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MASTER THESIS

Estrogen action in growth plate cartilage

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Abstract

Estrogen's role in the human body is pivotal for a multitude of biological processes and it acts as an initiator or modulator to bring about its physiological effects. It plays a significant role in the bone mineral metabolism and linear growth in both sexes. Linear growth in bones depends on the activity of the growth plate which comprises of resting, proliferative and hypertrophic zones and all of these have estrogen receptors. The effects of estrogen have been associated with two classical receptors (ER-α and ER-β). Recently a trans-membrane estrogen receptor, GPER (G-Protein Coupled Estrogen Receptor) has been discovered and its presence in the resting and the hypertrophic zones of the growth plate has been confirmed. Presence of this receptor in the growth plate brings forward the idea of its selective modulation for manipulation of growth. The aim of this project was to do a pilot study comprising of a series of experiments utilizing IGF-1, GPER agonist (G1) & antagonist (G15) in a specialized *ex-vivo* model of post-natal mice metatarsal organ culture. We confirmed the already established findings that IGF-1 is the most potent growth stimulator and report for the first time that bones exposed to G1-GPER agonist at the dose concentration of 10 nM grow significantly more than the controls. No effect of G15-GPER antagonist was seen on the growth plate even at high dose concentration of 1 μM. However the exact mechanism of GPER action in the growth plate needs to be evaluated in an in-depth in-vivo study.

Important terms and abbreviations

ALS	Acid Labile Subunit
BMP	Bone Morphogenic Peptide
BMD	Bone Mineral Density
BSA	Bovine Serum Albumin
DMSO	Dimethyl Sulfoxide
ER-α	Estrogen Receptor-alpha
ER-β	Estrogen Receptor-beta
Ex-vivo	Experiment done on a tissue in artificial environment with minimum alteration of conditions
GH	Growth Hormone
GPER	G-Protein Coupled Estrogen Receptor
IGF-1	Insulin like Growth Factor-1
IGFBP	Insulin like Growth Factor Binding Protein
PBS	Phosphate buffered saline
PTHrP	Parathyroid Hormone related Peptide
SD	Standard Deviation
SE	Standard Error

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Introduction

Bone and cartilage tissue in our body protects our vital organs such as heart, brain and lungs and also serve to give us mechanical support and mobility. It is of paramount importance to have a fully developed skeletal system. The growth of the bones is dependent on various factors that take part in initiation of bone growth and then its modulation. Growth plates are structures present at the epiphysis and they are responsible for bone elongation. These epiphysial growth plates comprise of cells that undergo process of proliferation and differentiation resulting in bone elongation. After sexual maturation these growth plates disappear and the growth process ceases. The growth plate comprises of three distinct zones which are the resting zone, proliferative zone and hypertrophic zone (Figure 1). Resting zone is the one that is closest to the epiphysis and comprises of small, evenly rounded cells that are surrounded by extra cellular matrix (ECM) (Ballock and O'Keefe, 2003). Next to this zone is present the proliferative zone, which comprises of compacted and dividing chondrocytes that are present in a ladder shape pattern that is in line with the bone alignment and they synthesize type II and type XI collagen and next to it is the hypertrophic zone where mature chondrocytes are found (Ballock and O'Keefe, 2003). The chondrocytes in this region stop cell division and have a marked increase in alkaline phosphatase activity. Longitudinal growth in the growth plate is a result of endochondral bone formation that involves recruitment of resting zone chondrocytes to undergo active proliferation and then subsequently differentiation, apoptosis and finally mineralization. Various factors govern this transformation of chondrocyte recruitment and differentiation. These factors can be local e.g. Bone Morphogenic Protein (BMP) (Moser & Patterson, 2005), Fibroblast Growth Factor (FGF) (Ornitz and Marie, 2002), Parathyroid Hormone related Peptide (PTHrP) (Maeda *et al.* 2007) and Vitamin D (Boyan *et al.* 2003). Also the governing factors can be systemic e.g. Growth Hormone (GH), Insuline like Growth Factor 1 (IGF-1), Thyroid Hormone and sex steroids such as Androgens and Estrogen.

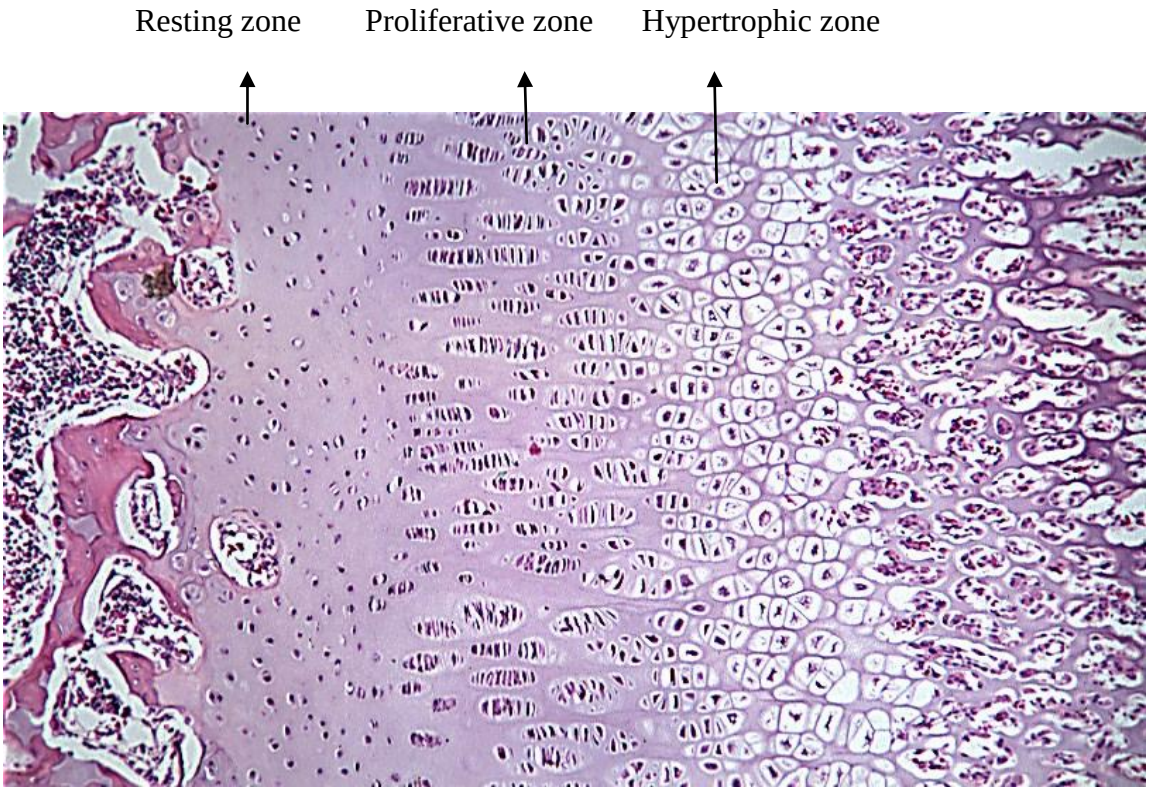

Figure 1: (Source: http://www.kumc.edu/instruction/medicine/anatomy/histoweb/bone/bone02.htm) Hematoxylin and Eosin stained epiphysial growth plate section showing resting, proliferative and hypertrophic zones of chondrocytes.

IGF-1 and its importance in bone physiology

Production of IGF-1, previously known as Somatomedin C, is stimulated by GH (Growth Hormone) in the liver (Melmed, 1999). It is essential for growth of all tissues in the body and produced in extrahepatic tissues (Ohlsson *et al.* 2009). In the growth plate, synthesis of IGF-1 is mediated by interaction of NF-ƙB p65 with Stat5b in growth plate chondrocytes (Wu *et al.* 2011). It has been shown that IGF-1 acts on the bone growth as a local factor and also as a systemic factor (Liu *et al.* 1993). More than 90 % of IGF-1 circulates in the form of a complex that comprises of IGF binding proteins (IGFBP3 and IGFBP5) and Acid Labile Subunit (ALS) (Boisclair *et al.* 2001). Individual genetic ablation of one of these components (IGF Binding Proteins and Acid Labile Subunit) results in development of minor skeletal abnormalities suggesting that the remaining free IGF-1 in the serum overcompensates (Yakar *et al.* 2009). However, IGF-1 total knockout mice models have shown marked growth impairment and bone destruction (Yakar *et al.* 2009). Hence IGF-1 presence in the body is very important in order for the bones to reach their desired potential in terms of growth and development. IGF-1 binds to its receptor

IGF-R and brings about its effects (Butler AA *et al.* 1998). It has been shown that IGF-1 actually causes acceleration in the aging of the target cell. Increased life span has been seen in cells in which ablation of daf-2 gene (gene encoding IGF-R) was introduced (Dorman J.B *et al.* 1995). Thus the growth achieved by IGF-1 is due to accelerated attainment of senescence by the cells in the growth plate.

Estrogen and its importance in bone physiology

Estrogen (17 β-estradiol) takes part in a multitude of physiological processes either as initiator or modulator. The effects of estrogen in the body are highly documented and its roles have been intensively studied. The effect of estrogen has been associated with the presence of two classical nuclear estrogen receptors namely Estrogen Receptor-α (ER-α) (Green *et al.* 1986; Greene *et al.* 1986) and Estrogen Receptor-β (ER-β) (Kuiper *et al.* 1996). Both of these are considered as factors that bring about ligand-activated transcription. These factors are located in the cytosol where they bind estrogen molecules and then this Estrogen-ER complex is translocated to the nucleus and there it interacts with nuclear response elements which are present on the target gene promoter areas. ER-α and ER-β are both expressed in the human growth plates (Nilsson *et al.* 2003A). ER-α is present in the osteoblasts and mature osteocytes (Zaman *et al.* 2006) while ER-β is present in osteoblasts, osteoclasts and osteocytes (Braidman *et al.* 2001).

Previously estrogen's main physiological actions were generally considered to be important in terms of female physiology. It brings about pubertal growth, development and maintenance of female secondary sex characters thus playing the same role as played by androgens in the male physiology. But the role of estrogen in male bone physiology was highlighted in 1994 with the description of a case of estrogen receptor mutation (Smith *et al.* 1994). The subject of the case study was a 28 year old 46 XY male who had a height of 204 cm, a bone age of 15 years and the bone mineral density (BMD) of his lumbar spine was 3 standard deviations (SD) below mean for his age. The striking feature was that he was having open epiphyses and was still growing even though he was in the third decade of his life. On further investigation, it was revealed that this subject had a mutation in the ER-α gene. No response was seen on administration of transdermal ethinlyl estradiol. The bone morphometric analysis revealed that his bones had a markedly decreased mineral content with impaired bone structure but the periosteal

circumference remained unaffected. His serum androgen levels were normal and he had normal male genitalia with bilateral descended testis having a volume of 25 ml each. His sperm count was 18 million with viability being 18 % (normal is >50 %). All these parameters indicated that the mutation in the ER-α was the cause of his skeletal disorder. The role of estrogen in bone physiology was further highlighted in a case study of a similar phenotype with aromatase deficiency (Conte *et al.* 1995). The subject, a 46 XX female, had a mutation in the P450 (CYP19) gene that resulted in high levels of androgens with no serum estradiol. Her bone age was found to be 10 years at a chronological age of 14 and half. She was given estrogen therapy that resulted in bone development and also development of secondary sex characters.

These case studies clearly indicated that estrogen is not only important for female bone physiology but also plays a modulating role in male bone physiology. It is important for proper mineralization of the bones in both sexes and bone growth is impaired if the exposure to estrogen is low or if there is hampering of the estrogen action due to a mutation in ER-α.

A third estrogen receptor has now been discovered and it is not a nuclear receptor. It is a G-protein coupled receptor and it was initially given the designation of GPR30. It is from the 7-transmembrane G protein receptor family and it was cloned in the late 1990s (Carmeci *et al.* 1997). It was later shown to be localized on the endoplasmic reticulum and on activation by estradiol, calcium ion influx was shown to be induced along with synthesis of phosphatidylinositol 3, 4, 5 phosphate (Revanker *et al*. 2005). Also, adenylyl cyclase activation due to GPR30 has been elicited (Filardo *et al.* 2002). This new estrogen receptor is now termed as GPER (G-Protein coupled Estrogen Receptor) and its presence has been confirmed in tissues such as brain, pancrease, cardiovascular system and immune system (Prossnitz, E.R & Barton, M 2011). The GPER (GPR30) is also expressed in the human growth plate (Chagin *et al.* 2007b) and the expression is similar in both sexes. It is expressed by all three types of bone cells, osteoblasts, osteoclasts and osteocytes (Heino TJ *et al.* 2009). It is present in the resting and hypertrophic zone but not in the proliferative zone of the growth plate (Chagin & Savendahl 2007b). Interference with the functioning of the GPER causes disruption of known actions of estrogen on the growth plate (Windahl S. *et al* 2009). GPER expression in the growth plate declines as puberty progresses and its presence has been linked with roles such as

resting chondrocyte recruitment and hypertrophic chondrocyte differentiation (Chagin & Savendahl 2007b).

Presence of GPER in the growth plate and extensive documentation of estrogen action on bone growth during puberty has lead to assumption of the possibility that estrogen receptors can be targeted in order to manipulate bone growth. Non-selective estrogen therapy is not without side effects and attempts are now being made in order to come up with a modality that selectively targets estrogen receptors in the growth plate cartilage. The idea of growth modulation by targeting estrogen receptors has lead the researchers to look for and come up with SERMs (Selective Estrogen Receptor Modulators). The aim has been to target the estrogen receptors only in the growth plate with minimal stimulation of estrogen receptors elsewhere in the body. These attempts lead to the synthesis of ligands for GPER that do not have affinity for other estrogen receptors. One of these ligands is a selective agonist of GPER called G1 (Bolaga *et al.* 2006). The availability of this selective agonist for GPER has immensely enhanced the understanding of the relatively unknown mode of action of this estrogen receptor and currently its role is being scrutinized in various kinds of disease models and conditions that are influenced by estrogen action. Attempts were made then to come up with a selective antagonist for the GPER and a compound was synthesized that shows antagonistic properties on binding with GPER. This antagonist is called G15 (Dennis MK *et al.* 2009). Both G1 and G15 are based on a scaffold of tetrahydro-3H-cyclopenta[c]quinoline (Dennis MK *et al.* 2011).

Objective and rationale

This study comprises of a series of pilot experiments that were conducted in order to evaluate the agonist and antagonist compounds of GPER in an *ex-vivo* mice metatarsal organ culture model and it is the first testing of GPER-agonist and antagonists in a specialized organ culture model specifically designed to study bone growth. The aim has been to provide proof of concept that GPER can be evaluated as a possible drug target for growth manipulation in bones. The effects on growth are studied in metatarsal bones of 3-day old post-natal mice. The C57BL/6 strain of mice was used in these experiments. Organ cultures with IGF-1 were performed first in order to establish procedural parameters and also to reproduce already verified effects of IGF-1 on metatarsal bones

(Chagin *et al.* 2010). Subsequently, G1 and G15 effects were then studied in order to fully understand as to how much growth stimulation can be achieved compared to IGF-1 which is the most potent growth stimulator. This study is part of a bigger project and the overall objective of the project is to ascertain the roles of estrogen in the growth plate and to determine its potential with regards to manipulation of growth. This study also serves as to lay ground work for discovery of alternate modalities to growth hormone (GH) treatment in cases of growth stunting and also a non-invasive medical alternative to growth plate removal surgery in cases of too much growth.

Materials and method

For this study a threefold step-wise experiment plan was devised. First series of experiments involved *ex-vivo* treatment of metatarsal bones with IGF-1. It was done in order to reproduce already verified effects and to check other procedural parameters and gain familiarity with the technique. After the IGF-1 experiments, two subsequent series of experiments were planned with G1-GPER agonist and G15-GPER antagonist.

Mice Metatarsal Organ Culture

It is a model in which longitudinal growth can be studied in real-time in an *ex-vivo* culture of fetal or postnatal rat/mice metatarsal bones and has been established in the host laboratory (Martensson *et al.* 2004).

Micro dissection of Metatarsals

Ex-vivo culture of metatarsals from 3-day old post natal mice was performed in order to see the effects of IGF-1, GPER agonist and antagonists on growth plate. The mice were ordered 23 days before the planned date of experiment. The gestation period of mice is approximately 20 days. On the day of the experiment the mice were sacrificed by administration of CO_2 and then micro dissection was carried out to obtain metatarsal bones. DMEM/F12 (without phenol red) was used as the media for these experiments and was purchased from Invitrogen, Inc. Media without phenol red was used as phenol red itself acts as a weak estrogen (Y Berthois *et al.* 1986). The media for dissection was supplemented with 20 μg/ml gentamycin (Invitrgoen, Inc) while the media for culturing was supplemented with 0.2% endotoxin free fraction V BSA (Bovine Serum Albumin

purchased from Sigma-Aldrich, Inc), 50 µg/ml ascorbic acid (ICN, Inc), 1 mM β sodium glycerophosphate (Sigma-Aldrich, Inc) and 20 µg/ml gentamycin (Invitrogen, Inc).

Stock solutions for supplements: 10% BSA in PBS (Phosphate buffered saline)
1 M β sodium glycerophosphate
50mg/ml ascorbic acid in distilled water

In the lab the metatarsal dissection was carried out microscopically. Hind limbs were used in order to obtain the metatarsals for all experiments. First the hind limbs were separated from the main torso with the help of scissors. Then the hind limb was placed inside a Petri dish containing dissection media. Incisions in the skin were made on the dorsal and ventral side of the limbs and then the skin was peeled off using forceps leaving only the bones and the connective tissue. The connective tissue was removed by using 30 gauge insulin syringes as knife under the microscope to clear out the bones and to cut precisely the metatarsal-phalangeal junction. Special care needs to be taken at this step as the bones are very fragile and growth plates can easily break if not handled carefully. The middle three metatarsals were used in these experiments. Once the bones were liberated, they were put into 24 well plates for culture. This study was approved by the ethics committee at Karolinska Institute.

G1 Experiment

The limbs of the animals were dissected out under the microscope and then the metatarsals were obtained by removing the connective tissues using an insulin syringe as knife. The metatarsals from each animal's right paw were collected in one Petri-dish containing culturing media and the metatarsals from left paw were collected in another Petri-dish. The right paw bones were used as treated and the left paw bones were used as controls. After dissecting out all the paws, the collected bones were put in individual wells of a 24 well plate and treated with 1 mL of media along with 1 uL of stock solutions of G1. The stock solutions were prepared in DMSO.

Stock solutions: 1 mM, 100 µM, 10 µM

The final treatments obtained: 1 mL media + 1 µL 1mM (stock) = 1 µM
1 mL media + 1 µL 100 uM (stock) = 100 nM
1 mL media + 1 µL 10 uM (stock) = 10 nM

These final treatments were selected by random. Bones from two animals were taken for each treatment with right paw bones being “treated” and left paw bones being “controls”. The controls were treated with DMSO vehicle. The time period for the culture was 16 days with length measurements and media change done as per following schedule,

Days: 0, 2, 4, 7, 9, 11, 14, 16

The bones on these time points were retreated with the same concentration of G1 along with media change. On day 16 the experiment was terminated with final measurements taken. The bones were then placed in 4 % formaldehyde overnight for preservation. After overnight placement in formaldehyde the bones were shifted to 70 % ethanol for storage.

Bone length measurements

Digital images of the cultured bones were taken using a stereo microscope (Nikon SMZ-U) that was connected to a digital camera (Hamamatsu C4742-95) and a desktop computer. Analysis of the images and measurements were done using the software ImajeJ (developed by NIH). The pictures were compared with scale of 1000 µm (micrometer) in order to calculate their length. The measurements were then put in an excel sheets where further analysis was performed.

Analysis

First the increase in lengths of the bones at each time point of the duration of culture was calculated. This was stratified according to treatment categories and control categories. Then averages of increase in bone length for each treatment group and controls were calculated for each time point in the culture. Then the standard deviation was calculated for all observations of increase in length for each day of media change and measurements. From standard deviation, standard error was calculated by using the following formula,

SE = Standard Deviation (SD)/ Sqaure root (n)

where n = number of observations

The data was then plotted on a line graph with increase in length (micrometers) readings placed on the y-axis and duration of culture (days) on the x-axis. Error bars were placed on each time point where bone lengths were measured. To check for significance of results, unpaired student t-test was used in excel sheets to calculate the p-values.

Apart from a line graph, a bar graph was also plotted with error bars for G1 results representing the increase in bone lengths in percentages compared to specific controls. The controls were taken as 100 percent and the increase in length was taken at midway and end of the experiment to show the increase in length in percentages of the treated group.

G15 Experiment

For G15 experiment a slight modification to the existing protocol was made. The modification was that all the bones were pooled together after dissection and then assigned to different treatment groups by random. The G15 stocks and treatment dosages were prepared in the same way as G1 treatments. Five bones were placed in each group and then measured accordingly. The media was changed with retreatment of bones done as per following schedule, day 0, 3, 5, 7, 10, 12 and 14. The rest of the procedure, analysis, calculations and presentation of data were done as described earlier.

IGF-1 Experiment

Same approach was applied to IGF-1 experiment as applied to G15. The bones were pooled and then placed in two groups of "treated" and "controls". The treated group was exposed to IGF-1 (1µg/ml) and the controls were treated with vehicle. Two experiments were performed and the media was changed according to following schedule, day 0, 2, 4, 7, 9 and 12 in experiment 1 while in experiment 2, it was 0, 3, 5, 7, 10, 12 and 14. The rest of the procedure, analysis and calculations were done as described earlier.

Results

Effects of IGF-1 on bone growth

IGF-1(1 µg/ml) treatment causes increase in bone growth and its effects on the bone growth are remarkable compared to controls. The difference in growth between the IGF-1 treated bones and controls was significant and this difference was maintained throughout the culture duration of the experiment. The difference on day 12 (Figure 2) in experiment 1 and day 14 (figure 3) in experiment 2 is significant (***$p < 0.001$ vs control, unpaired student t test) (Figure 2 and 3). Data represents mean ± standard error with n=4 (figure 2)

and n=5(figure 3). IGF-1 causes an increase in the cell cycle rate thereby achieving senescence early. It exhausts the growth potential of the growth plate. The final difference between treated and controls was approximately 750 µm in both experiments.

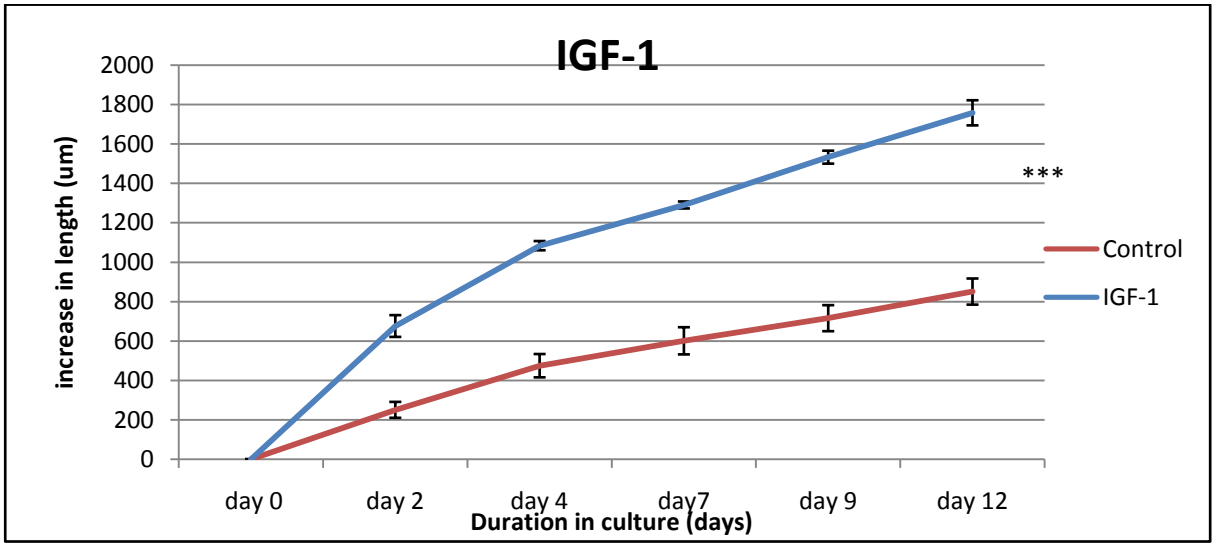

Figure 1: Effects of IGF-1 on linear bone growth. Metatarsal bones were taken from 3 day post natal mice and cultured in DMEM F12(without phenolred). Bones were pooled together and then assigned to treatment and control group on random. The blue line represents the average increase in longitudinal growth of treated bones in micrometers while the red line represents the controls. The duration of the culture is on the x-axis. N= 4, ***p less than 0.001

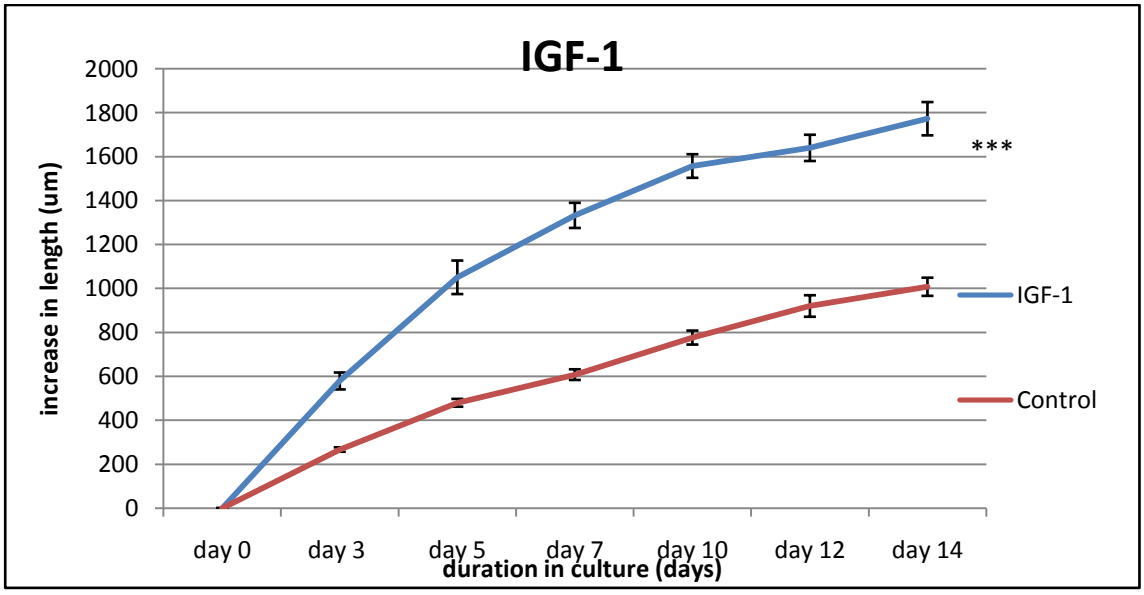

Figure 3: Effects of IGF-1 on linear bone growth. Metatarsal bones were taken from 3 day postnatal mice and cultured in DMEM F12 (without phenolred). Bones were pooled together and then assigned to treatment and control groups on random. The blue line represents the average increase in longitudinal growth of treated bones in micrometers while the red line represents the controls. Duration of the culture is on the x-axis. N = 5, ***p less than 0.001

G1-GPER Agonist

The effects of G1 agonist on bone growth were tested. G1 agonist is a stimulatory ligand of GPER. In our experiment we found that G1 stimulated bone growth (Figure 4). The most efficient concentration was 10nM ($p<0.01$ versus control). At higher concentrations of 100 nM and 1 µM, no stimulatory effect was observed and growth pattern was very much similar to vehicle treated control bones (Figure 5, 6). Data represents mean ± standard error. n=6, **p<0.01, versus control (unpaired student t-test).

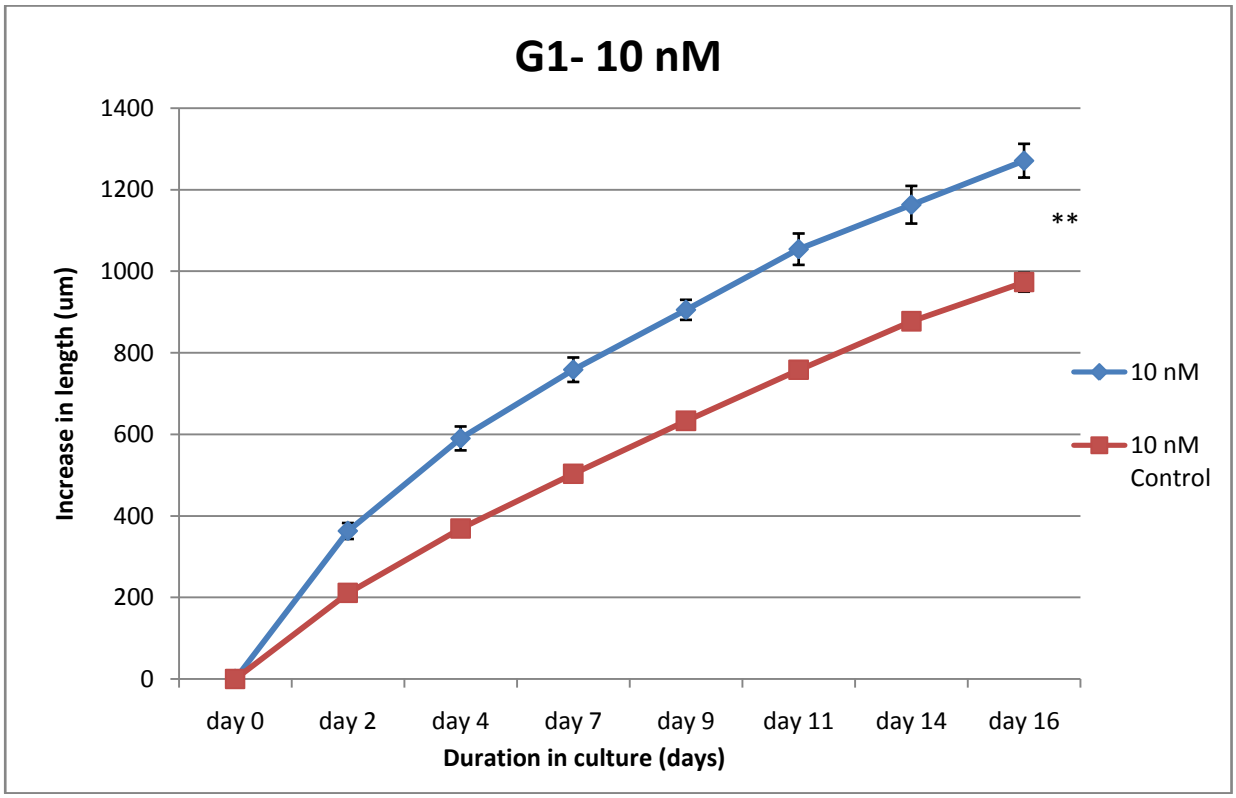

Figure 4: Effects of G1 10 nM; GPER agonist on metatarsal growth. Metatarsal bones were taken from 3 day postnatal mice and cultured in DMEM F12 (without phenolred). Bones from one paw were taken as "treated" and bones from the other paw were taken as "control". The blue line represents the average increase in longitudinal growth in the treated group and the red line represents the control group. X-axis represents the duration of the culture. N= 6, **p less than 0.01.

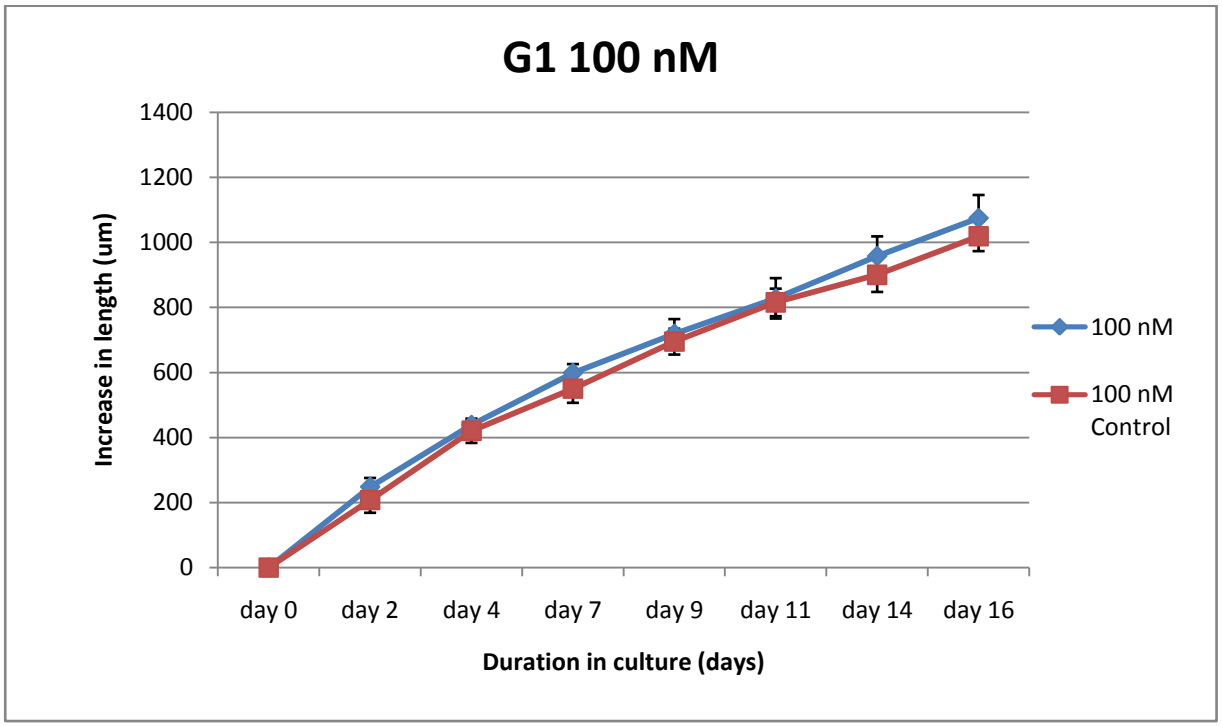

Figure 5: Effects of G1 100 nM; GPER agonist on metatarsal growth. Metatarsal bones were taken from 3 day postnatal mice and cultured in DMEM F12 (without phenolred). Bones from one paw were taken as "treated" and bones from the other paw were taken as "control". The blue line represents the average increase in longitudinal growth of treated bones in micrometers while the red line represents the controls. The duration of the culture is represented on the x-axis. N=6, p> 0.05

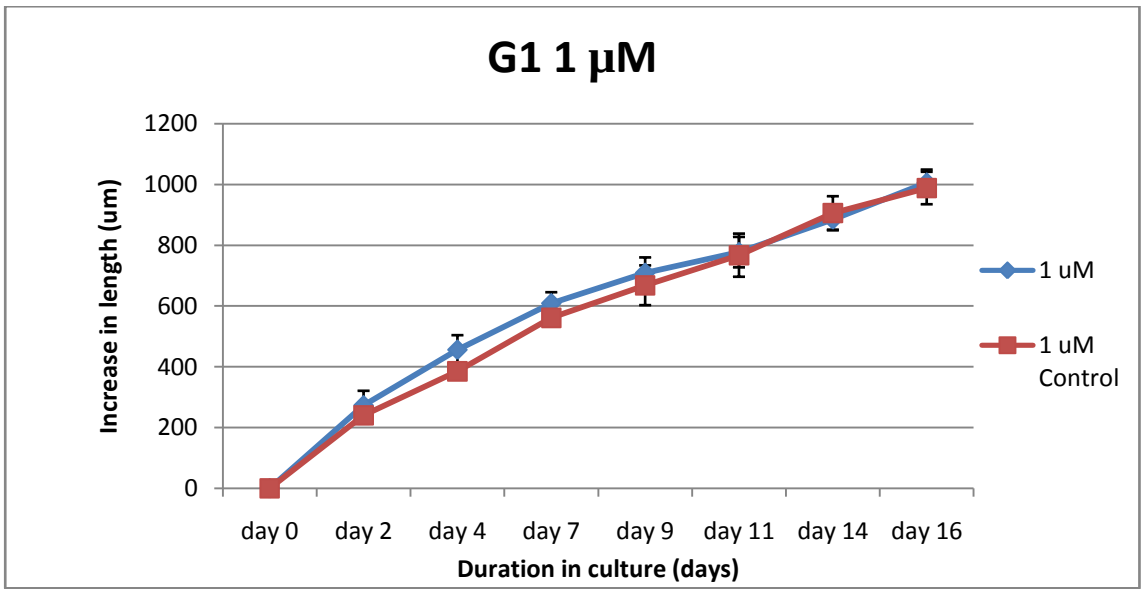

Figure 6: Effects of G1 1 µM; GPER agonist on growth. Metatarsal bones were taken from 3 day postnatal mice and cultured in DMEM F12 (without phenolred). Bones from one paw were taken as "treated" and bones from the other paw were taken as "control". The blue line represents the average increase in longitudinal growth of treated bones in micrometers while the red line represents the controls. X-axis represents the duration of the culture n=6, p>0.05

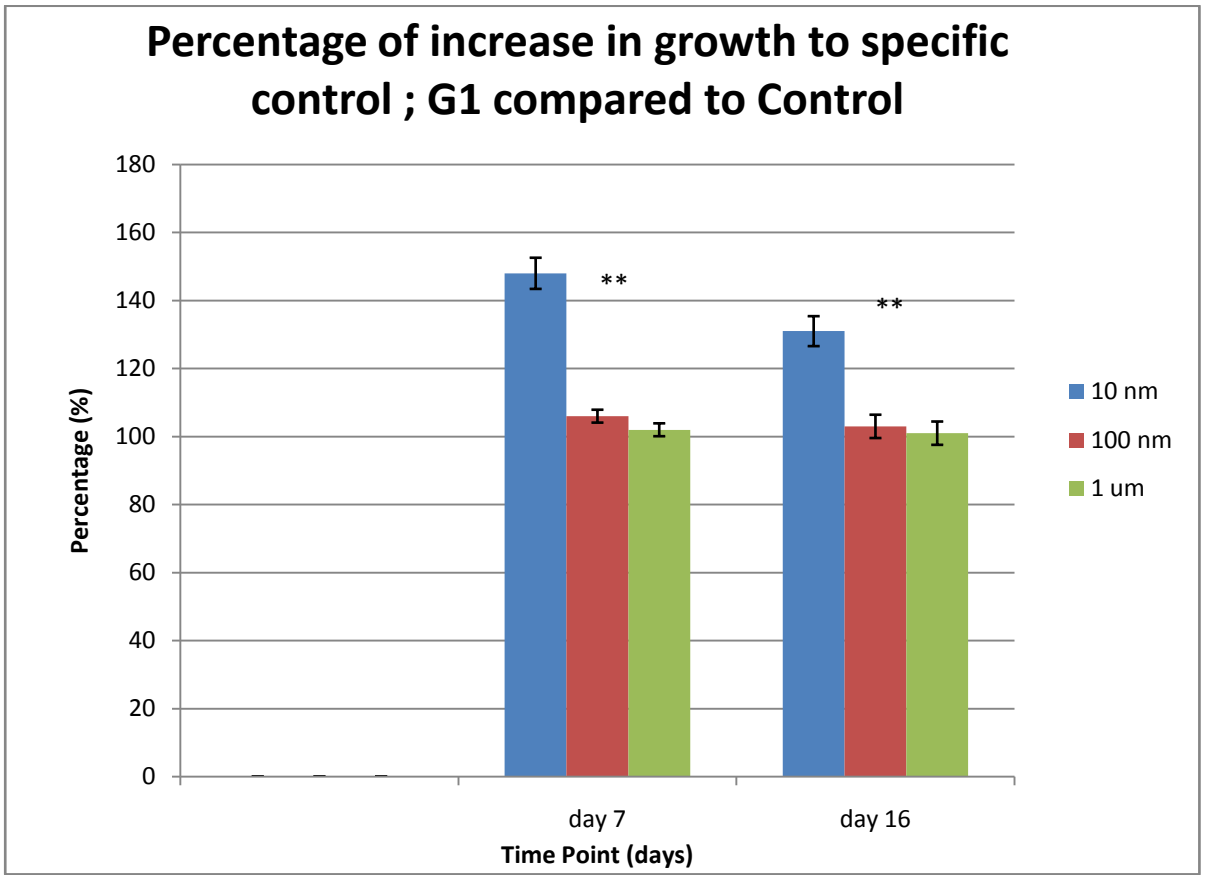

Figure 7: Representation of increase in growth of G1 treated metatarsals as percentage compared to specific controls. Controls taken at 100% with increase in growth in G1 treated presented as percentage on two distinct time points. The blue bar represents the percentage average of increase in longitudinal growth by 10 nM treatment group. The red and green bars represent the 100 nM and 1 µm treatment group. N=6 with P-value < 0.01 (**)

In figure 7, the overall increase in growth of the three treated groups is presented as percentages of specific control with controls taken at 100 %. It is clear that at 10 nM dose, the average increase in length is 48 % more compared to controls on day 7 and this difference is 32 % on day 16. Taking figure 4, 5 together with figure 6, it can be seen that the difference in growth rate is maintained throughout the culture duration (approximately 250 um) at dose concentration of 10 nM. No significant effects are seen at higher dosages of 100 µM and 1 mM.

G15-GPER Antagonist

There was no significant difference seen in the metatarsals treated with G15 compared to controls. G15 is a ligand that serves as a GPER antagonist. All the treatment groups and

the control group had the same growth rate during all time points in the culture and there was no significant difference in bone length at any of the dosages compared to the controls (figure 8) (p >0.05 vs control). Data represents mean ± s.e.m, n=5, p>0.05 versus control (unpaired student's t-test).

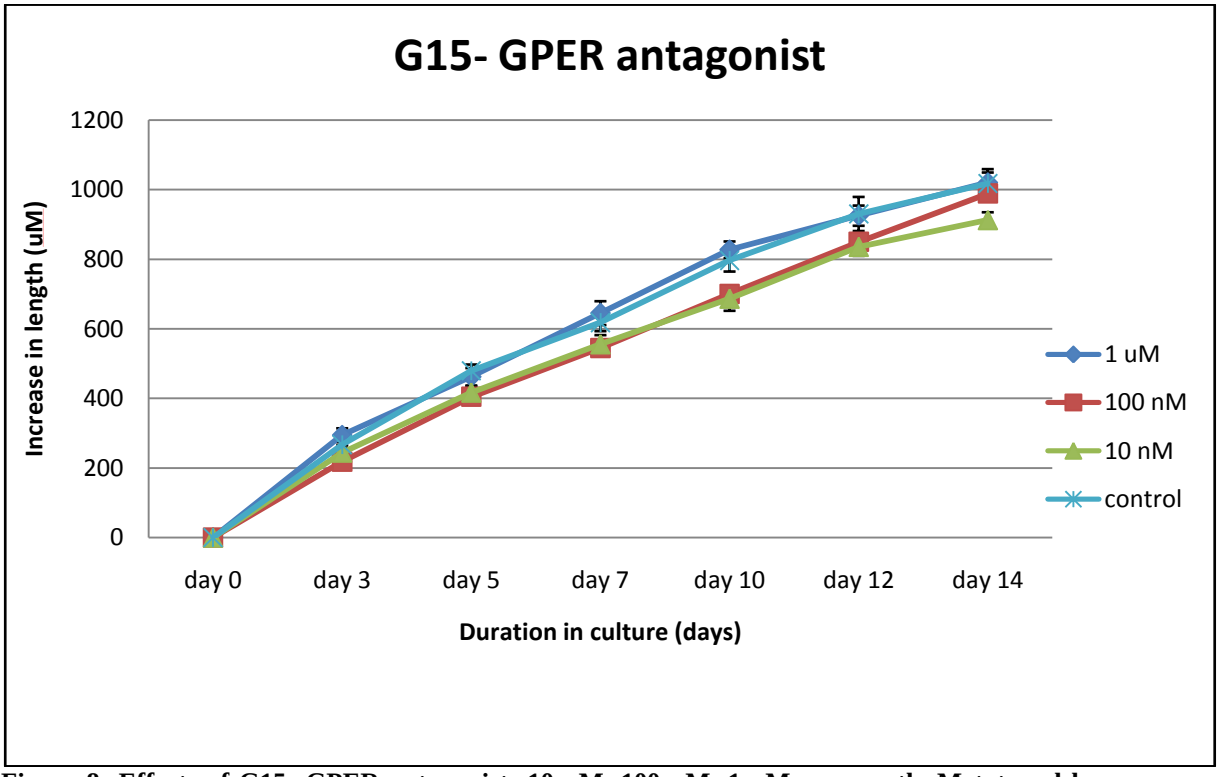

Figure 8: Effects of G15- GPER antagonist: 10 nM, 100 nM, 1 uM on growth. Metatarsal bones were taken from 3 day postnatal mice and cultured in DMEM F12 (without phenolred). All bones were first pooled together and then assigned to different treatment groups and control group on random. Duration of culture is represented on the x-axis. N= 5 with P-value >0.05

Discussion

This pilot study is the first proof of concept of bone growth stimulation through GPER achieved by the G1-GPER agonist. Data from this experiment points in the direction of a new drug target that maybe evaluated for the treatment of growth disorders. The presence of GPER in the resting and hypertrophic zones of the growth plate and the decline of its expression as puberty progresses points to a possible role in chondrocyte recruitment and

hypertrophic chondrocyte differentiation (Chagin & Savendahl 2007). This decline that was observed in the resting zone takes place in parallel in both boys and girls however in the hypertrophic zone the decline in girls has been seen to occur much earlier compared to boys. Since pubertal growth spurt occurs much earlier in girls compared to boys, hence this can be an indicator that GPER takes part in pubertal growth spurt and subsequent growth plate closure.

This study demonstrates that at the concentration of 10 nM, G1 was able to stimulate growth in *ex-vivo* culture of metatarsal bones that was significantly more than the controls (Figure 3). It is surprising that the maximum effect on bone growth is observed at the smallest dose concentration in our treatment range. There was no observation of any kinds of effect at higher dosages of 100 µM and 1 mM. But since the dosage selection was made on random hence it cannot be concluded that 10 nM is the most effective dose to see GPER agonistic activity of G1 in growth plate. In contrast to the agonist, the G15-GPER antagonist treatment of the metatarsal bones did not reveal any significant effect compared to controls. One possible reason for this lack of activity can be the fact that G15 even though has antagonistic relation with GPER, still it has been reported to have binding affinity for ER-α (Dennis MK. *et al*. 2011). This cross reaction of G15 with ER-α may have been the reason behind lack of activity in treated groups compared to controls. Other reason could be that since the treatment dose concentrations were selected randomly hence it could be that range of effective dose concentrations may not have been covered.

A new GPER-antagonist has been developed that has been shown to have minimum cross reactivity with ER-α while retaining its antagonistic activity towards GPER and it has been given the designation of G36 and is an isosteric derivative of G1 (Dennis MK. *et al.* 2011). G36 has not been evaluated in terms of its GPER antagonistic effects on growth plate. It will be interesting to see the effects of G36 in our model. An antagonistic potential may render it to be considered as a suitable drug candidate for a non-invasive treatment for excessive growth.

Already verified effects of IGF-1on growth plate (Chagin *et al.* 2010) were also observed in this study. The increase in growth that was seen in G1 treated bones (figure 3) is not as much compared to IGF-1 treated bones (figure 2, 3). Still it is substantial enough to suggest further probing into selective modulation via GPER by using in-vivo studies. As

mentioned earlier, IGF-1 brings about its effect by accelerating the cell cycle thus attaining senescence. So basically, to achieve its effects it exhausts the growth potential of cells. Because of that, phenomenon of catch-up growth is seen in control bones in prolonged cultures. It is highly desirable to come up with a growth modulator that does not affect the senescence of cells. Work is being done to evaluate the potential of GH therapy in combination with IGF-1. No promising data has emerged so far showing that GH in combination with IGF-1 has any synergism. It is a speculation that IGF-1 may be shelved in the coming days and may not be available for treating growth stunting. GH treatment will then be the only option available.

Also, on the other end of the spectrum, surgical removal of the growth plate is being offered as therapeutic option to selective number of cases that have too much growth. This increased acceleration of growth during pubertal development maybe a consequence of either an increased GH production or constitutional tall stature due to inheritance.

High-dose estrogen treatment has been used for too much growth as well as it effectively reduces adult height in extremely tall girls by inducing closure of epiphyseal end plates (Goldzieher, 1956; Venn *et al.* 2008). But the side effects can range from decreased fertility in adult life to premature ovarian failure (Hendriks *et al.* 2011). It may also induce the risk of deep venous thrombosis (Weimann and Brack, 1996) and also may render the subject vulnerable to breast cancer and gynecological cancers (Genazzani *et al.* 2001).

Hence, modulation of bone growth via GPER agonist and antagonist can be an alternative to the above mentioned treatments. An agonist will be a cheaper alternative to GH treatment which is highly expensive. An antagonist will give a preferable non-invasive treatment modality as an alternative to growth plate removal surgery. Also, as articular and growth plate chondrocyte regulation is pretty similar, hence this new modality maybe modified for a wider spectrum of disorders that may include osteoarthritis and osteoporosis.

GPER is a relatively recent discovery and not much is known about its role in the human physiology. GPER role is being documented in the context of other disease models as well. It has been shown in-vivo GPER knockout mice models that deletion of GPER gene results in impaired glucose tolerance and reduced skeletal growth and it is required for normal glucose homeostasis and bone growth (Mårtensson U.E *et al.* 2008). It has also

been localized in the human and rat heart and its stimulation has been shown to be cardio protective (Patel VH *et al*. 2010). These findings are suggestive of a wider spectrum of roles in the human physiology that GPER may be contributing to and targeting GPER may not just be beneficial for bone growth and modulation but also, it might lead us to discover newer and better suited therapeutic agents for a range of medical disorders.

Conclusion

This pilot study has for the first time put forward satisfactory data showing that G1 treatment with 10 nM concentration increases growth in *ex-vivo* culture of mouse metatarsal bones compared to controls. This effect needs to be validated further by careful planning of metatarsal organ cultures over a prolong period of time in order to ascertain how persistent the effect is (3 month culture). This pilot study opens a gateway to further studies that aim to evaluate and ascertain the mechanism of action of GPER in the growth plate. These studies should be focused on evaluating the effects of G1 on chondrocyte proliferation, differentiation and apoptosis in-vivo and should also be able to answer questions such as optimum dose range for G1, its therapeutic window and its effects on other tissues that contain GPER. The signaling pathways of GPER can be studied by utilizing G-protein and protein kinase C knockout mouse models. We did not see any effects of G15 on the growth plate in our cultures with possible reason being cross reactivity with ER-alpha. The new GPER antagonist G36 which has been reported to have very low cross reactivity profile with ER-alpha needs to be evaluated.

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