plate is important for the growth of bone in vertebrates. The regulation of longitudinal growth at the growth plate occurs generally through hormones and growth factors. Triggered by these factors the chondrocytes in the growth plate proliferate or differentiate, thus forming different zones in the growth plate in which each cell population has a different morphology and biochemical process. (Figure 2) These zones are resting, proliferative, prehypertrophic, hypertrophic and calcification zone.[4, 8]

![Figure 2](image_url)

Figure 2: Left: the interior of a typical long bone showing the proximal ends with a growth plate and a distal end. Right: Schematic overview of the growth plate showing all known different zones.[2]
Zone
Reserve : The cells exist single or in pairs and are surrounded by an abundant extra cellular matrix. Their proliferation is slow.
Proliferative : In this zone the resting cells are triggered to proliferate, resulting in longitudinal growth.
Prehypertrophic : After proliferation the cells will start increasing their cell mass and produce matrix.
Hyperthrophic : Matrix calcification occurs in between the columns of cells and the calcified matrix becomes scaffolding for bone deposition.

Table 1: The different zones and their prospective roll.

1.2 Regulating proteins

There are numerous proteins regulating the formation of bone. Generally it as been accepted that proteins PTHrP and Ihh are major regulators of human bone development. [9-12]

1.2.1 PTH

Parathyroid hormone (PTH), which is also designated parathyrin, consists as an 84 amino acid single chain peptide. It’s known to functions as regulator for calcium metabolism by raising blood levels of calcium through various mechanisms. PTH is also known to stimulate bone formation to increase bone mass and strength in rats and humans. Within the PTH molecule, the essential activity is associated with the first 34 amino acids at the amino terminus of the molecule. The gene responsible for encoding of PTH in humans can be found on chromosome 11p15.3-p15.1.

Parathyroid hormone related protein (PTHrP) is an autocrine/paracrine[13] factor that is structurally related to PTH. PTHrP is found to be only synthesised locally in the perichondrium and proliferating zone of the growth plate and considered to be a limiting factor for chondrocyte differentiation in fetal murine growth plate and keeping cells in the proliferating pool. [10, 12]. PTHrP regulates the endochondral bone development. PTHrP also has its PTH/PTHrP receptor (PPR1) that he shares with PTH. The PPR1 is mainly expressed by chondrocytes of the early hypertrophic zone, but recent studies have also found receptor expression by chondrocytes of other growth plate zones.[14] The molecular weight for PTHrP is not consistent for all living creatures. [15] The molecular weight of PTHrP for mouse, rat and human is around the 26 kDa.

The gene responsible for encoding of PTHrP in humans can be found on chromosome 12p12.1-p11.2. PTHrP can exist in a precursor and mature form. [16-18]

1.2.2 Ihh

Indian Hedgehog (Ihh) originally discovered in Drosophila, belongs to a family of morphogens. [19]
Ihh is a member of the vertebrate hedgehog family that currently consists of Sonic Hedgehog (Shh) (alternatively designated Vhh-1), Desert hedgehog (Dhh) and Indian hedgehog (Ihh).

The Drosophila segment polarity gene hedgehog (hh) encodes a precursor protein which undergoes auto cleavage to generate amino and carboxyl terminal peptides of 19 kDa and 25 kDa molecular weight respectively. Both proteins are secreted and appear to function in embryonic and imaginal disc patterning. Each contain amino terminal signal peptides and apparently function as secreted proteins involved in the mediation of various cell-cell interactions. [20]

Ihh is expressed not only in cartilaginous growth plate during limb development [12], but also during fracture healing in bone callus [21, 22]. Ihh is a key molecule that regulates chondrocyte proliferation and differentiation during endochondral bone formation and is expressed by early hypertrophic cells [12, 22, 23]. Ihh achieves these functions by inducing a series of downstream factors, including its receptor patched (Ptc), a 12-pass transmembrane protein [20], PTHrP [12], and more that will not be addressed in this thesis.

Over expression of Ihh in fetal chicken long bones blocks chondrocyte differentiation. [12] This is caused by an up-regulation of PTHrP expression in periarticular perichondrium in fetal long bones.

1.3 PTHrP – Ihh feedback loop

Both PTHrP and Ihh have a close interaction with each other. There are many control loops active during bone growth, but the PTHrP – Ihh loop is considered to be the most important.

Figure 3 shows that PTHrP originates from the perichondrial cells and chondrocytes at the end of long bones (1) and acts on the receptors of proliferating chondrocytes keeping them proliferating and thereby, delaying the production of Ihh.

Studies have shown that cells release their proteins in a way that creates gradients. Believing that this will also be true for the production of PTHrP from perichondrial cells and chondrocytes, at a certain point the concentration of PTHrP reaches a critical level in which the PTHrP will no longer have a delaying effect on the production of Ihh. When this occurs, Ihh expression is increased again. Zhao Q et al. calculated this critical level to be at 0,1 nM. He also found the effective concentration to be between 0,1 and 10 nM.[24]

Different studies have also shown that Ihh acts on its receptor on chondrocytes to increase the rate of proliferation (2) and, through a poorly understood mechanism stimulates the production of PTHrP at the ends of bones (3). Ihh also acts on perichondrial cells to convert these cells into osteoblasts of the bone collar (4). [9]
1.4 Mechanical Loading

It is suggested that mechanical loading influences bone formation and adaptation. Different research groups over the past few years developed experimental models to correlate the effect of mechanical loading with bone formation.[25, 26] All compression experiments caused by axial loading of the growing rat ulnas showed longitudinal growth suppression. [27, 28] This suggests that mechanical loading might suppress endochondral ossification through alteration in metabolism of the cells or increasing damage in the cells. Sustained distraction showed an increase of growth, but the loading effect was greater.[29] These models suggest that mechanical loading of bones influences regulatory processes in the growth plate.

Theoretically, effects of mechanical loading on bone development can be direct, via deformation or compression of the tissue, or indirect inducing changes in (paracrine) signalling. Both PTHrP and Ihh experiments have been shown sensitive to mechanical perturbation and suggests that the latter effect is present. Experiments involving deformation of chondrocytes showed up regulation of PTHrP and IHH production.[30, 31] The relative importance of these effects in total bone development is yet unclear. One of the questions our research group is interested in is what happens during mechanical loading of the cells?

To be able to determine the relevance of changes in PTHrP and Ihh and the influence on growth we asked ourselves the following 2 questions: Are PTHrP and/or Ihh cell concentrations changed with mechanical loading? And, is the change significant to explain for the total changes seen in growth.

Figure 3: Schematic representation of the different zones and their protein production, influencing each other. [9]
2 Aim of study

The aim of this study was to determine PTHrP and Ihh protein synthesis in different growth plate zones of fetal epiphyseal growth plate cells.

Two sub-aims are defined:

- Isolate and separate epiphyseal growth plate cells from different growth plate zones.
- Verify the location of proteins PTHrP and Ihh in histological slides.
3 Methods and Materials

For specification of the materials mentioned below, see “Appendix A, Material Specification.”

3.1 Isolate fetal epiphyseal growth plate cells

The development and growth including the many regulatory proteins in vertebrates are mostly expressed during the embryonic and adolescent stage. Past studies had shown the need of large amounts of cells for use during separation experiments.[14] Because of these reasons I used porcine fetal tissue obtained from the local slaughterhouse (Ballering Export B.V., Son en Breugel, The Netherlands). The porcine embryo is large in comparison with embryos from commonly used laboratory animals such as rats or mice and would potentially yield more cells to work with. The use of bovine fetal tissue was not allowed because of strict regulation regarding Mad Cow Disease and Creutzfeld-Jakob syndrome. An other reason for the use of porcine fetal tissue was the knowledge that the porcine and human genome is considered to be very similar and so future correlation could be made easier.

Cell isolation was done in an airflow cabinet to reduce the chances of contamination by micro-organisms. The distal and proximal growth plates were removed from the femur and humerus. Foetuses were superficially washed by spraying 70% ethanol on them. Legs were removed using a scalpel. The epidermis was first cut open using one scalpel and a new scalpel was used after to cut the inner tissue. After the legs were removed the femur length was measured for determining age.

To expose the epiphyseal growth plate for each leg, an incision was made above the cartilage cap, the bones were twisted till the cartilage cap was exposed. Using the scalpel tendons were cut through and the cartilage cap was tipped off using two thumbs. The cartilage cap was then placed in a solution of DMEM (1 g/l glucose, no L-glutamine) containing: 1/50 Penicillin/Streptomycin (10000 µg/ml); 1/50 Gentamycin (10 mg/ml) and 1/50 Amphotericin B (250 µg/ml), for the duration of the cartilage cap extractions. For the rest of this thesis it will be referred to as 2x DMEM.

After the removal of the cartilage caps of all the legs, the cartilage caps were placed on a sterile surface and excess tissue was removed using new scalpels. The cartilage caps were cut above the epiphyseal growth plate to reduce the chance of detecting proteins that are not from the epiphyseal growth plate (for example from the secondary ossification centre). The epiphyseal growth plate was longitudinally cut in small cubes. Subsequently the cubes were transferred into a cell culture flask with a solution of DMEM (1 g/l glucose, no L-glutamine) containing: 3/5 Collagenase Type I (2000 units/mg); 1/1000 Deoxyribonuclease; 1 from bovine pancreas (2000Kunitz Units); 1/100 Penicillin/Streptomycin (10000 µg/ml); 1/100 Gentamycin (10 mg/ml) and 1/100 Amphotericin B (250 µg/ml) and incubated overnight at 37°C, 5% CO2 under constant slow shaking, 150 rpm. For the rest of this thesis it will be referred to as 1x DMEM.
Next, fragments of epiphyseal growth plate were separated from single cells using a sterile 40µm cell strainer. The fragments of epiphyseal growth plate left on the cell strainer were discarded and the single cell solution transferred to sterile 50 falcon tubes and centrifuged for 5 seconds at 1000 rpm. The reason for this short spin is to separate the cells from any heavy particles present in the solution. The supernatant was transferred to sterile 50 ml falcon tubes and centrifuged 7 minutes at 1000 rpm. Following this, the supernatant was collected and stored directly at -80°C until further use. The pellet containing mostly epiphyseal growth plate cells was washed using 1x DMEM and centrifuged 7 minutes at 1000 rpm. The pellet was dissolved in 2ml of 1x DMEM and cells were counted using a Marienfeld Neubauer improved counting chamber with 1/50 Trypan Blue 0,5% (w/v) in physiological saline.

After counting, the ~ 2 ml epiphyseal growth plate cell solution was directly used to separate cells from different zones. Therefore a Percoll Discontinuous (step) gradient obtained from Sigma was used.

3.2 Separate the epiphyseal growth plate cells on their different growth plate zones.

Different research groups had shown that using continuous- and discontinuous Percoll gradient chondrocytes from different stages of differentiation would have different buoyant densities and showed to settle between the different Percoll concentration layers.[14, 32, 33] Alini et al. had shown that a successful separation of chondrocytes by using a discontinuous Percoll gradient containing Percoll concentrations of 1,01 till 1,07 g/ml.[32] During this project 2 discontinuous Percoll gradients were used. A discontinuous Percoll gradient of 1,01 till 1,07 g/ml and a discontinuous Percoll gradient of 1,01 – 1,03 – 1,05 and 1,07 g/ml of Percoll to increase the yield of cells per layer. The gradients were created according to “Appendix B, Percoll Calculation”.

I started off by breaking a glass pipette just under the bottle neck and made sure that brake point was sloped. (figure 5) I added 1.5 ml of the lowest Percoll gradient to a 15 ml Falcon tube. After this the “broken” glass pipette was inserted (figure 5) and using a slowly steady stream the following higher concentrations of Percoll were pipetted through the “broken” glass pipette.

When the last Percoll (highest) gradient was added an additional volume of Percoll (highest) gradient was added to push any lower gradient through the glass pipette. To remove the pipette I used a finger tip to block the entry point of the glass pipette to prevent any left over gradient mixing when the pipette was lifted.
By using a slowly steady stream the ~2 ml epiphyseal growth plate cell solution was added on top of the Percoll gradient. After addition, the Percoll gradient with epiphyseal growth plate cell solutions was centrifuged for 50 minutes, 500g at room temperature.

After centrifuging the different layers (figure 8) were transferred to sterile 15 ml falcon tubes and labelled with 1 being the most upper layer. To each tube, 5 ml of 1x DMEM was added and gentle mixed. All tubes were centrifuged for 7 minutes at 1000 rpm and washed again. After washing 1 ml of 1x DMEM was added to each tube and the cells were counted using a Marienfeld Neubauer improved counting chamber with 1/50 Trypan Blue 0,5% (w/v) in physiological saline.

After counting, the tubes were centrifuged again for 5 minutes at 1000 rpm. Supernatant was discarded and the pellet was washed with 1 ml ice cold PBS (stock solution was 200 ml of deionised water containing 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4 at 25°C) and centrifuged for 5 minutes, 1000 rpm at ~ 0°C using a pre chilled rotor bucket. Supernatant was discarded and 1 ml ice cold complete RIPA Buffer (with freshly added components) per 2.0x10^7 cells in suspension was added to each tube. The tubes were incubated on ice for 30 minutes and random vortexing. After incubation the tubes are centrifuged for 10 minutes, 1000 rpm at ~ 0°C using a pre chilled rotor bucket. Supernatant was transferred to sterile eppendorfs and stored immediately at -30°C until further use.

To be able to verify if the discontinuous Percoll gradient was separating the different differentiated chondrocytes according to previous described papers[14, 32, 33] Alkaline Phosphatase Staining of the different Percoll layers was performed in combination with staining of Alkaline Phosphatase on histological slides. Past research had shown that the concentration of Alkaline Phosphatase, an enzyme that is closely associated with mineralization, progressively increases in growth plates from the resting zone through the hypertrophic zone of the epiphyseal growth plate.[34, 35]

100 µl of each Percoll gradient layer was added to a 96 flat bottom wells plate and 200 µl of Alkaline-dye mix (Sigma) was added to each well. The 96 flat bottom wells plate was left for 30 minutes in darkness for incubation. After 30 minutes the samples were macroscopically and microscopically checked for any purple colorization.

For Immunohistochemical verification, 10 µm cryo slides were prepared from the same porcine fetal tissue. The cryo slides were placed on polysine coated microscope slides and left overnight at -80°C. The next day the cryo slides were stained with Alkaline Phosphatase Staining kit (Sigma) and DAPI (Sigma). After 30 minutes incubation in darkness, the slides were viewed using a Zeiss Axiovert 200M AxioCam HRm with normal and FL 365/80 filter.
3.3 Detect intra cellular proteins PTHrP and Ihh in cells from the different growth plate zones?

Proteins of each Percoll gradient layer were separated on molecular weight by Gel Electrophoresis. Subsequently, Western Blotting was used, in which the proteins separated in the gel could be transferred to nitrocellulose paper for Immunochemical staining.

To determine the efficiency of the procedure different optimization experiments had to be run. One of the experiments was to determine if there were any proteins present in the different Percoll layers after lyses of the cells with RIPA lyses buffer. I decided to run a 12% polyacrylamide/Bis-SDS gel with cell lysate of the different Percoll layers and incubated the 12% gel in Coomassie Brilliant Blue. This would visualize total proteins present in the different Percoll layers and give an impression of the amount of proteins present in the cell lysates. As can be seen below all Percoll layers showed different concentrations of total proteins. This proves that the lyses of cells using RIPA lyses buffer worked well and that there were proteins available to be used with the gel electrophoresis experiments.

![Figure 6: Impression of the different layers and the presence of total protein. This Coomassie Brilliant Blue staining was done on 7 Percoll layers (L1 till L7). Precision Plus Protein Standards of Bio-Rad was used as marker (M). Additional testing using 4 Percoll layer cell Lysate was not done, because this would have only pooled the cells and produce more proteins per Percoll layer.](image)

Two PTHrP antibodies and four Ihh antibodies were found and evaluated. These were not positively tested for porcine but they were for human PTHrP and Ihh. As the porcine genome and human genome are very equal we selected those for our measurements.
<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Prod. #</th>
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</tr>
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<tbody>
<tr>
<td>PTHrP (N-19)</td>
<td>sc-9680</td>
<td>F0204</td>
<td>Santa Cruz</td>
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<tr>
<td>PTHrP (N-20)</td>
<td>sc-9685</td>
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<tr>
<td>Anti-h/mIhh N-term</td>
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<tr>
<td>Anti B-Actin</td>
<td>GTX28226</td>
<td>-</td>
<td>GeneTex Inc.</td>
</tr>
</tbody>
</table>

Secondary Antibody

| Bovine anti-goat IgG HRP | sc-2350 | E2505 | Santa Cruz |
| Goat anti-mouse HRP conjugate | L9704447 | 96035 | Bio-Rad |

Table 2: Antibodies used during this study.

To be able to get an indication if the right protein was present in the Percoll layers, positive controls had to be used during the gel run. Unfortunately none of the positive results gave 100% signal even though this was stated by the manufacturer of the antibodies and positive controls. In total 4 positive controls were found and used during this study. (table 3)

<table>
<thead>
<tr>
<th>Positive Control</th>
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<th>Manufacturer</th>
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<tbody>
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<td>NIH/3T3 Whole Cell Lysate</td>
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<td>C2205</td>
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<tr>
<td>MCF7 Whole Cell Lysate</td>
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<td>L1603</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>3T3 Whole Cell Lysate</td>
<td>In-House</td>
<td></td>
<td>Birgit Faber</td>
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</table>

Table 3: Positive controls used during this study.

One additional antibody, β-Actin, was used during the study. β-Actin is a very stable housekeeping protein that is consistent in different species.[36] Because this protein shows to have a protein concentration very equal to each cell, a detection of this protein and intensity of the signal gives an indication of the amount of protein present in the cell lysate and a comparison between different experiments would be possible.

The best signals for detection of proteins PTHrP, Ihh and β-Actin were obtained with the following antibodies and concentration. (Table 4)
**PTHrP**
Primary Antibody

<table>
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Secondary Antibody
Bovine anti-goat IgG HRP

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Positive Control
3T3-L1 Cell Lysate

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<td>sc-2243</td>
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<td>Santa Cruz</td>
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**Ihh**
Primary Antibody
Ihh (C-15)

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<td>F1305</td>
<td>Santa Cruz</td>
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Secondary Antibody
Bovine anti-goat IgG HRP

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<tr>
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Positive Control
NIH/3T3 Whole Cell Lysate

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</thead>
<tbody>
<tr>
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<td>C2205</td>
<td>Santa Cruz</td>
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**B-Actin**
Primary Antibody
Anti B-Actin

<table>
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<th>Lot. #</th>
<th>Manufacturer</th>
<th>Concentration</th>
<th>Dilution Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTX28226</td>
<td>-</td>
<td>GeneTex Inc.</td>
<td>1,2 mg/ml</td>
<td>1/500</td>
</tr>
</tbody>
</table>

Secondary Antibody
Goat anti-mouse HRP conjugate

<table>
<thead>
<tr>
<th>Prod. #</th>
<th>Lot. #</th>
<th>Manufacturer</th>
<th>Concentration</th>
<th>Dilution Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>L9704447</td>
<td>96035</td>
<td>Bio-Rad</td>
<td>N/A</td>
<td>1/500</td>
</tr>
</tbody>
</table>

Table 4: Optimal Primary and Secondary Antibody concentrations obtained after trails. Note that these concentrations were optimised using Pierce SuperSignal West Dura Extended Duration Substrate # 37071.

The following protocols were used for Gel Electrophoresis, Western Blotting and Immunochemical staining.

The electrophoresis gels were created and ran using the Mini-PROTEAN 3 electrophoresis chamber and constructed according to the Bio-Rad instruction sheet. A 12% polyacrylamide/Bis-SDS running gel was used (table 5). The polyacrylamide/Bis-SDS running gel solution was directly pipetted between glass plates. A small layer of ethanol was added to equalize the surface and let to polymerise for 45 minutes at room temperature. After polymerisation, the ethanol was tipped off and the stacking solution was added with combs and let to polymerise for 15 minutes at room temperature.

<table>
<thead>
<tr>
<th>12% Running-gel*</th>
<th>Stacking-gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% Acrylamide/Bis</td>
<td>4,9 ml</td>
</tr>
<tr>
<td>MilliQ-water</td>
<td>4,1 ml</td>
</tr>
<tr>
<td>1,5 M Tris-HCl pH 8.8</td>
<td>3,0 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>130 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>8 µl</td>
</tr>
<tr>
<td>10% APS</td>
<td>30 µl</td>
</tr>
<tr>
<td>Stacking-mix**</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2,0 ml</td>
</tr>
</tbody>
</table>

Table 5: Electrophoresis gel concentrations

* For 2 gels
** Containing (13,3 ml Acrylamide/Bis; 25,0 ml 0,5 M TRIS-HCl pH 6,8; 1,0 ml 10% SDS-solution; 60 ml MilliQ-water)
According to the instructions of the Bio-Rad sheet, the gels were placed in the frames and the Elfo-Under (TG) and Elfo-Upper (TGS) 1x Solutions were added.

Before removing the combs and adding the samples, the samples were first boiled for 4 minutes and put on ice for a minute. After the samples were added the chamber was connected to a power supply and run at a constant voltage of 200 V till the sample buffer front was 1 cm above the glass edge. After running, the gels were ready to be blotted.

Because proteins in a gel without any fixation would disperse, the Western Blotting was performed directly after. For this Western Blotting procedure a Mini Trans-Blot Cell was used according to the instruction sheet of Bio-Rad. The gels were removed from between the glass plates of the Mini-PROTEAN 3 electrophoresis chamber and positioned according to the figure below.

![Figure 7: Western Blot construction.][37]

After building the blotting sandwich and positioning it in the Mini Trans-Blot Cell, the blot was run under constant voltage of 100 V for 60 minutes. After the 60 minutes the nitrocellulose paper was removed and left dry overnight in the fridge. The next morning the nitrocellulose was ready to be used for Immunochemical staining.

The nitrocellulose paper was first incubated for 20 minutes in a 50 ml Falcon tube containing 3 ml of Block Buffer (containing 5% Marvel Dried Skimmed Milk; 0.05% Tween-20 in 1x PBS (0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4 per 200 ml)) under constant rotation. After 20 minutes the Block Buffer was discarded and new Blocking Buffer was added containing Primary Antibody and left overnight under constant rotation. (The same Block Buffer was used as the day before, and was stored in the fridge overnight). The Block Buffer with Secondary Antibody was discarded and the

---

[37] Figure 7: Western Blot construction. [Image 148x378 to 448x564]
nitrocellulose paper was washed with Wash Buffer for 90 minutes under constant rotation and every 20 minutes replacing the Wash Buffer. The last wash was done using 1x PBS for 10 minutes.

The nitrocellulose paper was then incubated with Detection Reagents 1 and 2 at a 1:1 ratio and used 0.125 ml of Working Solution per cm² of membrane with Pierce ECL Western Blotting Substrate or SuperSignal West Dura Extended Duration Substrate for 1 or 5 minutes respectively under constant rotation. (Two staining kits were tested during this project) After incubation the nitrocellulose paper was placed in a Bio-Rad VersaDoc 3000 imaging system and analysed using a Nikon Nikkor 50mm f/1.4D Auto focus Lens and Bio-Rad Quantity One 1-D analysis software.

3.4 Verify the location of proteins PTHrP and Ihh in histological slides.

To visualize/determine the positions of proteins PTHrP and Ihh two Immunohistochemical protocols were used: a non-fluorescent assay and a fluorescent assay.

For the non-fluorescent assay the following protocol was used. 5 µm slides were prepared of porcine fetal proximal femur and embedded in paraffin. The slides were deparaffined using the below protocol.

2 x 5 minutes Paraclear (xylol)
2 x 2 minutes Ethanol 100%
2 minutes Ethanol 96%
2 minutes Ethanol 80%
2 minutes Ethanol 70%
2 minutes Ethanol 50%

Followed by 15 minutes wash with 1% H₂O₂ in methanol to wash out peroxidase from the tissue. The slides were rinsed 3 times with TBS (containing 100 ml 0.5M TRIS-buffer and 900 ml NaCl 0.9% pH 7,6) and followed by a rinse of TTBS (containing 0.1% Tween-20 in TBS). The slide was incubated for 8 minutes at 37°C with 0.04% pepsin (Sigma) and 0.5% Milk Powder in 1x PBS pH 2.0 (used 0.8 ml 6M HCl, 0.24 g Marvel Dried Skimmed Milk and 0.0192 g pepsin to 48 ml 1x PBS) for antigen retrieval. The slide was then rinsed 3 times with TTBS and incubated for 5 minutes in TritonX-100 1% followed with 5 minute incubation in 1x PBS. The slide was then incubated for 1 hour with 0,5% Milk Powder in TTBS. To reduce the amount of Antibody to be used, a circle was drawn around the tissue using a PAPPEN and let to dry. Primary Antibody was added to 1x PBS and incubated overnight at 4°C.

The slide was washed 6 times 5 minutes in 1x PBS followed by 60 minute incubation with Secondary Antibody in 1x PBS in dark at room temperature. Next the slide was washed 3 times 5 minutes with 1x PBS. The slide was now ready to be stained using DAP. DAP was added to the slide with 3 µl H₂O₂ 30% and left for maximal 30 minutes. If background of tissue started to color, the slide was rinsed with tap water and embed in Aquamount (Mowiol) for storage. The slide was now ready to be analyzed using a light microscope.
For the fluorescent assay a slightly different protocol was used. For this protocol 5 µm cryo slides were prepared of porcine fetal proximal femur. The cryo slides were placed on polysine coated microscope slides and left to dry overnight at room temperature. The next day the cryo slides were treated with 1% Triton X-100 in PBS for 5 minutes at room temperature. The slides were rinsed for 5 minutes with PBS. Primary Antibody was added diluted in PBS and left for 30 minutes at room temperature. Slides were rinsed after 3 times with PBS. Secondary Antibody was added diluted in PBS and left for 30 minutes at room temperature. After, the slides were rinsed again 3 times 5 minutes with PBS followed by embedding using Aquamount (Mowiol) for storage. Slides were now ready to be viewed using a Fluorescent Microscope.
4 Result & Discussion

During this project many optimization trials were run. After establishing the optimum values, like antibody concentration and exposure times, 3 trails were run and the result can be found below. An Alkaline Phosphatase Staining was performed with the last trail (3).

4.1 Isolate fetal epiphyseal growth plate cells.

Before dissection, the femur length of all embryos was measured, as a measurement of age.

<table>
<thead>
<tr>
<th>Embryo</th>
<th>Femur Length (mm)</th>
<th>Weight (grams)</th>
<th>Cell count (Cells/ml)</th>
<th>Total amount (Cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trail 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>48.5 ±400</td>
<td>±400</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>41.8 ±400</td>
<td>±400</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>44.9 ±400</td>
<td>±400</td>
<td>29 x 10^7</td>
<td>58 x 10^7</td>
</tr>
<tr>
<td>4</td>
<td>51.8 ±400</td>
<td>±400</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>47.2 ±400</td>
<td>±400</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trail 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>32 ±600</td>
<td>±600</td>
<td>13 x 10^7</td>
<td>26 x 10^7</td>
</tr>
<tr>
<td>2</td>
<td>37 ±600</td>
<td>±600</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>43 ±600</td>
<td>±600</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trail 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>47 ±500</td>
<td>±500</td>
<td>5,6 x 10^7</td>
<td>11 x 10^7</td>
</tr>
<tr>
<td>2</td>
<td>50 ±500</td>
<td>±500</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>45 ±500</td>
<td>±500</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 6: Porcine fetal femur length, weight and cell count last trails.

After dissection and tissue incubation overnight the obtained cells were counted.

During the pilot studies it was noticed that the removal of the epiphyseal growth plate was easier for embryos between 250 and 500 grams.

4.2 Separate the epiphyseal growth plate cells on their different growth plate zones.

The use of the 7 layer discontinuous Percoll gradient (1,01 till 1,07 g/ml) showed faint bands appearing at the different borders. (Figure 8 left) The 4 layer discontinuous Percoll gradient reduced to 1,01 – 1,03 – 1,05 and 1,07 g/ml concentrations showed increased visibility of the bands. (Figure 8 right)
Microscopic observation of the different Percoll layers showed that there was a decrease of cell size starting from the top layer continuing to the bottom layer in both gradients. This is supported by Alini et al. and Weisser et al. which showed that cells at different stages of differentiation have different buoyancies.[14, 32]

With the 7 layer discontinuous Percoll gradient, the cells separated between the different Percoll layers were very difficult visible and so made it very hard to isolate for detection of proteins PTHrP and Ihh at later stage. Therefore, in the rest of the study the 4 layer gradient was used.

![Image](image.png)

Figure 8: Discontinuous Percoll gradients 7 and 4 layered.

After the different Percoll gradient layers with epiphyseal growth plate cells were separated they were counted and morphology checked. As can be seen in figure 9 and table 7, all Percoll layers had differences in cell count and morphology.
Figure 9: Impression of the different layers after separation using 4 layer discontinuous Percoll gradient.

<table>
<thead>
<tr>
<th>Percoll layer</th>
<th>Cell count (Cells/ml)</th>
<th>Additional info</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Trail 1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>$0.14 \times 10^6$</td>
<td>Cell death and debris; small cells</td>
</tr>
<tr>
<td>2</td>
<td>$4.6 \times 10^6$</td>
<td>Small cell death and debris; large cell size</td>
</tr>
<tr>
<td>3</td>
<td>$29 \times 10^6$</td>
<td>Small cell death and debris; mix cell size but on the average smaller then layer 2</td>
</tr>
<tr>
<td>4</td>
<td>$19 \times 10^6$</td>
<td>Small cell death and debris; mix cell size but on the average smaller then layer 3</td>
</tr>
<tr>
<td><strong>Trail 2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>$0.5 \times 10^6$</td>
<td>Cell death and debris; small cells</td>
</tr>
<tr>
<td>2</td>
<td>$2.2 \times 10^6$</td>
<td>Small cell death and debris; large cell size</td>
</tr>
<tr>
<td>3</td>
<td>$12 \times 10^6$</td>
<td>Small cell death and debris; mix cell size but on the average smaller then layer 2</td>
</tr>
<tr>
<td>4</td>
<td>$8.3 \times 10^6$</td>
<td>Small cell death and debris; mix cell size but on the average smaller then layer 3</td>
</tr>
<tr>
<td><strong>Trail 3</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Not enough cells</td>
<td>Removed layer</td>
</tr>
<tr>
<td>2</td>
<td>$0.4 \times 10^6$</td>
<td>Small cell death and debris; large and smaller cell size</td>
</tr>
<tr>
<td>3</td>
<td>$3.4 \times 10^6$</td>
<td>Small cell death and debris; mix cell size but on the average smaller then layer 2</td>
</tr>
<tr>
<td>4</td>
<td>$20 \times 10^6$</td>
<td>Small cell death and debris; mix cell size but on the average smaller then layer 3</td>
</tr>
</tbody>
</table>

Table 7: Cell count of the 4 separate Percoll layers of last trials.
In all the three discontinuous Percoll gradient trails, it can be seen that most layers consist of mixtures of cell sizes. However it can also be seen that the mixture of cell sizes decreases in size when continuing downwards in the discontinuous Percoll gradient. Good recovery with high viability was best in layers 2 till 4. Because of the low cell count and much debris in layer 1, this layer was not studied further. An explanation for this could be because of the high degree of cell death and/or the presence of different cells not being part of the epiphyseal growth plate. This is supported by Alini et al.[32]

After the counting, some cells of Percoll layers 2 till 4 were used for Alkaline Phosphatase staining. The staining showed that each Percoll layer stained differently. Macroscopic observation showed a decrease, from layer 2 till 4, of purple staining. (Data not shown) Microscopic observation also showed a decrease of purple staining. In figure 10, the amount of purple coloured cells in layer 2 and 3 look almost the same, but table 5 shows that the amount of cells in layer 2 is almost 10 times less. Thus, layer 2 has a higher ratio of staining than layer 3. These findings support the findings of previous research groups that showed that alkaline Phosphatase activity increases with maturational development.[14, 32, 33] Also, on a cryo section of the proximal growth plate of a femur, Alkaline Phosphatase progressively increases from the resting zone to the hypertrophic zone of the growth plate. (Figure 11)
Figure 11: Alkaline Phosphatase staining of porcine fetal tissue cryo slide at 10x. Notice that the purple staining is progressively increased from the resting zone through the hypertrophic zone.

The drop in Alkaline Phosphatase observed at the onset of mineralization has also been reported by other research groups.[38]

Larger cells consist of a relatively lower amount of heavy particles and a larger quantity of water. Because of this these cells have a lower buoyant density that can be verified using a FACS experiment. In house study using FACS had shown that buoyant density of a cell is related to cell size. This also supports the assumption that layers 1 till 4 presumably correlate with the hypertrophic, early hypertrophic, proliferation and resting zones respectively after separation using a discontinuous Percoll gradient.[39]

4.3 Detect intra cellular proteins PTHrP and Ihh in cells from the different growth plate zones?

Using the antibodies and concentrations described in paragraph 3 Methods and Materials signals for almost all antibodies were obtained.
Figure 12: Results of trail 3.

* Blots PTHrP and 4x diluted PTHrP were incubated with both secondary antibodies Bovine anti-goat IgG Conjugate (sc-2350) (1:500) and Precision StrepTactin-HRP Conjugate (161-0380) (1:15000).
* Blots Ihh and 4x diluted Ihh were incubated with both secondary antibodies Bovine anti-goat IgG Conjugate (sc-2350) (1:1000) and Precision StrepTactin-HRP Conjugate (161-0380) (1:15000).
* Blot B Actin was incubated with both secondary antibodies Goat anti-Mouse HRP conjugate (1:500) and Precision StrepTactin-HRP Conjugate (161-0380) (1:15000).
* Primary PTHrP (N-19) sc-9680 lot # F0204 (1:50); Ihh (C-15) sc-1196 lot # F1305 (1:100); B Actin (1:500)

Exposures times were respectively 100; 25; 50 and 150 seconds and used 1.5 ml of each substrate (Pierce SuperSignal West Dura Trial Kit Prod # 37071 lot # GH97151) with 5 minute incubation.

M1: Precision Plus Protein All Blue Standards #161-0373 Bio-Rad (2.5 µl per lane)
M2: Precision Plus Protein Unstained Standards #161-0363 Bio-Rad (1 µl per lane)
C1: 3T3-L1 Cell Lysate # sc-2243 lot # L1603 Santa Cruz (10 µl per lane)
C2: NIH/3T3 Whole Cell Lysate # sc-2210 lot # C2205 Santa Cruz (10 µl per lane)
L: Percoll gradient layers 2, 3 and 4 (10 µl per lane)

All controls and samples were first boiled for 4 minutes and shortly put on ice.
For β-Actin, all layers and control showed a band appearing around the 42 kDa as expected. Except for layer 2 all other layers and control had more bands around different heights. This was thought to be aspecific binding of the antibody, phosphorylated β-Actin and/or β-Actin iso-forms. In this experiment, this was not considered to be important, only the band around the 42 kDa. The intensity and presence of the 42 kDa band would indicate that the samples did contain protein and that there would be a chance of finding the proper protein in the total protein concentration. Note that layer 1 was not included in any of the experiments because of the lack of cells and so its proteins.

For PTHrP, only layers 3, 4 and control gave bands. Layer 2 didn’t have any visible bands. This was consistent with the poor band intensity with β-Actin and the low cell count for this layer, see table 7 and figure 9. There is also a possibility that the signal is too low for detection or that this layer doesn’t express PTHrP.

The positive control showed bands around 60, 43 and (very faint) 26 kDa weight. Danks et al. found that Sparus pituitary extract contained PTHrP and iso-forms around the 29, 26 and 14 kDa weight. Danks showed that the 14 kDa was similar to the 1-84 amino acid form and 26 kDa to the 1-141 amino acid form with a pre-pro portion attached. The 29 kDa according to Danks could have been a dimer of the 14 kDa molecule or an alternative 3’ splice product or possible post-translation glycosylated form.[40] Manufacturer of the positive control 3T3-L1 Cell Lysate showed that mature PTHrP would have a molecular weight of 26 kDa.[41] Considering that the differences between the different bands, but maintaining the ratios between the 3 bands for each animal, could be explained by the difference in animal. I suggest that the bands around the 60, 43 and 26 kDa are dimer of 26 kDa, iso-form and mature PTHrP respectively. To prove this assumption, isolating the three different proteins bands and sequencing them is advisable.

As figure 12 showed, layers 3 and 4 clearly indicate the presence of possible PTHrP iso-forms but no visible PTHrP band around 26 kDa. Trail 2 showed a faint band around the 26 kDa. (Figure 13 L3) The possible weakness of the 26 kDa band could be explained by the lack of enough PTHrP in this age of cell zone, concentration of antibody and/or the poor affinity between the antibody and protein. Concerning all past results I concluded that PTHrP, in either iso-forms and/or mature form, was visualised using this technique.
For Ihh, layers 2, 3, 4 and control gave bands. Again layer 2 showed to have only one strong band at the beginning of the lane and was not well interpretable due to low cell number. The control only showed faint bands. According to Medill et. al. and manufacturer of Ihh (C-15), Ihh consists of 2 proteins with an amino and carboxyl terminal peptides of 19 kDa and 25 kDa molecular weight respectively.[17, 41] If these 2 proteins would still be one during the experiment bands around the 44 kDa would be expected. As can be seen in figure 12, bands can be found around the 25 kDa and 44 kDa weight, indicating the presence of Ihh. The other bands were thought to be aspecific binding of the antibody, phosphorylated Ihh and/or Ihh iso-forms. In this experiment this was not considered to be important, only the bands around the 25 and 44 kDa.

Trail 2 and 3 showed clear differences between the different Percoll layers for PTHrP. As can be seen in figure 14, layer 2 (trail 1) showed no visible band around the 26 kD compared to layer 3 and 4 of the same trail. Layer 2 and 4 (trail 2) showed no visible band around the 26 kDa compared to layer 3. This indicated the difference in PTHrP concentration in the different zones. The signal appearing in layer 4 for trail 1 and not for trail 2 could be explained by their different embryonic ages at dissection.
As was mentioned in the introduction, PTHrP is generally accepted to be expressed in and around the proliferative zone. As can be seen in figure 14, the suspected PTHrP signal (26 kDa) is shown in the lower layers L3 and L4 corresponding with low buoyant cells like resting and proliferative zone cells.

Ihh is generally accepted to be expressed in and around the (early)hypertrophic zone. As can be seen in figure 15, the Ihh signal (25 kDa) is shown in the layers L2, L3 and L4 corresponding with medium till low buoyant cells like (early)hypertrophic, proliferative and resting zone cells. This was not as expected. Even though the signal is seen in a more mature cell zone, it still has signals in cell zones not considered to express Ihh. An explanation could be that the age of the embryos might have played a role, or that the suspected 25 kDa band is not that of Ihh but another protein. One way to be able to get a better indication of the different expressions of PTHrP and Ihh in the different growth plate zones is to increase the number of Percoll concentrations and pull the zones from each other. This might separate zones that are overlapping in one layer of Percoll. The use of antibodies that are more specific for either PTHrP and/or Ihh is also advisable.
4.4 Verify the location of proteins PTHrP and/or Ihh in histological slides.

During the pilot studies different histological slides were Immunohistochemical stained using fluorescent and non-fluorescent assays. As can be seen in appendix D, mouse tissue was used as a positive control in accordance with the antibody manufacturers. The positive control showed presence of possible IHH proteins throughout the sample. The porcine sample unfortunately didn’t indicate the presence of IHH. This was also supported by western blotting in which this particular antibody did not give any signals (data not shown). The result obtained from the Immunohistochemical staining of trails 1 till 3, for Ihh, see paragraph 4.3, showed many signals. It was clear according to the pilot studies and trails that none of the antibodies were 100% specific for porcine Ihh. Therefore, using these antibodies, signals from various sources would be detected with and not only mature Ihh. No additional Immunohistochemical stainings for IHH were performed afterwards.

For the detection of PTHrP in porcine fetal tissue it was with more certainty that the bands seen in figure 14 would all be different PTHrP forms. As can be seen in Appendix D, the pilot studies indicated the presence of probably different forms of PTHrP in mouse tissue (positive control). The porcine sample indicated the possible presence of different forms of PTHrP in the growth plates and perichondrium. Keep in mind, according to the western blots, that these results are not 100% certain of the presence of PTHrP and that additional experiments like gel band purification followed by protein sequencing would give certainty if all signals are PTHrP related. Because of time limitation additional Immunohistochemical staining on porcine fetal tissue using other antibodies including PTHrP (N-19) sc-9680 lot # F0204 could not be performed.
The isolation of the epiphyseal growth plate cells out of the femur and humerus both distal and proximal didn’t show to be difficult but did show to be easier for embryos between 250 and 500 grams and the amount of cells isolated would also increase in numbers when using larger tissue. (Table 6) This technique for isolation of porcine fetal epiphyseal growth plate cells is recommended for further use.

When using the discontinuous Percoll gradient for separation of the different epiphyseal growth plate zones, layers 1 till 4 presumably correlate with the hypertrophic, early hypertrophic, proliferation and resting zones respectively. This assumption is supported by the Alkaline Phosphatase staining, Alini et al., Weisser et al.[14, 32, 33] and in house study using FACS.[39] This technique for separation of porcine fetal epiphyseal growth plate cell zones by their different differentiation stages is recommended for further use.

The results of Gel Electrophoresis, Western Blotting and Immunochemical staining data suggest that proteins PTHrP and Ihh were detected in some of the epiphyseal growth plate zones. Additional testing is needed to give a 100% accuracy. The techniques used for detection of porcine proteins PTHrP and Ihh out of porcine fetal epiphyseal growth plate cells are suitable for further use, but require further optimization. The best immunochemical staining signals for PTHrP and IHH were obtained using antibody sc-9680 (F0204) and sc-1196 (F1305) respectively.

The results also indicate that there is a difference in expression of PTHrP and Ihh protein in the different epiphyseal growth plate zones. PTHrP is shown to be expressed around the proliferative zone. This is generally supported.[9, 17] Ihh is shown to be expressed in and around (early)hypertrophic, proliferative and resting zone. This assumption is partly supported. Even though the signal is seen in a more mature cell zone, it still has signals in cell zones not considered to express Ihh.

Detection of the porcine proteins PTHrP and Ihh using histological slides is not advisable using the antibodies shown in table 2 against PTHrP and Ihh due to potentially aspecific bindings of the antibodies.

Overall I can conclude that the study could not achieve its aim to determine PTHrP and Ihh protein synthesis in different growth plate zones of fetal epiphyseal growth plate cells because of the many signals obtained for both proteins PTHrP and IHH during Immunochemical staining and not being able to verify them using Immunohistochemical staining. However this study did show that the techniques used are recommended for further use but require further optimization.
6 Recommendations

The age of embryos can be estimated from the Crown-Rump length, described in Appendix C. Unfortunately, this method was only obtained at the end of this study. As a result, embryonic age could not be used here to interpret data. It is recommended to always monitor Crown-Rump length.

After separation of the different epiphyseal growth plate zones, the amount of cells isolated per zone was sometimes too small. The trails showed that at least a minimum amount of 3 porcine fetuses of around 500 grams each (~$11 \times 10^7$ cells) would be necessary for easy separation and further experiments. I would recommend 5 porcine fetuses of around 500 grams each (~$58 \times 10^7$ cells). It is important to keep in mind, that 5 porcine fetuses from the same parent was in my study more an exception then rule and you will have to be certain that the porcine fetuses are of the same parent otherwise this might create a rogue in the result. The use of bigger embryos like bovine could be considered. Unfortunately current regulation does not allow the use of fresh bovine foetal material. The use of smaller embryos like rats and mice could be considered because of the presences of more antibodies on the market for these organisms then porcine. However, this would mean that more embryos are needed to reach the minimal requirement of cells and also mean pooling embryos from different parents.

The detection of PTHrP and Ihh in porcine fetal epiphyseal growth plate cell zones showed the likely presence of the 2 proteins. It is recommended to run additional experiments like protein sequencing to get a 100% conformation if PTHrP and Ihh are present.

The detection of the porcine proteins PTHrP and Ihh using histological slides was not successful. It is recommended that new more specific antibodies against porcine PTHrP and Ihh should be found and used to localize the two proteins in Immunohistochemical staining.

Detecting PTHrP and Ihh mRNA’s in the different epiphyseal growth plate zones with PCR would enhance interpretation possibility. Note that, detection of mRNA’s doesn’t always indicate the expression of the related protein.

Because future studies will address experiments in which increase and decrease of proteins PTHrP and Ihh during mechanical loading of epiphyseal growth plate are to be determined, an experiment was performed to check if a reduction of total protein in each Percoll gradient layer would still give a visible signal on the Western Blot after Immunochemical staining using antibodies against porcine proteins PTHrP and Ihh. As can be seen in figure 12 (4x diluted), both proteins still gave a faint signal at different molecular weight levels. The results showed that using only ~$11 \times 10^7$ cells would give a very low detection signal with 4x dilution factor. This again pointed to early described recommendation of ~$58 \times 10^7$ cells. Of course if more reduction of signal would occur after mechanical loading, more cells are needed to be able to detect a signal.

To be able to detect any differences between the different mechanical loading experiments after immunochemical staining, I recommend the use of the Quantify One intensity calculator tool software of Bio-Rad (VersaDoc). Using this
tool the software will be able to compare any intensity differences between bands and give a percentage indication of the change.

This study only focused on the intracellular detection of proteins PTHrP and Ihh. It is known that these proteins are released into the environment. The extracellular concentration is likely much less than intracellular. To be able to get a detection of these proteins extracellular the same technique as for intracellular can be used, with a possible addition of a concentrating technique using e.g. Millipore Centricon centrifugal filter devices with 3,000 NMWL Ultracel YM membranes.
References


## Appendix A, Material Specification.

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Appendix B, Percoll Calculation

Calculations
Using the below formula the different concentrations of Percoll gradients can be calculated.[42]

\[
V_0 = V \left( \frac{d - 0.1d_{10} - 0.9}{d_0 - 1} \right)
\]

Figure 16: Formula

Vo = Volume of Percoll required (undiluted Percoll) in ml
V = Volume of final working solution in ml
d = Desired density of final working solution
do = Density of Percoll undiluted
d10= Density of 1.5 M NaCl (1.058) or 2.5 M sucrose (1.316)

E.g. you want to create 15 ml of a 1.01 g/ml Percoll concentration for a 1.01 – 1.07 g/ml Percoll Discontinuous (step) gradient.
1.130 g/ml Percoll solution is present in the ordered bottle.
1.5 M NaCl solution is also needed.

Using the formula above:
\[
V_0 = 15 \times \left( \frac{(1.01 - 0.1 \times 1.058 - 0.9)}{(1.130 - 1)} \right)
\]
\[
V_0 = 0.485 \text{ ml undiluted Percoll}
\]

Volumes to add:
In a 15 ml tube the following volumes are added:
\[
\frac{1}{10} \times 15 \text{ ml} = 1.5 \text{ ml (1.5 M NaCl “sterile”) (see Percoll information sheet)}
\]
Undiluted Percoll = 0.485 ml
d\text{H}_2\text{O “sterile”} = 13.015 ml

The other concentrations can be calculated the same way.
Appendix C, Calculation of Date of Concept for porcine using the Crown to Rump length

Using the following graph and table an estimated conceptual dating for porcine can be calculated.[43] Courtesy of Dr. Arie van Nes, University of Utrecht, Department of Farm Animal Health.

<table>
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<th>Crown-Rump length (mm)</th>
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<td>20</td>
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Table 8: Porcine Crown-Rump length and age relationship.[43]
Appendix D, Histological slides

All the below histological cryo slides were incubated using primary antibody overnight at 4°C. Anti goat IgG (sigma 9787) was used as a secondary antibody for 60 minutes at room temperature, followed by 4 minutes incubation using DAB. A positive DAB colouring is characterized by brown colouring of the tissue.

Histological staining using Anti-h/mIhh N-term (AF1705) primary antibody on 10µm Porcine tibia tissue. Negative control gave the same result and is not shown. Note, that the negative control after prolonged incubation showed brown colouring that was not caused by immunoprecipitation but likely by the DAB colouring itself. This could explain for the light brown colourization around the secondary ossification centre. With this in mind, I suggest that IHH, in this sample, is not present, too low to detect or has no affinity with the antibody.

Histological staining using Anti-h/mIhh N-term (AF1705) primary antibody on 10µm mouse foot tissue. It shows that IHH is detected throughout the tissue in positive control.
Histological staining using PTHrP (N-20) (sc-9685) primary antibody on 10µm Porcine tibia tissue. Positive result shows a colouring around the growth plates and perichondrium.

Histological staining using PTHrP (N-20) (sc-9685) primary antibody on 10µm mouse foot tissue. It shows that PTHrP is detected throughout the tissue in positive control.