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Postnatal Regulation of Bone Growth by Muscle IGF-1

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Interplay between muscle and bone is known to play an important role in growth modifications. Muscle loading from muscle hypertrophy promotes adjacent bone growth via Insulin-like Growth Factor-1 (IGF-1). Yet, in the absence of muscle hypertrophy, bone growth is not completely aborted indicating potential direct muscle paracrine role of muscle IGF-1 on bone. Maximizing growth potential and enhancing bone growth in mature bone in craniofacial skeleton is a significant benefit in orthodontic treatment. In this study, potential anabolic effect of muscle IGF-1 on post-natal mandibular growth is investigated. Methods: Four wild-type (WT) mice each at age of 6 weeks and 10 weeks; four dominant negative muscle specific IGF-1 receptor mice (MKR) each at age of 6 weeks and 10 weeks with one side masseter muscles injected with AAV-IGF-1 at 2 week of age were utilized. Four WT female and four WT male at age of 26 weeks with AAV-IGF-1 injection at age of 18 week were utilized. Muscle fiber size, mandibular bone lengths (sagittal and vertical) and condylar growth plate were evaluated from each animal. Results: Supplemental IGF-1 increased vertical mandibular bone growth in 6 weeks old MKR mice while 6 weeks old WT mice showed more increase in horizontal mandibular bone growth. There was no significant difference in mandibular growth in 10 weeks old WT and MKR mice. There was no significant effect of IGF-1 on muscle fiber size in both WT and MKR mice. Condylar growth plate analysis showed more mature form of chondrocytes with IGF-1 supplement. Conclusion: Post-natal muscle IGF-1 promoted bone growth in the absence of muscle hypertrophy.

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“POSTNATAL REGULATION OF BONE GROWTH BY MUCLE IGF-1”

by

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Thesis Defense
Master of Science in Oral Biology

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School of Dental Medicine
Department of Orthodontics

Abstract

Interplay between muscle and bone is known to play an important role in growth modifications. Muscle loading from muscle hypertrophy promotes adjacent bone growth via Insulin-like Growth Factor-1 (IGF-1). Yet, in the absence of muscle hypertrophy, bone growth is not completely aborted indicating potential direct muscle paracrine role of muscle IGF-1 on bone. Maximizing growth potential and enhancing bone growth in mature bone in craniofacial skeleton is a significant benefit in orthodontic treatment. In this study, potential anabolic effect of muscle IGF-1 on post-natal mandibular growth is investigated. Methods: Four wild-type (WT) mice each at age of 6 weeks and 10 weeks; four dominant negative muscle specific IGF-1 receptor mice (MKR) each at age of 6 weeks and 10 weeks with one side masseter muscles injected with AAV-IGF-1 at 2 week of age were utilized. Four WT female and four WT male at age of 26 weeks with AAV-IGF-1 injection at age of 18 week were utilized. Muscle fiber size, mandibular bone lengths (sagittal and vertical) and condylar growth plate were evaluated from each animal. Results: Supplemental IGF-1 increased vertical mandibular bone growth in 6 weeks old MKR mice while 6 weeks old WT mice showed more increase in horizontal mandibular bone growth. There was no significant difference in mandibular growth in 10 weeks old WT and MKR mice. There was no significant effect of IGF-1 on muscle fiber size in both WT and MKR mice. Condylar growth plate analysis showed more mature form of chondrocytes with IGF-1 supplement. Conclusion: Post-natal muscle IGF-1 promoted bone growth in the absence of muscle hypertrophy.

Introduction

There has been a long, yet continued study on muscle and underlying bone interactions. Muscle can influence bone in two general ways. First, changes in either mass or strength of muscle can affect bone growth mechanically through forces generated by muscle loading. Second, muscle is a source of local growth factors, like Insulin-like Growth Factor-1 (IGF-1) that act on bone and provide a chemical influence on bone growth.

Since the functional matrix hypothesis postulated that epigenetic factors like skeletal muscle regulates skeletal tissue modifications (Moss, 1968), Frost developed the mechanostat hypothesis, emphasizing that mechanical loads on bone largely determines bone physiology while non-mechanical factors only account for a small part (Frost, 1987). According to the hypothesis, when bone grows or muscle force increases, surrounding tissue is strained, and together with other signals and non-mechanical factors, a regulatory feedback loop is established between the muscle strain and effector cells. Therefore, the mechanical strain dictates bone remodeling and strength while chemical agents like hormones are just modulators (Schoenau, 2005). Supporting the idea, Duchenne muscular dystrophy (DMD) patients who inherently have weaker muscle show decreased bone mineral density and small bones in comparison to aged matched healthy people (Bianch, 2003), (King, 2014). Additionally, reduced muscle strength in Myotonic Dystrophy (DM) and DMD patients lead to less biting force, and this has been shown to be involved in dentofacial morphology change and malocclusion by craniofacial bone growth retardation (Kiliaridis, 1998).

On the contrary, importance of chemical effects on bone has also been demonstrated. A study introduced muscle specific IGF-1 transgenic overexpression in mdx mice, and restored muscle strength and hypertrophy (Barton, 2002). This suggests that local muscle IGF-1 alone can remediate the muscle paracrine function, which may also affect adjacent bone physiology. Another study showed IGF-1 depletion in mice retarded postnatal bone development and growth rate resulting in 30% general body size reduction when compared with wild type littermates (Baker, 1993).

While it is now well accepted that both mechanical and chemical factors of muscle influence bone growth, morphology and density, it is still not clear to what extent the chemical effect alone can influence those properties. It is clinically significant to determine if chemical supplementation can overcome inherent muscle deficiency. In orthodontic patients, asymmetric facial skeleton is often the cause of malocclusion that requires skeletal correction, usually orthognathic surgery in addition to orthodontic treatment. Significant numbers of adult orthodontic patients compromise their treatment when orthognathic surgery is required because of the cost, time commitment and other existing or potential health complications. In adolescents and children, most surgery is delayed until skeletal growth is complete so that the outcome is more predictable.

However, if the growth potential could be maximized or stimulated, while young patients are still in growth period, surgery may become unnecessary.

Insulin like growth factor 1 (IGF-1) is a single polypeptide chain involved in growth, development and metabolism of bone and muscle. While almost all cells in

the body have IGF-1 receptors, and hence are affected by IGF-1, it is known to play a key role in muscle hypertrophy, repair and strength (Adams and McCue 1998). One way is to act directly on muscle cells to produce more protein synthesis and increase mass via PI3K-Akt-mTOR signaling pathway (Li, 2002). Another way is IGF-1 binds to increase activation of satellite cells, which can re-new themselves and replace the reservoir satellite pool or differentiate into myoblasts and form new muscle fiber (Barton, 2010).

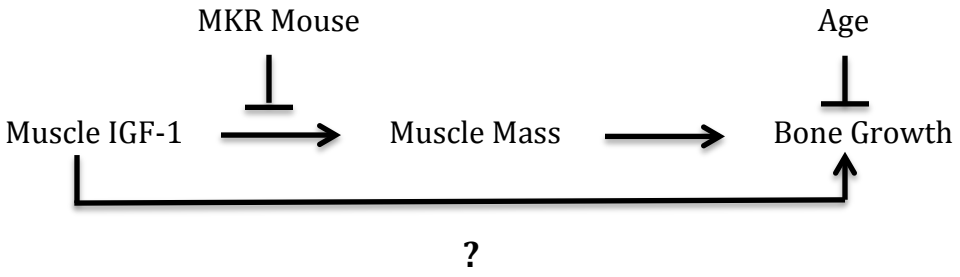
Circulating IGF-1 is primarily produced in the liver, yet most tissues, including skeletal muscle, produce local IGF-1 that plays a paracrine role on any given tissue. It has been supported that bone physiology relies on both paracrine and endocrine IGF-1. While muscle size and growth were not compromised in endocrine IGF-1 restricted mice, bone density was affected to be inferior to normal mice (Govoni, 2007). Additionally, boosting circulating levels of IGF-1 by increasing liver production increases muscle weight, proposing that circulating IGF-1 can enter local tissue and have anabolic effects (Elis, 2011). Thus, IGF-1 might act directly on muscle and bone, and indirectly on bone via enhancing muscle mass.

So far, studies have demonstrated that muscle mass provides a mechanical loading for bone growth and strength in conjunction with local growth factors secreted by muscle, including IGF-1. Discovery of the extent of muscle IGF-1 influence on muscle and bone, either by mechanical or chemical, or both, would benefit in orthodontic patients for potential growth modifications during active growth period. Further more, it is not yet elucidated if postnatal autocrine/paracrine IGF-1 supplemented at different stages of growth would affect

muscle and bone growth. Hence, this study aimed to see: 1) IGF-1 effect on muscle hypertrophy and bone growth; 2) effect of IGF-1 on bone growth in the absence of muscle hypertrophy; 3) the effect of age on increased muscle IGF-1 and mass alterations of bone growth.

We anticipate that post-natal IGF-1 will have direct effects on bone growth independent of muscle hypertrophy and increased loading. Therefore, we will test the null hypothesis that there is no effect of IGF-1 on bone growth. In this study, viral IGF-1 was delivered into masseter muscles of wild type (WT) and transgenic mice, which have muscle specific dominant negative IGF receptor blocked (MKR) to compare IGF-1 effect in mechanical and chemical ways on bone. Groups of mice were sacrificed at 6, 10, 26 weeks of age and evaluated for the effects of age on paracrine role of IGF-1 (Fig. 1).

Study Design



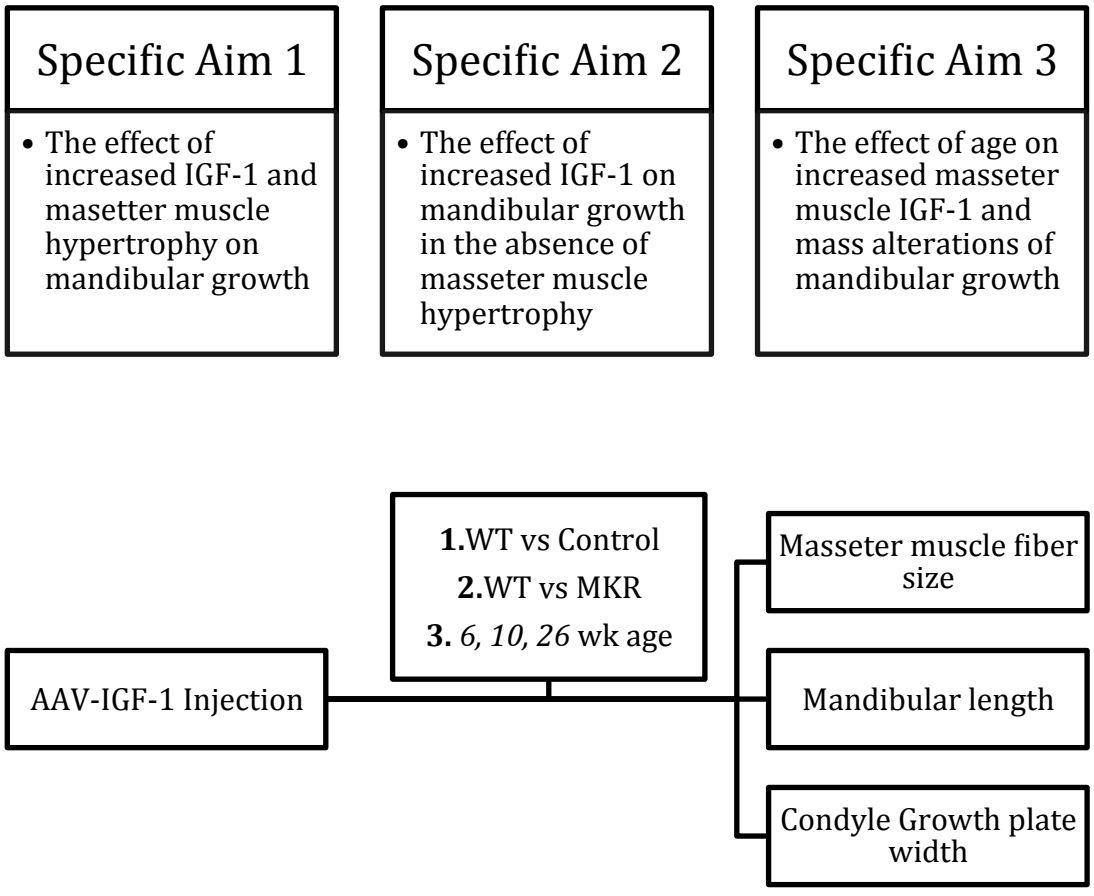


Fig. 1

Fig. 1. In this study, roles of muscle hypertrophy and age on direct IGF-1 effect on bone are investigated using dominant IGF-1 receptor blocked (MKR) and different age mice. Viral IGF-1 (AAV-IGF-1) is injected into one side of masseter muscles. Masseter muscle fiber size, mandibular growth and condylar growth plate width from each experimental group are analyzed.

Materials & Methods

Viral construction and injection

A recombinant AAV plasmid (pSUB201) was constructed that contains the myosin light chain 1/3 promoter/enhancer, rat IGF-IA cDNA, and simian virus 40 polyadenylation signal for viral production, as previously described (Barton, 1998). Recombinant AAV serotype 8 (rAAV-2/cap8: AAV-2 genomes pseudopackaged into AAV-8 capsids) was prepared by the Institute for Human Gene Therapy Vector Core (Philadelphia, PA) following published procedures.

Animals

All experiments involving animals were approved by University of Pennsylvania Animal Care and Use Committee. The animals utilized for this study were C57Bl/6 (WT) mice and the MKR mice, which harbors a dominant negative IGF-I receptor transgene under the muscle creatine kinase (MCK) promoter (Fernandez, 2001). Anesthetized WT mice of 2 and 18 weeks of age and 2 weeks old MKR mice were injected with AAV-IGF-1 (Barton, 1998) 100 µl of 10% glycerol/PBS containing $\approx 10^{10}$ rAAV particles into the interstitial space of the anterior muscle compartment of the right or left masseter.

Groups that were injected at 2 weeks of age (WT and MKR) were sacrificed at two different time points: after 4 weeks (four WT and four MKR) and after 8 weeks (four WT and four MKR). Therefore, 6 and 10 week groups refer to the age of sacrifice. 18 weeks old mice group (WT) at the time of injection were sacrificed after 8 weeks, and hence referred to as 26 week group. They were divided into four males

and four females. For each group, control samples without IGF-1 treatment were sacrificed according to the strain (WT and MKR) and age matched experimental group (Fig. 2).

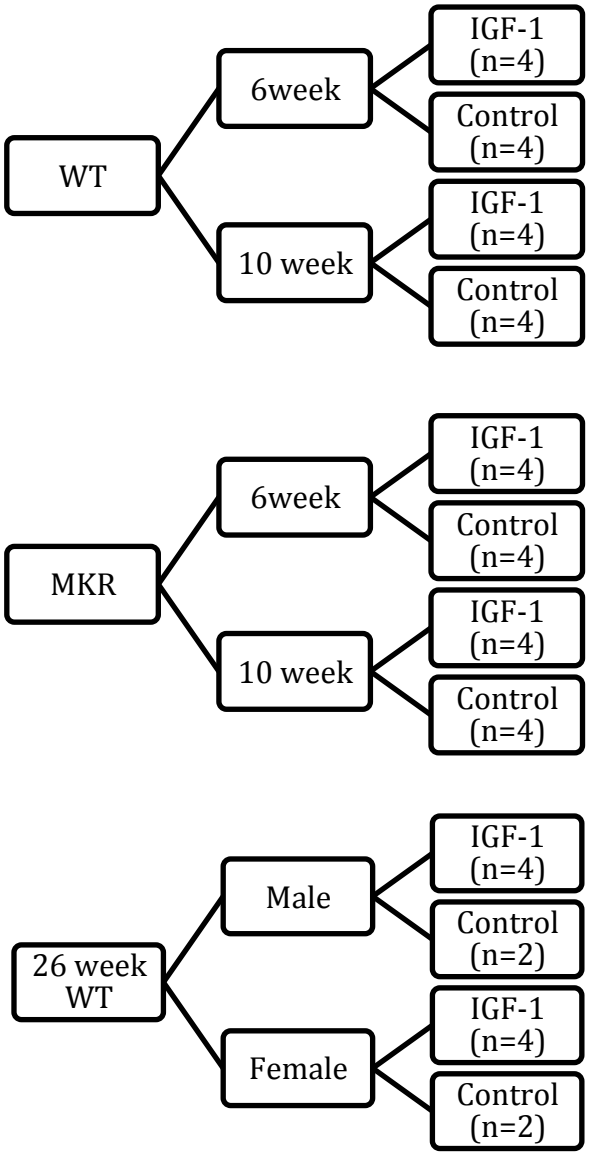


Fig. 2

Fig. 2. Experimental groups of animals in the study are shown. Wild type (WT) and dominant muscle IGF-1 receptor negative (MKR) mice at 6, 10 and 26 weeks of age were used. There were IGF-1 treated on the right side masseter (IGF-1) and control groups.

Immunohistochemistry of masseter muscle

At the time of sacrifice, a portion of left and right superficial masseter muscles were harvested for evaluation of the fiber size. Each muscle was frozen in OCT embedding compound (Tissue Tek, Torrance, CA) in melting isopentane, and 10 μm cryosections were prepared. For immune-staining procedure, sections were blocked in 5% bovine serum albumin (BSA)/phosphate-buffered saline (PBS) and incubated overnight at 37 °C in 5% BSA/PBS containing a rabbit anti-laminin (rabbit Ab-1, Neomarkers, Fremont, CA). After 24 hours, thoroughly washed with PBS sections were incubated in 5% BSA/PBS containing Alexa Fluor IgG secondary antibody (1:2,000; Invitrogen) for 1 hour in the dark at room temperature. Slides were then mounted with Vectashield DAPI media (Vector Laboratories, Burlingame, CA) and examined for fluorescence using a Leica DMRBE fluorescent microscope (Leica, Bannockburn, IL) equipped with a Micro Max digital camera (Princeton Instruments, Trenton, NJ) interfaced with Image Pro Plus software (Media Cybernetics, Bethesda, MD). Images were exported and merged together to make a composite image for each sample using Photoshop (Adobe Photoshop CC, 2012). Fiber size analysis was done using in house developed software (Smith and Barton, 2014) measuring each fiber diameter.

IGF-1 content

Upon removal of superficial masseter muscles, small portions of right and left deep masseter muscle were removed, weighed, and then frozen at -80°C. IGF-1 content in muscle was evaluated following procedures in an ELISA kit specific for

rodent IGF-1 (MG100, R&D Systems, Minneapolis, MN). IGF-1 content was calculated as ng IGF-1 per gram wet weight tissue (ng/g).

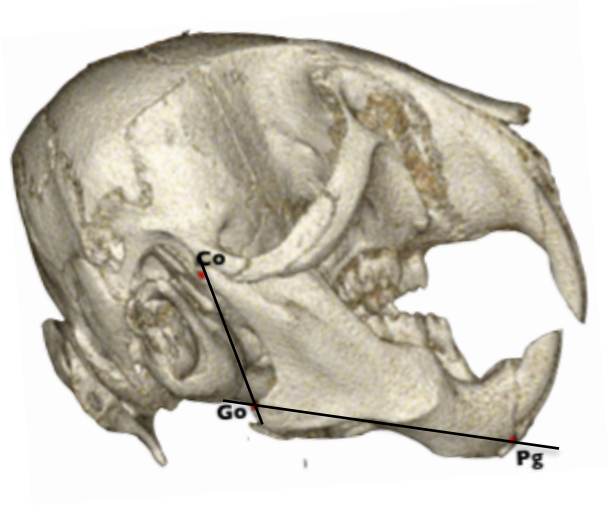


Fig. 3

Fig. 3. uCT scan 3D image of craniofacial skeleton and mandibular landmarks: the most postero-superior point of the mandibular condyle (Co), the most postero-inferior point of the lower border of the mandible (Go) and the most antero-inferior point at the lower border of the mandible (Pg).

Craniofacial uCT Imaging and Analysis

After sacrifice and masseter tissue harvest, the heads of the wild type and MKR mice were removed at 6 and 10 weeks old. The heads were placed in 4% paraformaldehyde at 4°C until micro-CT imaging. Each sample group was imaged by μCT (Scanco vivaCT 40) at 60μm resolution.

Skull micro-CT images were exported to image processing software and craniofacial dimensions were measured manually on micro-CT images. Scanned images in DICOM format were first cropped and down sized using FIJI (ImageJ, NIH) and then using OsiriX (www.osirix-viewer.com) 3D images were constructed. Three mandible landmarks were identified on 3D skull image (Fig. 3): the most postero-

superior point of the mandibular condyle (Co), the most postero-inferior point of the lower border of the mandible (Go) and the most antero-inferior point at the lower border of the mandible (Pg). Length of mandibular ramus was determined by measuring Co and Go and the distance between Go and Pg determined the length of the mandibular corpus (Ramirez-Yanez, 2005). After landmarks were identified on 3D images, X, Y and Z coordinates were recorded, then linear measurement values were calculated mathematically via Microsoft Excel software (Microsoft Corp., Redmond, WA.).

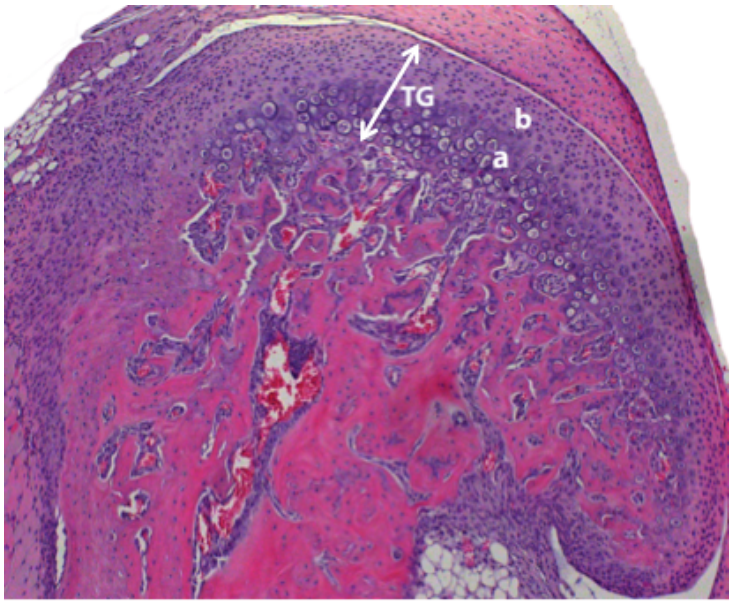


Fig. 4

Fig. 4. Condyle. H&E stain. a, hypertrophic zone b,proliferating zone TG, Total growth plate

Condylar growth plate analysis

Additional histological analysis of decalcified condyle sections was performed to measure growth plate and hypertrophic zone widths. The heads of mice sacrificed at 6 week and 10 week were decalcified by leaving in 10% EDTA pH

6.95 in Tris and KOH solution at 4°C for 4 weeks. When decalcification was complete, the heads were cut in half sagittally and each side were separately embedded in paraffin. Each prepared samples were sectioned at 5 μm and stained with hematoxylin and eosin (H&E). Images were viewed using light microscope x10 resolution and photos were taken (Leica, Bannockburn, IL) equipped with a Micro Max digital camera (Princeton Instruments, Trenton, NJ). Hypertrophic and proliferating regions of condyles were measured and added up to give total growth plate widths (Fig. 4) (Adobe Photoshop CC, 2012). Measurements were taken at four different locations and the values were averaged.

Results

Fiber diameter

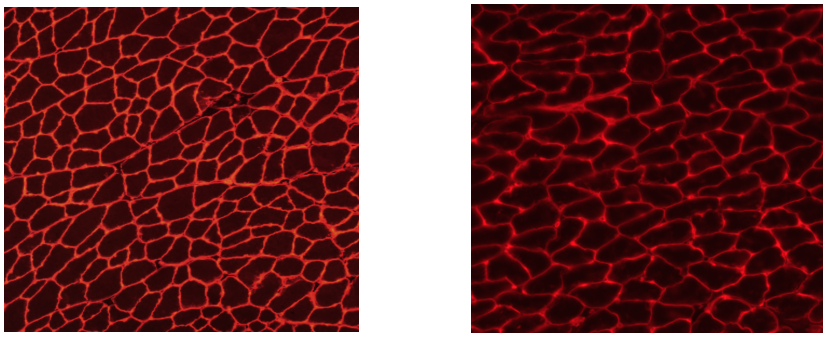
To evaluate the effect of IGF-1 on muscle fiber size, masseter muscle fiber diameter was compared on a small piece of the masseter muscles. For each group, mean fiber diameter of masseter muscle was calculated for comparison analysis. Muscle fiber size within the same sample varied significantly (Fig. 5) and mean values from one sample to another showed high variance.

For example, it was observed in control groups, 6 week WT fiber diameter was larger than 6 week MKR fiber diameter on both right side (62.685μm and 41.185μm) and left side (57.215μm and 43.6μm) that was statistically significant (Fig. 6(a)). However, within each animal, the mean fiber diameter difference between right and left sides was 8μm in both WT and MKR group (Fig. 6(b)), which was approximately 15-20% variability on the mean fiber size. In 6 week WT and

MKR group, IGF-1 treated group showed smaller fiber size than the control group on both IGF-1 treated and control sides. Further, in IGF-1 treated 6 week old groups, MKR mice had significantly larger mean fiber diameter than 6 week WT group on both IGF-1 treated and control sides. Within each group, no significant differences observed between IGF-1 treated (right) and control (left) sides in both WT and MKR mice (Fig. 7(a)).

In control groups, 10 week MKR mice showed smaller fiber diameter than WT, but not significantly. In IGF-1 treated groups, no significant differences were noted in mean fiber size between MKR and WT for both IGF-1 treated (right) and control (left) sides (Fig. 7(b)).

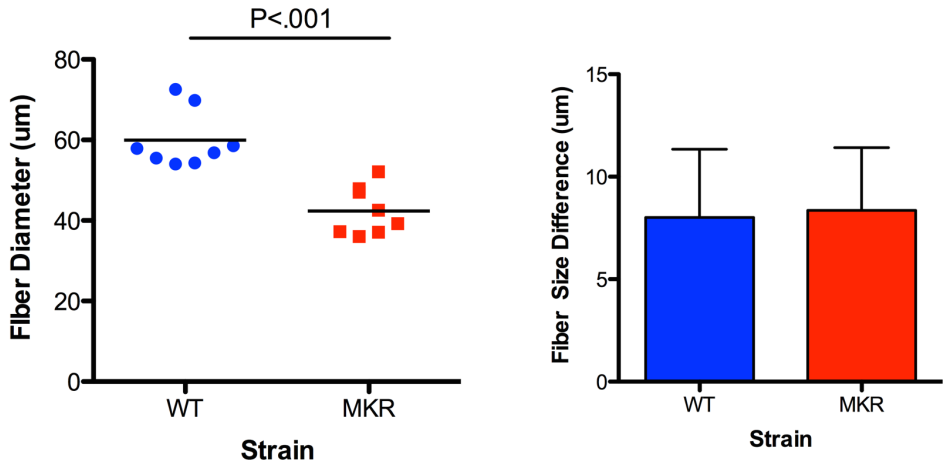
In 26 week of age group, there was no significantly different fiber size between control and IGF-1 treated groups for both male and female. Also, there was no significant difference in fiber diameters within individual sample of right and left. Taken together, while previous studies were able to detect changes in muscle mass after viral IGF-1 injection, the inherent variability in fiber size prevented us from making a meaningful measurement of fiber size in this study.



(a) (b)

Fig. 5

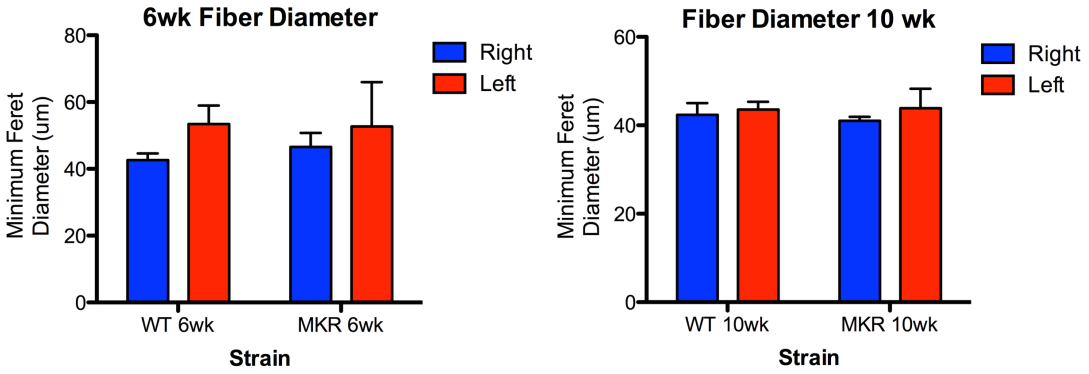
Fig. 5. (a) WT (b) MKR muscle fibers in anti-laminin stain. Fiber size discrepancy within the sample is high.



(a) (b)

Fig. 6

Fig. 6. (a) WT fiber diameter was larger than MKR (n=4) in 6 week control (n=4) groups. (b) Fiber size difference between right and left sides were about 8μm in both WT and MKR.



(a) (b)

Fig. 7

Fig. 7. (a) 6 week MKR (n=4) fiber diameter was increased more than WT (n=4) with IGF-1 supplement. No significant difference between IGF-1 treated (Right) and control (Left) sides within the group. (b) 10 week group showed no significant difference with IGF-1 supplement either between WT and MKR or IGF-1 treated (Right) and control (Left) sides within the group.

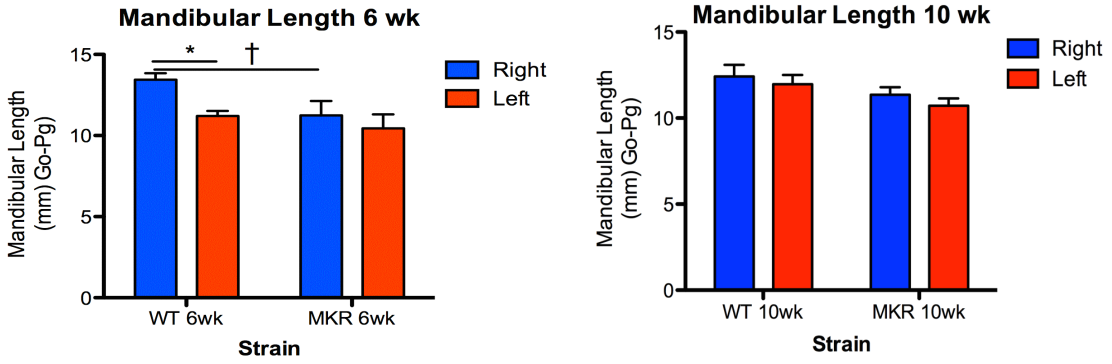
Craniofacial uCT Image and Analysis

To determine effect of IGF-1 on skeletal bone tissue, mandibular lengths were compared between control and IGF-1 treatment groups. Further, direct effect of IGF-1 on mandible without masseter muscle mass increase was evaluated using

MKR and WT mice. All measurements from 3-4 mice per experimental group were averaged and used the mean values for comparison analysis. Unfortunately, skeletal analysis in 26 week age group of mice could not be determined in this study.

Mandibular body length (Go-Pg):

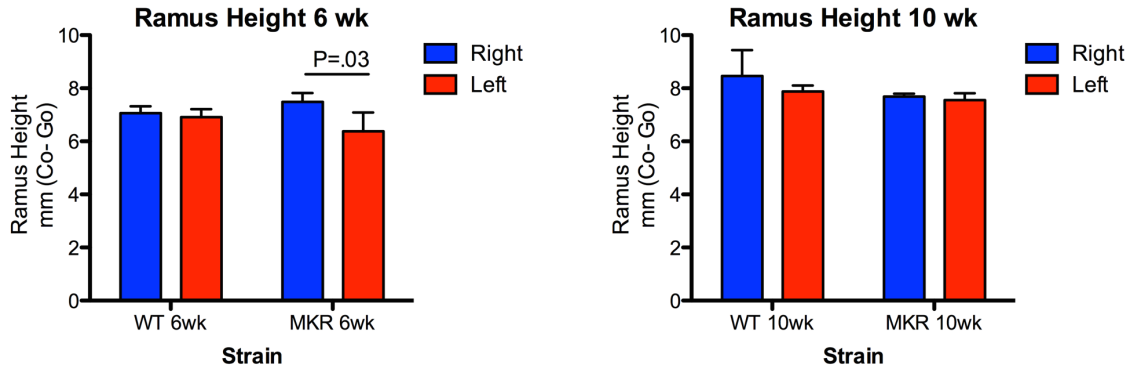
In the 6 week WT group, IGF-1 treated side (right) increased significantly in mandibular length when compared to untreated (left) side, whereas in 6 week MKR mice, there was only 7% increase on the IGF-1 treated side (Fig. 8(a)). Also, on IGF-1 treated side, there was a significant increase in WT group when compared to MKR group. In 10 week WT and MKR groups, there was no significant difference in IGF-1 treated (right) and untreated (left) side (Fig. 8(b)).



(a) (b)

Fig. 8

Fig. 8. (a)Mandibular length (Go-Pg) increased in 6 week group on IGF-1 treated (Rigth) side in WT (n=4) but not significantly in MKR (n=4). (b)Mandibular length (Go-Pg) increase in 10 weeks is not significant on both IGF-1 treated (right) and control (left) side of WT (n=4) and MKR (n=4).



(a) (b)

Fig. 9

Fig. 9. (a) Ramus height (Co-Go) increased with IGF-1 supplement (Right) in 6 week MKR (n=4) group but not in WT (n=4). (B) Ramus height (Co-Go) in 10 week group (n=4) showed no difference between IGF-1 treated (Right) and control (Left) in both WT (n=4) and MKR (n=4).

Mandibular ramus height (Co-Go):

In the 6 week MKR IGF-1 treated group, IGF-1 injected (right) side was significantly increased by 17% in comparison to the control (left) side (Fig. 9(a)), but there was no significant increase in 6 week WT on IGF-1 treated side. Within the 10 week WT group, IGF-1 treated side showed slight increase but not significantly. 10 week MKR group, no difference was observed between IGF-1 treated and control sides (Fig. 9(b)).

Taken together, IGF-I treatment altered mandibular length in WT animals, and less so in MKR animals; further, IGF-1 treatment altered mandibular ramus height only in 6 week old MKR mice.

Condylar growth plate Analysis

To determine the effects of IGF-1 on maturation stage of the condyle, condylar growth plate widths were measured from right and left condyles of each sample.

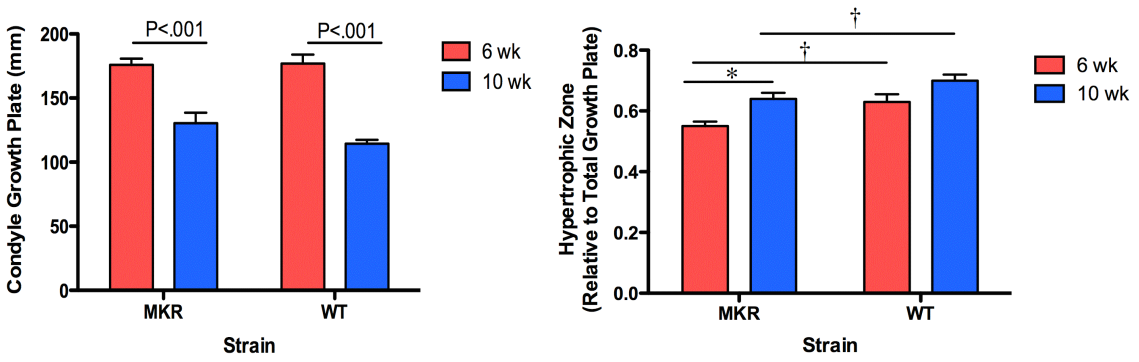
Total growth plate width in both WT and MKR mice was dependent on age. There was a significant total growth plate width reduction in 10 week WT and MKR group in comparison to 6 week comparable groups (Fig. 10(a)). There was 22% reduction of total growth plate in 10 week WT group when compared to 6 week WT group and 16% in MKR groups. A similar trend was observed for both treated and control sides.

Within the same group, although it was small, total growth plate width was smaller on IGF-1 treated side than control side in 6 week WT, by 4%. However, 6 week MKR group showed no significant difference between control and treated sides of total growth plate width. Similarly, both 10 week WT and MKR groups exhibited no significant difference of total growth plate between right and left.

On IGF-1 treated sides (right), hypertrophic zone width was increased in 10 week group than 6 week, in both WT and MKR groups (Fig. 10(b)). There was significant difference in hypertrophic zone widths when comparison was made between WT and MKR. Both in 6 week and 10 week groups, MKR mice showed reduced hypertrophic zone width than WT mice (Fig. 10(b)). The control (left) side values were very close to the right side's and showed the comparable results.

Both WT and MKR 10 week mice showed significantly less total growth plate width but increased hypertrophic width than 6 week on IGF-1 treated side (Fig. 11)

and control side. Taken together, the effects of age and mouse strain on the condylar growth plate were most evident, and the potential differences associated with IGF-1 treatment were only observed in the 6 week WT groups.



(a) (b)

Fig. 10

Fig. 10. Total growth plate width and relative hypertrophic width comparison on IGF-1 treated (right) side. (a) With the same groups, total growth plate width was significantly decrease in 10 weeks old mice (n=4) than in 6 weeks old mice (n=4) both in MKR and WT. (b) Hypertrophic zone width was increased in 10 weeks old mice (n=4) when compared to 6 weeks old mice (n=4) both in MKR and WT.

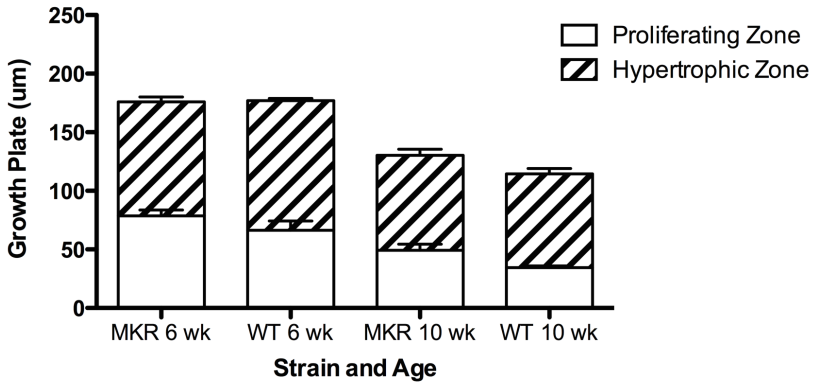


Fig. 11

Fig. 11. Comparison of mean total growth plate width and relative mean hypertrophic zone width in 6 week and 10 week WT and MKR mice. Total growth plate width was reduced in 10 week both WT and MKR mice and hypertrophic zone width was decreased in 10 week WT and MKR mice.

Discussion

The insulin-like growth factors (IGFs) are a family of low molecular weight peptides that resemble insulin both in their structure and in their effects. Studies have shown IGFs regulate skeletal muscle growth and differentiation, and participate in attached bone physiology. In the orofacial area, likewise its effects on hind limb muscle and long bone the IGF system is involved in the growth and development of mastication muscle and craniofacial bone. Previous study shows lack of IGF-1 in masseter muscle decreases its muscle mass and disproportionate development of craniofacial skeleton (McAlarney, 2001). In this study, effects of local IGF-1 supplement on masseter muscle and mandibular growth is evaluated and further, suggests direct paracrine role of muscle IGF-1 on bone growth. IGF-1 effects appear to be confined to active growth period and not in mature tissue.

Fiber diameter

Reduced myofiber size noted in control groups of 6 week MKR in comparison to WT on both right and left sides (Fig. 6(a)) seems in agreement with the premise that MKR mice demonstrate reduced skeletal muscle growth in comparison to wild type (Fernandez, 2002), (Kim, 2005). However, within each control animal, mean fiber diameter difference between right and left was about 15% of the mean fiber diameter in masseter muscle in both WT and MKR mice (Fig. 6(b)).

In 10 weeks old mice, however, there was no significant difference between WT and MKR controls and further, IGF-1 addition did not produce significant effect on fiber sizes in either WT or MKR mice (Fig. 7(b)). It is possible that as mice become more mature in developmental stage, compensatory mechanism may have

restored reduced muscle size in MKR mice, thus diminishing the difference to WT. Supporting that notion, in 26 week old WT mice did not show any anabolic IGF-1 effect on muscle size. This proportional difference is significantly larger than the differences observed between treated and control masseter muscle mass comparison in previous study, and it suggests fiber diameter difference does not correlate IGF-1 effect on muscle hypertrophy, or that a small sample of the masseter is not representative of the entire composition. In transgenic animals with IGF-1 manipulation the masseter exhibits 10-15% changes in fiber size, when sampling the entire muscle. In this case, we used some of the muscle for sectioning, and another part of IGF content. Thus, the native variability in fiber size compounded with the potential variability of the effects in IGF delivery suggests that obtaining meaningful data from these samples is impossible with our current N.

However, another important fact that emerged was that effects were seen at 6 weeks but lost at 10 weeks of age. This transient pattern of IGF-1 effect confined to early in development was also seen across bone growth comparisons. Mandibular length difference between IGF-treated and control was significant in 6 week group but not in 10 week group. Fernandez et al. also noted in a previous study that muscle mass decrease was less in adulthood than between 0-3 weeks of mice age (Fernandez, 2002). One interesting result from fiber diameter comparison is the increased diameter in 6 week MKR than in WT when muscle specific IGF-1 was exogenously added (Fig. 7 (a)), because previous studies showed muscle fiber size in MKR mice did not change with IGF-1 receptors blocked (Kim, 2005). Further more, while IGF-1 delivery was only on the right masseter muscle, both right and

left masseter myofiber sizes were increased. These data, however, are unlikely to be a valid indicator of IGF-1 effect on masseter muscle fiber size, due to high variability within the masseter muscle fibers mentioned previously. Lack of consistent relationship between fiber size and IGF-1 effects presented in this study again suggest that diameter measurement may be an accurate representation of muscle fiber size. Instead, muscle fiber cross sectional area compared in previous study showed that there is a decrease in fiber cross section, but no significant reduction in myofiber diameter of skeletal muscle in MKR mice (Fernandez, 2002). Another possibility is that masseter muscle comprises a number of different types of muscle fibers, and their shapes and size differences are substantial (Fig. 5). It is also apparent in control animals with large intrinsic variance in fiber size even within the same individual (Fig. 6(b)). So far, in previous studies hind limb muscle fiber size has been shown to be affected by IGF-1. Here, we show muscle size analysis in masseter muscle may be more complex than limb muscle and difficult to analyze just by measuring fiber diameter because of different muscle fiber composition and functions.

Craniofacial uCT Image and Analysis

By measuring the skull landmarks directly on micro CT scanned 3D images, mandibular lengths were compared in two aspects: mandibular length (Pg-Go) showing sagittal growth and ramus height (Co-Go) that indicates vertical growth. These are the most crucial areas of interest in orthodontic treatment when skeletal component is included in treatment plan. The overall trend was that on injected side

there was more growth both in mandibular length and ramus height. Regardless of IGF-1 receptors blocked or not, there was an increased growth in IGF-1 treated side when compared to the control side. Based on the data from the previous study that MKR mice muscle mass does not increase with IGF-1 supplement, this result suggests that there is a direct IGF-1 effect on mandibular bone growth without mechanical loading from increased muscle mass. IGF-1 increased sagittal growth of mandible in 6 week is shown by increase in Go-Pg length (Fig. 8(a)), more in WT than MKR, and more on the IGF-1 treated side than control side. This may reflect that sagittal growth is more affected by muscle strength and loading than chemical effect of IGF-1. In 6 weeks old mice, MKR group on IGF-1 injected side showed more vertical growth than WT (Fig. 9(a)) that may indicate vertical growth is promoted by IGF-1 chemical effect acting directly on the bone. Studies have shown vertical growth of mandible is more likely to be affected by masticatory muscle strength (Sciote, 2012) and muscle loading inhibits growth of condyle (Von den Hoff, 2008). These support our data that reduced muscle loading in MKR increased vertical growth of mandible and local IGF-1 input even more, surpassing the matched WT. Both mandibular length and ramus height differences via IGF-1 addition became unnoticeable in 10 week group (Fig. 8(b) and Fig. 9(b)), probably due to a similar reason as the transient fiber size changes mentioned above. Overall, IGF-1 effect on bone growth together with mechanical strength from masseter muscle is multifactorial because while IGF-1 promotes growth on condyle, muscle loading via increase in muscle strength and mass will inhibit condylar growth.

Condylar growth plate analysis

The growth plate reflects longitudinal growth results from chondrocyte proliferation and differentiation. Longitudinal growth rates are high during active growth –during the first year of life and growth spurt (puberty) in humans, and at the end of puberty, growth plates fuse, thereby ceasing longitudinal growth. IGF-1, together with other hormones and growth factors, is now considered as an important regulator of chondrocyte proliferation and differentiation.

In the results shown, it was clearly demonstrated that older mice (10 week) showed reduced total growth plate width than younger (6 week) mice as expected from mature growth plate fusion. Further more, IGF-1 effect on total growth plate width reduction suggests that IGF-1 promoted growth on bone and exhibit more mature stage of growth, as less growth plate width is observed in mature bone. Another distinguishing feature from growth plate histology was the hypertrophic zone that was increased relative to total growth plate in older mice group. This indicates that as bone growth matures, hypertrophic zone width increases in relation to decreasing total growth plate width. There was a significant difference in hypertrophic zone width observed between WT and MKR. WT mice showed a larger proportion of hypertrophic zone than MKR in both 6 week and 10 week groups. This could imply that in WT mice mechanical loading induced IGF-1 production, and hence more increase in hypertrophic zone width than in MKR, which is in agreement with the previous thought IGF-1 promotes bone growth to show a more mature stage of growth plate. There have been debates on IGF-1 in chondrocytes. Interestingly, IGF-1 null mice show a 30% decrease in linear dimension of the terminal hypertrophic chondrocytes, suggesting a role for IGF-1 in the regulation of

chondrocyte hypertrophy but not in proliferation (Wang, 1999). From these findings, Le Roith et al. proposed IGF-I was responsible for chondrocyte differentiation and IGF-II, controlled by GH, was involved in the regulation of chondrocyte proliferation (Le Roith, 2001). Yet, another study in rats found IGF-1 in all zones of the growth plate, with the highest expression levels in the proliferating and the pre-hypertrophic zones of chondrocytes (Reinecke, 2000). Hence, from this study, it was shown that IGF-1 was involved to induce more mature form of bone histology, yet it is not clear whether IGF-1 affects proliferating or differentiating stages of chondrocytes development.

Conclusion

From this study, exogenous local IGF-1 effect on muscle hypertrophy could not be clearly determined because of inherent high myofiber diameter discrepancy in masseter muscle. However, paracrine role of muscle IGF-1 directly on surrounding bone is demonstrated by an increase in mandibular vertical growth, while indirect IGF-1 effect via muscle loading increased horizontal growth. Age is shown to be an important factor to consider for effective IGF-1 anabolic influence in skeletal muscle fiber. Future studies should be directed towards potential direct IGF-1 on bone growth by evaluating the older mice mandibular growth based on the primary premise that IGF-1 is most likely to be effective only in early stage of post-natal development from this study.

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