Enhanced Bone Formation during Distraction Osteogenesis in FGFR3 Deficient Mice

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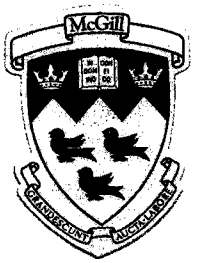

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List of Abbreviations

ACH	Achondroplasia
BMC	Bone Mineral Content
BMD	Bone Mineral Density
BMP	Bone Morphogenic Protein
BMPR	Bone Morphogenic Protein Receptor
BSA	Bovine Serum Albumin
DNA	Deoxyribonucleic Acid
DBM	Demineralized Bone Matrix
dNTP	Deoxyribonucleic Tri-phosphate
cDNA	Complementary Deoxyribonucleic Acid
Col1	Collagen Type I
Col2	Collagen Type II
Col10	Collagen Type X
DO	Distraction Osteogenesis
Erk	Extracellular signal-regulated kinase
FGF	Fibroblast Growth Factor
FGFR3	Fibroblast Growth Factor receptor three
FGF18	Fibroblast Growth Factor 18
HSPG	Heparin Sulfate Proteoglycans
IHH	Indian Hedge Hog
IGF	Insulin Growth Factor
MAPK	Mitogen-activated protein kinase
μCT	Micro Computed Tomography μCT
MT	Mutant
MMA	Methylmethacrylate
OSX	Osterix
Ocn	Osteocalcin
PBS	Phosphate Buffered Saline
PKC	Protein kinase C
P-STAT1	Phosphorylated Signal Transducer and Activator of Transcription
PTH	Parathyroid Hormone
PTSBD	Posttraumatic Segmental Bone Defects
RNA	Ribonucleic Acid
mRNA	Messenger Ribonucleic Acid
STAT	Signal Transducer and Activator of Transcription
Smurfs	Smad ubiquitin regulatory factors
Smad	Homologue of MAD (Mothers against Decapentalegic)
TCA	Tri-chloro Acetic Acid
TGF-β	Transforming Growth Factor-β
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
WT	Wild Type

Abstract

Enhanced Bone Formation during Distraction Osteogenesis in FGFR3 Deficient Mice

Distraction Osteogenesis (DO) is a technique for bone lengthening and filling of bone defects following trauma, infection or resection of tumors. DO consists of an osteotomy of the bone to be lengthened, followed by controlled distraction of the bone segments with an external fixator until the desired lengthening is obtained (distraction phase). This is followed by the consolidation phase, during which the external fixator is kept in place until the newly formed bone in the distracted zone consolidates. This phase is long and may cause numerous problems. Ongoing research aims at finding a method to accelerate the consolidation of the newly formed bone.

Fibroblast Growth Factors (FGF) play a significant role in bone development and repair. FGF18 has been shown to be the only FGF member to be expressed throughout both the distraction and the consolidation phases of DO. It was also reported that FGF18 is the physiological ligand of FGFR3. Therefore, we hypothesized that FGF18 and FGFR3 may have an important role in DO.

To test this hypothesis, we investigated DO in FGFR3 deficient mice (FGFR3-/-). (FGF18 deficient mice are not viable). A miniaturized DO apparatus was applied to the tibia followed by an osteotomy. Distraction began after a 5-day latency period at a rate of 0.2 mm/12 hours for 12 days.

Samples were collected at 3 time points comparing the mutants (FGFR3-/-) to their wild type littermates: end of distraction (17 days post-surgery), mid-consolidation (34 days post-surgery), and end of consolidation (51 days post surgery). The samples were analyzed using X-ray, DEXA, microCT, histology, biomechanical testing and Real-Time PCR.

Our results revealed that FGFR3 deficient mice showed accelerated bone formation compared to the W.T. littermates at mid-consolidation where the parameters measured revealed increased bone mineral density, bone mineral content and trabecular number in the mutant tibial samples. The newly regenerated bone consolidated faster in the FGFR3 knock-out mice and the bone was of better quality as revealed by biomechanical tests in which more force was needed to break the mutant bone because it exhibited higher resistance than the age matched wild-type sample. The marker gene expression patterns revealed an up-regulation of chondrogenic markers that suggest that the knock-out mice follow the endochondral ossification pathway during DO. All results were statistically significant.

These results show that signaling through FGFR3 acts to decrease bone formation during DO. Consequently, blocking FGFR3 may lead to accelerated bone formation in DO. This may have important clinical implications in attempts to improve the functional outcome of DO by decreasing the long duration that the external fixator has to be kept on.

RÉSUMÉ

Amerlioration de la formation osseuse pendant l'osteogenese par distraction chez les souris deficiente en FGFR3

L'Ostéogenèse par Distraction (OD): est une technique pour l'allongement et le remplissage des défauts osseux suite à un traumatisme, une infection, ou une résection de tumeurs. L'OD consiste en une ostéotomie de l'os à allonger, suivie par une distraction contrôlée des extrémités de l'os avec un fixateur externe, jusqu'à ce que l'allongement désiré soit obtenu (phase de la distraction).

Cela est suivi par la phase de consolidation, alors que le fixateur externe est gardé en place jusqu'à ce que l'os récemment formé dans la zone de distraction se renforce. Cette phase est longue et peut causer de nombreux problèmes. Les efforts en recherche visent à trouver une méthode pour accélérer la consolidation de l'os récemment formé.

Les *Fibroblast Growth Factors (FGF)* jouent un rôle important dans le développement et la réparation de l'os. Le FGF18 s'est révélé être le seul membre FGF à être exprimé dans toutes les phases de la distraction et de la consolidation en OD. Il a aussi été rapporté que FGFI8 est le ligand physiologique du recepteur FGFR3. Donc, nous avons emis l'hypothése que FGF18 et FGFR3 ont un rôle important dans l'OD.

Afin de tester cette hypothèse, nous avons étudié l'OD chez des souris ayant une déficience en FGFR3 (les souris ayant une déficience en FGF18 ne sont pas viables). Un appareillage miniaturisé pour l'OD a été appliqué au tibia, suivi par une ostéotomie. La distraction a commencé après une période de latence de 5 jours, à un taux de 0,2 mm/12 heures pour 14 jours.

Des échantillons ont été préléves chez les animaux contrôles et les animaux mutants: fin de la distraction (17 jours après la chirurgie), mi-consolidation (34 jours après la chirurgie) et fin de consolidation (51 jours après la chirurgie).

Ces échantillons ont été analysés par rayons X, DEXA, microCT, histologie, test biochimique et PCR en temps réel.

Nos résultats ont révélé que les souris ayant une déficience en FGFR3 démontrent une accélération de la formation osseuse en comparaison avec les animaux sauvages. Les paramètres mesurés ont révélé une amélioration de la densité minérale, du contenu minéral, et du nombre de trabécules dans les échantillons mutants. L'os récemment régénéré s'est consolidé plus vite chez les mutants et l'os était de meilleure qualité, comme le révèlent les tests biomécaniques dans lesquels plus de force était necessaire pour casser l'os mutant.

L'expression génique de marqueurs de la chondrogénèse était augmenteé, suggérant que les souris mutantes empruntaient la voie de l'ossification endochondrale pendant l'OD. Tous les résultats se sont avérés statistiquement significatifs.

Ces résultats montrent que la signalisation en aval de FGFR3 agit pour diminuer la formation de l'os pendant OD. Par conséquent, bloquer cette signalisation pourrait mener à une accélération de la formation de l'os dans OD. Cela peut avoir des implications cliniques importantes pour les tentatives d'améliorer les résultats fonctionnels de l'OD en diminuant la durée pendant laquelle le fixateur externe doit être gardé.

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1.0 Introduction

1.1 Distraction Osteogenesis

Distraction Osteogenesis (DO) is a limb-lengthening procedure which utilizes the body's own tissue engineering to generate new bone as a result of slowly separating two viable osteomized bone edges. DO involves an external apparatus, now known as the Ilizarov apparatus, which is fixed on a limb (arm or leg) during a surgical operation. After the apparatus is positioned in the desired location, an osteotomy or dissection of the bone is performed at the site that requires lengthening. The apparatus is fashioned as to be gradually distracted or pulled apart hence separating the bone and creating a distraction gap within the limb at the site of the osteotomy (Figure 1). This unnatural and stressful procedure influences bone formation at the molecular level. The process of DO triggers several osteogenic factors and induces new bone formation within the distraction gap. In addition to bone regeneration, DO causes a simultaneous expansion of soft tissue (blood vessels, nerves, skin, muscles, ligaments and periosteum), which is known as distraction histiogenesis.

1.1.1 Historical Background

The Ilizarov apparatus was named after Dr. Gavriil Ilizarov, a Russian orthopedic surgeon, whose contributions to the improvement of DO led to the development of the new principles of limb lengthening. Starting in the 1960's, Ilizarov treated thousands of patients in his native Siberian community. He noticed that after a delay of a few days after osteotomy, bone and soft tissue would regenerate when subjected to the mechanical tension of slow, gradual distraction. Mechanical tension is one of the key signals required for embryological bone formation and growth during morphogenesis. Since then, DO has

received widespread recognition as being the preferred method for limb-lengthening, correction of bone deformities and craniofacial reconstruction.

Modern DO evolved from the work of Ilizarov in his modest clinic in Siberia, where he conceptualized the basis of this new reconstructive method.

The DO procedure involves formation of surgical fractures with minimal tissue damage and manipulation of the fragments with rods supported by an external frame (Figure 1). The surgery combines minimally invasive surgery and application of a unique device which can manipulate bones and maintain fixation at the same time. This apparatus evolved into the mechanism of the Ilizarov ring fixator used by orthopedic surgeons today. Ilizarov discovered that combining minimal tissue dissection, slow transport of skeletal fragments, almost rigid fixation, and maintenance of skeletal loading, resulted in formation of both new bone and adjacent soft tissue. Accomplishing a stable, functional rehabilitated bone and soft tissue represents a major achievement in reconstruction surgery.

1.1.2 Phases of Distraction Osteogenesis

There are numerous clinical applications for DO such as filling of segmental bone defects, treatment of angular deformities and fracture non-unions. The treatment procedure following the surgical operation when the osteotomy is performed consists of three phases (Frost, 1989). The first phase is the latency phase which lasts 5 to 7 days; no distraction is carried out during this time, the patient is left to recover from the surgery and for the callus to reduce the swelling from the trauma of the operation (Frost, 1989).

The second phase is the lengthening or distraction phase which is continued until the distraction reaches the desired length. After a series of experiments conducted on dogs, Ilizarov found that the optimal amount of daily lengthening is 1 mm/day; hence 5 cm of lengthening will require 50 days of distraction. Unfortunately, the distraction rate can not exceed 1mm/day because this causes soft tissue damage within the nerves, muscles and vessels; and if the rate of distraction is slower than 0.5mm/day then the bone consolidates prematurely. The third and most crucial phase of the lengthening process is the consolidation phase. During this phase of DO, the external fixator has to be left in place following distraction, until the newly formed bone within the distracted gap becomes biomechanically solid enough to withstand the stresses of mechanical usage.

The consolidation phase is very lengthy; almost 1 month for every 1cm lengthened. For example a lengthening of 5 cm will require 5 months of consolidation in which the fixator needs to be kept on the patient. During this phase the new bone matures and is remodeled until it becomes indistinguishable from the original bone (Aronson et al., 1989; Hamdy et al., 1997; McKibbin, 1978; Welch et al., 1998).

1.1.3 Clinical applications of DO

DO has widespread clinical application within the orthopedic field such as treating bone defects following trauma, osteomyelitis, resections of malignant bone tumors and fracture non-unions. However, the clinical applications of DO are not only limited to orthopedic problems. DO is widely used in the field of reconstructive surgery, particularly in cases of craniofacial anomalies where mandibular, maxillary and alveolar DO is applied. Thus, people who suffer from diseases such as craniofacial microsomia, craniosyntoses, Nager's syndrome, Treacher Collins syndrome, Pierre Robin sequence,

temporomandibular joint post-traumatic or growth disturbances, and a variety of mandibular developmental disorders can benefit from DO using miniature, internal distraction devices (Cascone et al., 2005; Smith et al., 2005; Cohen et al., 1999).

DO is also used to treat posttraumatic segmental bone defects (PTSBDs) for patients who have had injuries of the extremities that may lead to long-term challenges. PTSBD patient usually exhibit bone loss due to an accident or trauma; usually these patients exhibit chronic nonunion and segmental bone defects, most common of the tibia. DO is one of several management alternatives used for PTSBD, which also include limb shortening, autologous nonvascularized cancellous bone graft, bone transport distraction osteogenesis, and free vascularized bone transfer (DeCoster et al., 2004). The Ilizarov technique was successful in treating PTSBDs, especially for large defects (up to 30 cm) in both adults and children. This technique requires skilled and highly specialized training and equipment, and like other DO procedures, it requires a long treatment duration which is often faced with frequent complications such as deformity and soft-tissue problems. However, despite these complications this form of DO is probably the most commonly applied procedure used for managing intermediate to large PTSBDs (DeCoster et al., 2004).

One of the most commonly used forms of DO is craniofacial distraction osteogenesis. Since 1992, following McCarthy's first ever application of DO principles to mandibular distraction on patients with hemifacial microsemia and Nager's syndrome (McCarthy et al. 1992), so appeared as a promising new method in reconstructive surgery in the human craniofacial skeleton (Swennen et al, 2001). However, craniofacial DO is plagued with many parameters that affect the treatment including: age, surgical

technique, distraction rate and rhythm, latency period, consolidation period and distraction device. Nevertheless, craniofacial DO is still used in many procedures which include: mandibular DO (mandibular lengthening and widening), alveolar reconstructions, bone transport of the jaw, maxillary DO, and mid-facial and/or cranial DO (Swennen et al, 2001).

1.1.4 Histological features

At the histological level, the features of DO are very similar to those of fracture healing. DO could be considered as a fracture where the callus is subjected to a controlled and specific mechanical environment. In order for new bone to be formed within the distracted gap, the bone needs adequate blood supply, rigid fixation, and a precise rate and rhythm of distraction. After the osteotomy is completed, a hematoma is formed. As distraction proceeds, the hematoma is organized into fibrous and fibro cartilaginous tissue in a longitudinal pattern along the direction of distraction. New bone starts to form as early as two weeks after the end of distraction. The new bone is formed from the periosteum, from the cortex at the site of the osteotomy and from the spongiosa, and proceeds from the osteotomy and cuts towards the center, always forming a fibrous, radiolucent interzone between the two advancing edges of the mineralization front. The newly formed bone is oriented parallel to the distraction force (Shearer et al., 1992; Aronson et al., 1990; Aronson et al. 1997).

Several histological studies have revealed the presence of many blood vessels between the newly formed columns of bone during distraction osteogenesis. However, if the osteotomy is poorly made, the fixator is unstable, or the distraction rate is too fast,

these factors may have a negative effect on vascularization and local blood supply. Hence, it may disturb the regeneration of tissues and cause delayed bone healing. "Sufficient evidence has emphasized the contributions of both the periosteum and local neovascularity to bone formation during distraction" (Choi et al., 2000).

Previous studies have suggested that either vascular endothelial cells or pericytes differentiate into osteoblasts or precursor cells; therefore it is possible that vessels could directly participate in new bone formation during DO (Choi et al., 2000).

However, the origin of the new blood supply for regenerating bone tissue still remains controversial. Choi et al.'s (2000) study concluded that newly regenerating bone tissue in the distraction gap receives blood supply from both the periosteum and the medullary canal. However this result contradicts the observations of Mosheiff et al. (1996), who observed that medullary vessels did not cross the osteotomy site during tibial lengthening by DO (Mosheiff et al., 1996). They recorded that important new vessels sprouted and penetrated new bone in the distraction gap from a large longitudinal artery located in the muscle bed at the posterior side of the tibia (Mosheiff et al., 1996).

Choi et al. (2000) observed a close temporal and spatial relationship between newly regenerated bone formation and vascular proliferation of the periosteum and medullary canal during DO (Choi et al., 2000). Newly formed osteogenic tissue progressively filled the distraction gap according to the reconstitution of the periosteal vascular network, and then it was followed by the interconnection of the medullary circulation (Choi et al., 2000).

Despite these controversies, it remains obvious that adequate neovascularization is essential for bone formation during DO. As such, the contribution of angiogenic factors such as VEGF (Choi et al., 2000), should not be neglected.

1.1.5 Type of bone formation in DO

Bone formation is an intricately balanced development that is coordinated by a network of signaling pathways.

There are two types of normal bone formation: 1-Intramembranous bone formation and 2- endochondral bone formation (Figure 2). The major difference between the two types is the presence or absence of an intermediate cartilaginous phase (Zelzer et al., 2003). During intramembranous bone formation mesenchymal precursor cells proliferate and differentiate directly into osteoblasts that produce a collagen matrix, called osteoid. The osteoblasts then begin to mineralize the osteoid, forming a primary immature bone tissue called woven bone, which slowly matures into lamellar bone (Gilbert et al., 2003).

On the other hand, endochondral bone formation involves mesenchymal cells that condense and differentiate into chondrocytes that secrete an avascular cartilaginous matrix, containing type II (Col2) and type X collagen (Col10). The hypertrophic, differentiated chondrocytes proceed to mineralize the surrounding cartilaginous matrix. This calcified matrix is then infiltrated by vascular tissue and the process of angiogenesis brings osteoclast precursors to the new bone. The osteoclasts excavate the hematopoietc bone marrow cavity while new osteoblasts are recruited to replace the cartilage scaffold with bone matrix.

Which of these processes contributes to new bone formation during DO? This remains a very controversial question, for there are several studies that suggest conflicting results. DO could either involve intramembranous or endochondral ossification, or even sometimes both at the same time. However, we should always keep in mind the factors that influence DO; such as stability of the fixator, timing, rate of distraction and species-related differences might determine the involvement of endochondral and intramembranous bone formation (Kusec et al., 2003). One study conducted by Kusec et al. (2003) on dogs, revealed that intramembranous ossification predominated the healing region occupying most of the distracted area. Furthermore, earlier studies conducted by Sato et al. (1999) on a rat model of DO suggest that the induction of ossification via DO produces an environment that suppresses the formation of cartilage (Sato et al., 1999). Hence, they believe that cartilage was progressively resorbed from the ends of the osteotomized bone and that new bone was formed directly by intramembranous ossification. On the other hand, Li et al. (1999) revealed different results in a rabbit model of DO (Li et al., 1999). There results showed cartilage remnants in the new bone trabeculae, where chondrocytes were buried into the bone matrix. They suggest that a mixture of endochondral and intramembranous ossification occurs during DO, and that intramembranous ossification is not a unique characteristic of distraction. Li et al. (1999) documented the presence of an overlapping cartilage-bone phenotype in the cells of the distracted region (Li et al., 1999). They also discovered that Collagen I mRNA and Collagen II protein were found together within the cartilage-bone overlapping regions. They explain the possibility of direct transformation of hypertrophic

chondrocytes into osteoblasts during DO, and propose the hypothesis that hypertrophic chondrocytes may transdifferentiate into bone cells.

The molecular mechanism of bone formation during DO is complicated and controversial, involving several different molecular pathways. It is the mechanical tension-stress and strain induced by the unnatural process of distraction that influences the expression of certain molecules. BMP-2 and BMP-4 expression is enhanced during DO, and is credited for most of the molecular mechanism of bone formation during DO (Li et al., 1999; Sato et al., 1999). Furthermore, the presence of TGF-β, IGF-1 and FGF-1 in the distraction callus may explain the proliferation of osteoblasts and their formation from precursor mesenchymal cells.

Several studies have shown the importance of a systemic regulatory biomechanical pathway during DO within a human population. This research revealed that bFGF (basic Fibroblast Growth Factor) was significantly increased in humans that underwent DO during the distraction and consolidation phases as compared with a control, non-distracted osteotomy fracture healing group (Weiss et al., 2002). Moreover, levels of Transforming Growth Factor-β (TGF-β1), Insulin Growth Factor-I (IGF-I), and human growth hormone (hGH) were significantly increased in the DO group especially during the distraction period as compared to the non-DO group, indicating a key regulatory function in mechanotransduction and angiogenesis during callus distraction (Weiss et al., 2002).

Immunohistochemical data collected from human distracted calluses reveal similar results. Cells stained positive for both TGF-β and IGF-I could be found in every region of the distraction callus (Eingartner et al., 1999). TGF-β induces osteoblast

differentiation and proliferation, as well as stimulates the appearance of extra cellular matrix (ECM) proteins (Baylink et al., 1993; Bonewald et al., 1990). TGF-β was found in osteoblasts and fibroblasts with the distraction callus within areas of rapid cellular proliferation (Eingartner et al., 1999). Mechanical stress during DO stimulates the expression of TGF-β during callus distraction in human patients (Holbein et al., 1995).

IGF-I is an important cytokine for several biological processes, and is found in chondroblasts during fracture repair, especially during early callus creation (Bourque et al., 1993; Schmid, 1995). Immunohistochemically IGF-I was observed in fibroblasts and osteoblasts in a distinct area of bone formation during DO, suggesting its important role during DO for stimulating cellular proliferation and inducing ECM formation (Eingartner et al., 1999).

Another study showed that matrix synthesis coincides with increased TGF-β expression during DO and increased Osteocalcin (Ocn) production coincides with bone matrix mineralization (Mehrara et al., 1999).

Recent research with various animal models of DO has shown endochondral ossification occurs significantly during DO (Hamdy et al., 1997). In addition, mice deficient for one allele of the Runx2/Cbfa1 transcription factor (which is crucial for intramembranous bone formation), underwent DO normally (Isefuku et al., 2004). These results suggest that the endochondral pathway of bone formation is important for regeneration and suggest that molecules expressed by the chondrocytes of the cartilaginous callus could significantly modulate bone formation during the process of DO.

1.1.6 Clinical problems of DO

DO requires a long time for the newly formed bone to consolidate. The external fixator has to remain in place for an extended period of time, which may lead to several side effects, medical and socio-economic problems for the patient. Some of the side effects of having an external fixator on a patient for long periods of time include pin tract infections, osteomyelitis, soft tissue swelling, compliance pain as well as psychological problems (Paley, 1990). Therefore, accelerating the process of distraction and consolidation by increasing the rate and quality of bone formation would shorten the length of time the external fixator has to be kept on the patient, hence improving the comfort of this treatment.

1.1.7 Attempts to accelerate bone formation during DO

There have been several attempts and approaches at accelerating osteogenesis during DO, including mechanical stimulus, low intensity ultrasound, injecting growth hormone, and bisphosphonate treatment. Some of these studies have showed promising results, but none have yet been shown to be effective in humans.

Low intensity pulsed ultrasound to accelerate bone formation

One of the most successful attempts researched for acceleration of bone formation is through ultrasound. Low-intensity pulsed ultrasound (LIPUS) has recently shown to be an extremely effective biophysical therapy in the process of fracture-repair (Heckman et al., 1994; Kristiansen et al., 1997; Leung et al., 2004; Pilla et al., 1990). Ultrasound has been applied in different cases for management of fractures, delayed unions, non-unions and distraction osteogenesis (Chan et al., 2006). The application of ultrasound in DO was

used as a mean to accelerate the ossification and maturation process in animal models (Chan et al., 2006; Mayr et al., 2001; Shimazaki et al., 2000), and also in patients (El-Mowafi et al., 2005; Gebauer et al., 2005). This process involves intramembranous bone formation as the dominant type of tissue formation, while endochondral ossification usually is less essential. The effects of ultrasound in DO have been studied in several animal models. In a series of rabbit studies where a distracted callus was treated with low-intensity impulse ultrasound, a larger, hard callus area as well as diminished value of fibrous tissue was found in ultrasound-treated bones (Shimazaki et al., 2000). However, these studies also showed no difference in bone mineral density or mechanical strength between the treated and untreated calluses. In another sheep study, endochondral bone formation was increased in the ultra-sound treated calluses, and biomechanical tests revealed an increase in stiffness of the bone at the osteotomy site (Claes et al., 2005). A recent study has shown that in rabbits, pulsed ultrasound stimulates bone formation most effectively during the distraction or elongation phase (Sakurakichi et al. 2004). Further research is needed to show whether additional ultrasound treatment during the distraction phase can further shorten the time necessary for callus consolidation. However, it is still unclear which mechanism translates the mechanical forces generated by the low-intensity pulsed ultrasound into new bone formation (Claes et al., 2005).

Mechanical Stimulus to accelerate bone formation during DO

Over the past several years many in vitro and in vivo studies have shown that proliferation and differentiation of bone derived cells is dependent upon mechanical loading (Brighton et al., 1991; Buckley et al., 1988; Harter et al., 1995; Neidlinger et al., 1994; O'Connor et al., 1982).

Meyer and associates (2001) have performed several experiments on rabbit models of DO in order to study the relationship between tension-stress and chondrogenesis during DO (Meyer et al., 2001). It has been an accepted fact that mechanical load influences the cellular microenvironment of bone tissue, and affects tissue differentiation, regulation and maturation (Meyer et al., 2001). This research investigates the effects of uniaxial strains on tissue during DO on a rabbit model of mandibular lengthening. Their results show that a strain of intermediate magnitude resulted in a callus formation with typical features of both endochondral and intramembranous bone formation. At lower magnitudes of strain, intramembranous bone formation predominated, however on higher than normal strain or hyper physiological strain revealed soft collagenous tissue within the distraction gap. Further research into the expression markers during DO revealed that the magnitude of mechanical stress clearly influenced the phenotypic differentiation of cells in the distracted gap tissue. Furthermore, studies of the molecular mechanisms of strain-related bone formation identifies mechanical strain as being a determining factor for chondrogenesis during DO (Engel et al., 1987; Gentili et al., 1993; Gundberg et al., 1984; Holland et al., 1987; Nakase et al., 1994).

Thus, it is the external mechanical stimulus applied during the unnatural process of DO that determines the differentiation process between chondrocytes and osteoblasts; and ultimately the type of bone formation involved during DO which is directly related to the magnitude of the strain applied (Meyer et al., 2001).

Bisphosphonates

Ample research has been done on the possible acceleration of bone formation during DO with bisphosphonates treatment after its significant success in treating osteoporosis. One such study conducted by Takahashi and associates (2006) studies the effects of bisphosphonates and its properties of inhibiting bone formation in a rabbit model of DO. Their results revealed that not only bone formation but bone resorption as well are highly activated in the regenerated bone in DO (Takahashi et al., 2006). Earlier studies on the aspects of DO have revealed that bone regeneration does play a role in this process; in the rabbit model it was shown that there are three radiologically characteristic zones. The zones include a central radiolucent zone, adjacent sclerotic zones and subsequent osteopenic zones. The sclerotic zones eventually migrate towards the central radiolucent zone according to the distraction process. However, it is the osteopenic zones that replace the sclerotic zones that are undoubtedly caused by bone resorption, and hence play a role during DO (Kojimoto et al., 1988).These zones correspond to the inflammatory, repair and remodeling phases in fracture healing. Hence, the newly formed bone has a very short life span and is rapidly absorbed resulting in osteopenic areas.

Bisphosphonates are useful therapeutic agents used to treat diseases with high bone turnover (Osteoporosis, Paget's disease, etc) (Khosla et al., 2005). Nitrogen-containing bisphosphonates (N-BPs) are very efficient anti-catabolic agents used to combat bone resorption (Khosla et al., 2005). Studies have shown that applying N-BPs during DO leads to increased bone formation in the area of the newly regenerated bone tissue (Smith et al., 2004; Little et al., 2001; Little et al., 2001). The study conducted by

Takahashi et al. (2006) further verifies the previous findings, for they used varying concentrations of injected N-BPs and noted significant modification of the regenerated bone and increased mechanical properties (Takahashi et al., 2006). However, these findings were conducted in rabbits, and further research needs to be seen in clinical cases in order to regulate the N-BP dosage and the time of DO (Takahashi et al., 2006).

Thus, with very little convincing evidence and research as to which is the ideal method for accelerating bone formation during DO, the most sensible pathway to follow is to study DO on a molecular and cellular level. The area most studied for bone regeneration and bone repair is the effects of molecular growth factors during the healing and remodeling process. Several growth factors and molecular pathways have been identified in bone development. We will discuss a few of them in our research and focus on the ones that we believe possess the most potential.

Growth Factors

Recently, there have been several attempts to try and accelerate the consolidation phase of DO using osteogenic growth factors. The growth factors include TGF-β (Transforming Growth Factor Beta), IGF (Insulin Growth factor), FGFs (Fibroblast Growth Factors) and BMPs (Bone Morphogenic Proteins). These growth factors have been extensively studied during post-natal development, embryogenesis and fracture healing; very little is known about their respective roles and pathways during the process of DO and its phases (Yeung et al., 2001). There has been no reported data concerning the expression patterns of FGF18 or FGFR3 during DO prior to our own studies.

Using a rabbit model of DO in previous studies, our team analyzed the expression of Bone Morphogenic proteins BMP-2, -4, and -7, and the expression of the BMP receptor proteins during distraction osteogenesis (Mandu-Hrit et al., 2006).

It was found that the maximum expression of BMP-2, -4, and -7 was during the distraction phase of DO, however their expression slowly declined and fell off during the consolidation phase. The peak point for the expression of BMP receptors was marked during the second week of distraction, yet the expression level gradually retreated towards the end of distraction phase and during the consolidation phase (Mandu-Hrit et al., 2006).

In another series of experiments, we investigated the expression of bone morphogenetic protein (BMP)-signaling Smads in distraction osteogenesis (DO). The expression of the BMP effect on Smad proteins 1, 4, 5, 6, 7 and 8 and Smad ubiquitin regulatory factors (Smurfs) 1 and 2 was analyzed in the distracted region using immunohistochemistry (Haque et al., 2006).

The expression of receptor-regulated Smads (R-Smads) 1, 5 and 8 revealed a significant increase during the distraction phase, followed by a steady decrease during the consolidation phase. Smad 4 showed significant expression during both distraction and the beginning of the consolidation phase. Smad 6 and Smad 7 were greatly expressed during the consolidation phase. Smurf 1 and 2 showed maximum expression at the end of the distraction period. The expression of all proteins was mostly detected in chondrocyte and fibroblast-like cells. The expression pattern of R-Smads correlates with the previously collected data for the expression pattern of BMPs 2, 4, 7 and their receptors.

These results suggest that BMPs and Smad proteins may play a critical role in the signaling pathways that relate the mechanical forces created by distraction osteogenesis to the molecular and biological responses (Mandu-Hrit et al., 2006).

Following the results of this research we went further to investigate the effect of accelerating bone formation in DO by the administration of exogenous growth factors early during the distraction phase of the lengthening process. The manipulation of the BMP signaling pathway was done by establishing a technique where recombinant BMP-7 (also referred to as osteogenic protein 1 or OP-1) is injected at the beginning of the distraction phase in a rabbit model of DO. The samples were collected and analyzed; the results revealed that as compared to the control, a two-fold increase in bone volume was clear for treated groups at three weeks post injection. These results suggested that early injection of OP-1 during distraction can accelerate bone formation by the activation of numerous pathways (Mandu-Hrit et al., 2005).

This study provides benefits for strategies to advance bone regeneration in DO. Despite this major finding, it is still very problematic to attempt any such experiments in humans. The dose that needs to be injected for clinical effect is almost a thousand time the total amount of BMPs in the human body, and hence represent a major concern. Thus there remains to identify new methods to accelerate bone formation during DO (Mandu-Hrit et al., 2005).

The investigation went on using the rabbit model of DO; samples were collected from the various phases of DO and the samples were analyzed for immunohistochemistry staining against molecules from the FGF family. In fact, FGF18 was observed to be the

only FGF family ligand and the only osteogenic growth factor that showed increased levels of expression during both distraction and consolidation phases. FGFR3 also showed increased expression during the distraction and consolidation phases, suggesting that it may transduce FGF18 signals during bone formation in DO (Mandu-Hrit et al., 2006).

1.2 Fibroblast Growth Factors (FGFs)

FGFs include a family of structurally and functionally related polypeptide growth factors that act in a paracrine and autocrine manner and exhibit a particular characteristic affinity for heparan (Gospodarowicz et al., 1984; Shing et al., 1984). This characteristic is crucial for interactions with signal-transducing receptors (Ornitz et al., 2000). The FGF family has been shown to induce angiogenesis (Gospodarowicz et al., 1979; Folkman et al., 1987; Klagsburn et al., 1991), vasculogenesis (Krah et al., 1994), wound healing and tissue repair (Davidson et al., 1985), which are particularly essential during the process of DO. In vertebrates, there are 22 members of the FGF family, that range in molecular mass from 17 to 34 kDa and share 13-71% amino acid identity which makes them highly conserved between species (Ornitz et al., 2001). Most FGFs share an internal core region of similarity, with 28 highly conserved and six identical amino-acid residues (Ornitz, 2000). Ten of these highly conserved residues interact with the FGF receptor (FGFR) (Plotnikov et al., 2000). The expression patterns and signaling pathways of FGFs propose that they have essential roles in development, and that the reliability of these signaling pathways requires a particularly tight regulation of FGF activity and receptor specificity (Ornitz et al., 2001). Fibroblast Growth Factors (FGF) and their respective receptors (FGFR 1, 2, 3 and 4) play a significant role in bone development and repair. FGF

receptors are a second family of polypeptides that mediate the multifarious activities of FGFs. FGFRs are a complex family of cognate signal-transducing receptors. The FGFs are heparin-binding proteins, and their interactions with cell-surface associated heparan sulfate proteoglycans have been shown to be essential for FGF signal transduction.

There have been studies conducted surrounding the biochemical properties and functions of FGFs that have focused on the specificity of interactions between FGFs and their receptors. Several factors that affect the stability of FGFs have been identified, and the composition and mechanism of the active FGF/FGFR signaling complex has been extensively studied. The FGFR tyrosine kinase receptors contain two or three immunoglobulin-like domains and a heparin-binding sequence (Lee et al., 1989; Johnson et al., 1990; McKeehan et al., 1998). The FGFR gene undergoes alternative mRNA splicing in order to specify the sequence of the carboxy-terminal half of immunoglobulin-domain that ultimately results in an isoform of the FGFR (Chellaiah et al., 1994; Naski et al., 1998; Yan et al., 1993). "This alternative-splicing event is regulated in a tissue-specific manner and dramatically affects ligand-receptor binding specificity" (Ornitz, et al., 2001). Therefore, since FGFs within the same subfamily have related receptor-binding properties and overlapping patterns of expression, functional redundancy is likely to occur (Ornitz et al., 2001).

Fibroblast growth factors (FGFs) and their receptors are involved in every single step of skeletal formation, from the first outgrowth of the limb to the endochondral lengthening of long bones and intramembranous bones of the skull. Mice and humans each have 22 FGFs in their respective genomes. FGF-7,-8,-17 and -18 expressions are

restricted to the perichondrium surrounding the growth plate. In the growing skull, expression of FGF-18 and -20 are found in differentiating osteoblasts. FGFR3 is expressed by proliferating chondrocytes in long bones, and at the osteoblastic front of sutures of the growing intramembranous bones of the skull (O'Rahilly et al. 1972; Kaufman, 1994).

There are several FGFs expressed in bone development that is why it is very difficult to identify the specific FGF ligand that binds to and activates FGFR3. However, it was observed that FGF18 deficient mice have a growth plate phenotype similar to that of the FGFR3 knockout mice. This suggests that the primary activator of FGFR3 in chondrocytes is FGF18, which is synthesized in the perichondrium surrounding the growth plate (Rodriguez-Vazquez et al. 1997; Ornitz, et al., 2001).

1.2.1 FGF18:

Some FGF ligands are only present during embryonic development such as FGF 3, 4, 8, 15, 17 and 19; many other FGF ligands such as FGF 1, 2, 9 and 18 are found and expressed throughout the lifespan (Chen et al., 2005). FGF18 has recently been under investigation since it has been shown to play a key role in skeletal growth and development along with its sister ligands FGF 1 and 2. Shimoaka et al. (2002), discussed FGF18's important mitogenic actions on osteoblasts and chondrocytes as being similar to those of FGF 2 and thus may therefore compensate the role of FGF 2 during skeletal development (Shimoaka et al., 2002). The same studies also revealed that FGF18 plays several roles in morphogenesis, angiogenesis and the development of a range of diverse cells.

Structurally, FGF18 is similar to FGF8 and 17, in humans it is located on chromosome 5q34 and it encodes a 207 amino acid protein sequence. FGF18 contains a 26 amino acid hydrophobic terminus that may act as a secretary signal peptide (Hu et al, 1998).

Several in vivo and in vitro studies have shown the requirement of FGF18 during bone development. FGF18 is involved in chondrogenesis in the growth plate, and osteogenesis in the cortical and trabecular bone (Liu et al., 2002). Research conducted on the chondrogenic defects of FGF18 deficient mouse embryos has shown increased chondrocyte proliferation and increased hypertrophic zones in the growth plate (Liu et al., 2002). The osteogenic defects of these embryos are delayed ossification, defects in joint development, and delayed calvarial suture closure (Moore et al., 2005; Ohbayashi et al., 2002). FGF18 knock-out mice experience premature death and do not survive to adult age. Neonatal FGF18 deficient mice all have skeletal abnormalities, including under developed fibula and curved tibia. The ribs were so deformed that the thoracic cage was significantly reduced in cavity volume and hence is the main suspected reason for early premature death (Liu et al., 2002).

FGF18 deficient mice have delayed ossification and decreased expression of osteopontin and osteoclacin which are the main osteogenic markers. These results were not observed in FGFR3-ablated mice, suggesting that FGF18 may also function through mediators other than FGFR3 during osteogenesis (Liu et al, 2002). Furthermore, the study mentioned above, conducted on Stat1 (which is an important factor in the signaling pathway for intramembranous bone formation) showed that Stat1 deficient mice had higher bone density and a decreased expression of FGFR3, but a high expression of

FGF18. Thus, the study suggested that FGF18 is responsible for the higher bone mass observed and thus may be an important modulator for both endochondral and intramembranous bone formation in adult mice. In addition, the decreased FGFR3 expression consequently implies that FGF18 could possibly function independently of FGFR3 (Xiao et al., 2004).

The function of FGF ligands is reliant on the spatial and temporal expression of their respective FGFR or receptors (Marie, 2003). Since there are only 4 known FGF receptors, from 22 other FGF family members, ligand binding is not uniquely specific and one receptor can be activated by several FGFs (Dailey et al, 2003). FGF signaling is mediated by FGFRs and other intermediary molecules. FGF binding to the receptors requires the presence of heparin sulfate proteoglycans (HSPG), and signaling occurs via tyrosine phosphorylation. HSPG is a structurally complex molecule that could either inhibit or activate different FGFs; they form a trimolecular complex with the FGF and FGF receptor on the plasma membrane which is important for signaling. It has been reported that FGF18 binds with high affinity to FGFR3. FGF18 activates the III c-type isoform of FGFR3, which forms through alternative splicing, and is a positive regulator of bone formation (Chen et al., 2005; Moore et al., 2005). Several studies conclude that FGF18 is a ligand for FGFR3 (Ornitz et al., 2002). In developing bone, FGFR3 is expressed in proliferating chondrocytes, and its activation in fact inhibits proliferation and differentiation of growth plate chondrocytes (Naski et al., 1998). Several studies indicate that FGF18 positively regulates osteogenesis and negatively regulates chondrogenesis via FGFR3 (Ohbayashi et al., 2002).

However, FGF18 ablated mice have a more severe phenotype than mice lacking FGFR3 (Liu et al., 2002) The FGF18 deficient mice experience delayed bone formation which causes their premature death. This in part may be due to the direct signaling to osteoblasts or hypertrophic chondrocytes or to a delay in vascular invasion of the growth plate. The most rational explanation is that FGF18 is signaling bi-directionally, to osteoblasts in the endosteum and primary spongiosa, and to periosteal mesenchyme. These signals to periosteal mesenchyme could either directly or indirectly regulate vasculogenesis. In addition, since recent data has revealed the expression of FGFR3 in mature osteoblasts (Xiao et al., 2004) it is therefore still uncertain which FGF receptor (FGFR1, FGFR2 or FGFR3) is actually responding to FGF18 expression in osteoblasts.

Several studies have revealed a similarity in phenotype between FGF18 and FGFR3 knock-out mice during embryonic development of long bones. Furthermore, several studies analyzing the growth plates of mice lacking FGF18 at the histological level have shown similar results to those of mice lacking FGFR3. These results revealed that both knockout mice strains exhibit an up regulation of Indian hedge hog (Ihh) expression and increased chondrocyte proliferation. These phenotypic resemblances suggest that FGF18 is a physiological ligand for FGFR3 in chondrocytes (Liu et al., 2002; Ohbayashi et al., 2002). Furthermore, in vitro studies show that FGF18 can activate FGFR3 and stimulate the proliferation of cultured articular chondrocytes (Ellsworth et al., 2002).

1.2.2 FGFR3:

Achondroplasia is an autosomal dominant inherited disease that is related to a mutation in the fibroblast growth factor receptor-3 (FGFR3) gene encoding one member of the FGFR subfamily of tyrosine kinase receptors. This mutation results in constitutive activation of the receptor (activating mutation). Several biochemical studies of FGFR3 combined with experiments in knock-out mice have demonstrated that FGFR3 is a negative regulator of chondrocyte proliferation and differentiation in the growth plate (Richette et al., 2007). These studies have prompted our group to investigate the expression of FGFR3 during DO in the rabbit model. Our data revealed that FGFR3 is continuously expressed throughout the distraction and consolidation phases, suggesting that it plays an integral role in new bone formation during DO (Mandu-Hrit et al., 2006).

FGFR3 knock-out mice exhibit prolonged growth of long bones and vertebrae. FGFR3 deficient mice also exhibit kyphosis of the neck vertebrae as well as the long bones. Although, FGF18 and FGFR3 knock-out mice exhibit similar phenotypes, FGFR18-ablated mice have considerably smaller long bones in comparison to the wild-type mice, more so than FGFR3 deficient mice (Ohbayashi et al., 2002). Research has shown that FGFR3 knock-out mice, in addition to expressing skeletal overgrowth are osteopenic due to reduced cortical bone thickness and defective trabecular bone mineralization. Valverde-Franco et al. (2003) further showed that young adult mice (4 months) lacking functional FGFR3 were osteopenic and developed severe osteomalacia. These abnormalities were not due to PTH or vitamin D deficiencies, which further implicate the importance of FGFR3 signaling in osteoblast biology and its potential responsibility for adult skeletal disorders (Valverde-Franco et al., 2003).

1.2.3 FGF Signaling in Osteoblasts:

Osteoblasts are also known as the bone-forming cells are responsible for bone formation through-out an organism's life. The rate of this bone formation is dependent on the differentiation and maturation of osteoprogenitor cells into functional osteoblasts (Marie 2003; Triffitt, 2001). Osteoblast differentiation genes are regulated by several signaling factors, including FGFs that control the proliferation and differentiation of numerous cell types (Jaye et al., 1992; Basilico et al., 1992).

FGF signaling through their receptors leads to FGFR dimerization, and phosphorylation of specific tyrosine residues that activate several signal transduction pathways (Szebenyi et al., 1999). Some of these FGF signaling pathways regulating gene expression in osteoblasts include mitogen-activated protein kinases (MAPK), extracellular signal-regulated kinases (Erks), p38 MAPK and protein kinase C (PKC) (Newberry et al., 1997; Mansukhani et al., 2000; Debiais et. al., 2001).

Erk activation mediates FGF-stimulated phosphorylation and transcriptional activity of Cbfa1/Runx2 (Xiao et al., 2002), which suggests the important function of the Erk pathway in FGF signaling during osteoblast differentiation (Kim et al., 2003).

Another pathway, the PKC pathway mediates FGF activity which is implicated by increased N-cadherin expression in osteoblasts (Debiais et al., 2001). The importance of the PKC pathway is also shown in the FGF/FGFR-stimulated expression of Cbfa/Runx2 (Kim et al., 2003). This finding is supported by other studies that suggest that PKC

activation lead to increased osteoblast differentiation and premature apoptosis induced by FGFR activation in osteoblasts (Marie et. al., 2002).

The literature implicitly places FGFR3 as an essential player in the process of new bone formation during distraction osteogenesis. We proceeded to continue studying its role during DO based on what we have observed in previous research.

1.2.4 Role of Stat1 in new bone formation

Signal transducers and activators of transcription (Stats) are a part of a critical pathway that is essential in FGF modulation of chondrocyte proliferation and endochondral bone formation during embryogenesis (Xiao et al., 2003).

The recent advances in molecular biology have provided a better understanding of intracellular signal transduction. FGFRs are involved in the activation of downstream pathways including the Ras-MAPK signaling pathway (Kanai et al., 1997), and the Stat pathway in chondrocytes (which is specifically carried out by the nuclear translocation of Stat1) (Su et al., 1997).In addition, Su et al., (1997) revealed that the nuclear translocation of Stat1 along with the expression of the cycline-dependent kinase inhibitor p21, prevent cell cycle progression and the growth arrest of chondrocytes (Su et al., 1997). Hence, Stat1 and p21 are the downstream molecules of FGFR3 intracellular signal transduction.

A fracture repair study revealed that both FGFR3 and Stat1 were strongly detected in prehypertrophic chondrocytes in the fracture callus. "This data shows that FGFR3-mediated Stat1 signaling plays a role during fracture repair" (Nakajima et al., 2003). Furthermore, apoptosis of hypertrophic chondrocytes is essential for the

replacement of cartilage with bone during the process of endochondral ossification. In fracture repair, Nakajima et al., (2003) revealed that apoptotic cells were localized to hypertrophic chondrocytes in which FGFR3 and Stat1 expression were observed together. Thus, FGFR3-mediated Stat1 signaling pathway induces apoptosis of hypertrophic chondrocytes, regulates chondrogenesis and promotes endochondral ossification thus contributing to fracture healing and repair (Nakajima et al., 2003).

Seven mammalian Stats that have been recently identified have shown a critical role in the biological response necessary to modulate cellular proliferation and differentiation (Kisseleva et al., 2002). In osteoblasts, activation of Stat3 and Stat1 by Interleukin (IL)-6-type cytokines promoted differentiation and prevented apoptosis (Bellido et al., 1997). Several studies have indicated a role for Stat1 in mouse dwarfism models and developmental events involving FGF/FGFR3-mediated endochondral ossification and chondrocyte differentiation (Li et al., 1999; Lievens et al., 2003; Sahni et al., 2001). In a recent study conducted by Xiao et al. (2003), the analysis of Stat1 deficient adult mice revealed increased bone mass and bone mineral content (BMC) as compared to the wild-type littermates. Further studies on the mechanisms modulating osteoblast function in the Stat1 deficient mice revealed a significant decrease in mRNA expression of FGFR3. Interestingly, FGF18 labeling in Stat1 deficient mice showed significant increase in hypertrophic chondrocytes at the growth plate level, as well as within cortical bone and trabecular bone surface osteoblasts (Xiao et al., 2003). These results suggest that FGFR3 signaling through Stat1 negatively regulates osteoblast function. Furthermore, Stat1 may be involved in the regulation of both intramembranous and endochondral postnatal bone ossification. Moreover, Stat1 is a negative regulator for

the maintenance of bone mass and bone formation. However, it is the role of FGF18 as a ligand that is directly implicated in the increased bone mass of the Stat1 deficient mice (Xiao et al., 2003).

2.0 Rationale and Objective

The results in the rabbit model of DO reveal that FGFR3 is continuously expressed throughout the phases of the process suggesting it might be a central factor during new bone ossification. We set out to examine the putative role of FGFR3 signaling during DO.

The long-term objective of this research is to find a way to accelerate bone formation during DO, so that the external fixator could not be kept on a child for long periods of time.

Hypothesis

We hypothesize that FGFR3 is involved in the process of bone regeneration during distraction osteogenesis. The lack of FGFR3 should affect the type, amount, or quality of the bone formed during DO.

Specific Aim:

The aim of this research is to study new bone formation during DO in an FGFR3 ablated mouse model.

3.0 Materials and Methods:

3.1 Operative protocol

All protocols were approved by McGill University Animal Care Committee. Distraction Osteogenesis surgery, as described by Jill Helms (Tay et al., 1998), was performed on adult FGFR3 knock-out mice, comparing the mutants (FGFR3-/-) to their wild type (W.T.) littermates. The surgical procedure began by transfixing two 0.25 mm pins, 90^{o} apart in both the distal and proximal end of the tibia using a hand-held, variable-speed drill. As part of a tiny Ilizarov apparatus, the two rings were fastened and tightened to the pins and 3 screws were used to link both rings from the proximal and tibial ends. Once the Ilizarov apparatus or external fixator was mounted in place, a transverse osteotomy or bone cut was performed at the center of the tibia using a scalpel. The bones were horizontally aligned and then the periosteum and skin were carefully sutured using internal sutures. The mice were kept under anesthesia using isoflurane throughout the entire surgery, and given subcutaneous injections of bupernorphine (1mg/kg) for analgesia. The mice were able to bear weight immediately after surgery and were monitored three times daily during the first three days post-surgery, then daily until sacrifice.

The average operation time was 35 minutes. Following the surgery the mice were allowed to ambulate freely within 24 hours of the surgery.

Following a 5 day latency period where the external fixator was kept in place, distraction of the bone took place twice a day, once in the morning and one time in the afternoon, at a rate of 0.2mm/12hours for the duration of 12 days for a total distracted length of approximately 5mm.

The callus region of the tibia was collected by sacrificing the mice in groups of 8 at time intervals of: 17, 34 and 51 days post surgery, which correspond to the end of distraction phase, mid-consolidation and end of the consolidation phase, respectively. Mice were euthanized by cervical dislocation while under anaesthesia. The external fixator device was carefully removed and the tibia was dissected and processed accordingly for analysis.

3.2 Sample Preparation:

The regenerated bone was analyzed at the radiological, histological, and molecular level. The samples were analyzed using Faxitron X-ray, DEXA, μCt and the following parameters were measured: BMD (bone mineral density) in grams/cm^2, BMC (bone mineral content) in grams, BV/TV (bone volume/tissue volume) and Trabecular number. The samples collected for histological analysis were placed in 4% PFA (4g Para formaldehyde powder dissolved in 100 ml 1X PBS, PH 7.5) and kept at 4 °C until further processing. Samples collected for biomechanical testing were carefully wrapped in gauze and placed in a falcon tubes completely immersed in 1X PBS and kept at -20°C until processed for biomechanical properties. The samples collected for RNA extraction were placed in RNAlater (Ambion RNA isolation kit from Applied Biosystems; Branchburg, New Jersey) and kept at -20°C until further processing. The samples collected for protein extraction were snap frozen with liquid nitrogen and placed at -80°C until further processing.

3.3 Histology:

The samples collected for histology were processed after they had been measured by DEXA and μCT scanning. The samples were prepared by being treated with a series of dehydration and hydration steps in which they were submerged in increasing percentages of ethanol. The samples were then placed in a clear glass flat-bottom sample tube and treated with increasing percentages (0 to 4.5%) of methylmethacrylate [MMA, 1N NaOH (10g NaOH pellets dissolved in 250mL deionized water), Di-butyl phthalate (13 to 17% in a final solution of 100mL, depending on the required MMA percentage),and Benzoyl peroxide (1 to 4.5g in a final solution of 100mL, depending on the percentage of MMA required)] The fixed tibias were embedded in 4.5% MMA. Sections of 5 μm were collected using a Leica microtome. The samples were placed at 55˚C over night to dry. The next day the samples were deplastified using Ethyl Glycol Monoethyl Ether Acetate (EGMA, F.W. 132.16). The samples were then dried and stained using Trichrome-Goldner for comparative histology. Trichrome-Goldner stain is a combination of 4 separate stains: Hematoxyline-Weigert [1% Hematoxyline powder in 95% ethanol, and Chloral-Ferric powder (5.8g) dissolved in 500mL deionized water with 1% HCL], Fushine- Ponceau stain [Fushine acid powder (0.167g) and Ponceau powder (0.667g) dissolved in 500mL deionized water with 1% Glacial acetic acid], Orange G [5% phophomolybdinum, and Orange G powder (10g) dissolved in 500mL deionized water], Vert -lumiere stain (0.03% Vert-lumiere powder (1.5g) dissolved in 500mL deionized water with 1% glacial acetic acid). Trichrome-Goldner stains mineralized tissue green. A microscope with an attached Olympus camera was used to capture and document histological images at 10, 20 and 40 fold magnification for analysis. Images

were analyzed to distinguish between mineralized and unmineralized tissue with the defined region of interest in the distraction gap.

3.4 Radiology, DEXA and Microcomputed Tomography (μCT)

Distracted tibias from the mice were dissected and fixed overnight in 4 % Para formaldehyde. Anterio-posterior and lateral X-ray views of the operated tibiae were taken using a Faxitron X-ray machine (Model: Faxitron ® MX-20).

Dual Energy X-ray Absortiometry, or DEXA scanning, was used to measure bone mineral density (BMD) and bone mineral content (BMC) of the samples. The DEXA scanner rapidly emits x-ray energy from two different sources towards the bone sample in an alternating fashion at a set frequency. The mineral density of the bone weakens, or prolongs the transmission of these two sources of X-ray energy through a filter onto a photon counter in a quantity related to the amount of bone mass present. Hence, the greater the BMD, the greater the signal picked up by the photon counter. The use of the two different X-ray energy sources greatly improves the precision and accuracy of the measurements for small tibial samples.

The bones were then analyzed by microcomputed tomography (μCT) in order to obtain quantitative static histomorphometry parameters for trabecular and cortical bone. Qualitative computed tomography (QCT) is the only technique that can directly measure bone density and volume but can distinguish trabecular from cortical bone. A SkyScan model 1072 desktop micro-CT instrument was used. Samples were scanned at a

magnification resulting in a pixel size of 9.57 μm. Using a rotation step of 0.9 degrees and an exposition time of 7500 ms (milli-second) for each step, a total of 206 images was generated for a total scanning time of 75 minutes. The cross-sections along the specimen axis were reconstructed using Cone-Beam Reconstruction Software (SkyScan), with a distance between each cross-section of 27.371 μm. CT-Analyzer and 3D Creator software (both from SkyScan) were used to analyze and perform 3D rendering respectively.

3.5 Biomechanical properties Analysis:

The biomechanical properties of the distracted bones were tested and compared between genotypes. The samples for biomechanical analysis were collected in normal saline solution and mounted in a modified Instron three point bending test apparatus. The ends of the tibia samples rest on two fulcra separated by 5 mm and a load is applied from above to the anterior mid-shaft, midway between the two fulcra. The load is applied at a constant rate of 5 mm/minute, until failure. An MTS digital acquisition module sampling at 250 Hz was used to obtain all the force-deformation curves. The final breaking force (maximum load) and final deformation (maximum displacement) was determined directly from the curve. Young's modulus (stiffness, K) was calculated as the slope of the force-deformation curve through its linear portion.

3.6 Real Time and Reverse-Transcription PCR:

3.6.1 RNA extraction:

The distracted tibial samples collected from the five time points were crushed and ground using a mortar and pestle, then suspended in 2 mL of TRIzol reagent (Invitrogen; Carlsbad, California) solution and further homogenized using a Polytron at high speed for no more than 30 seconds. Following homogenization, the tibial samples were centrifuged and 400 μL of chloroform was added to the supernatant to separate the RNA. 1 mL of isopropyl alcohol was added to the separated RNA. Once the RNA precipitated, the samples were centrifuged again. The collected pellet from each sample was washed with 70% ethanol and then vacuum-dried for 4 minutes and resuspended in 25 μL of RNAse-free water. Samples were stored in –80 ° C prior to reverse transcription.

3.6.2 Reverse Transcription:

The isolated mRNA was reverse-transcribed using The High-Capacity cDNA reverse transcription kit [(Cat: 4368813) Applied Biosystems; Branchburg, New Jersey)] following the manufacturer's instructions. The kit utilizes Random Primers and Multiscribe Reverse Transcriptase to successfully transcribe and translate tissue RNA into functional cDNA. After the master mix is made, equal amounts of RNA samples are added, and the tubes are loaded in the thermal cycler. The end result is cDNA transcribed from RNA, hence it only has the exons and not the introns and we could detect which are the genes expressed during the different stages of DO.

3.6.3 Real-Time PCR:

The expression of osteogenic factors, their receptors, their downstream effectors, and key bone and cartilage differentiation markers were quantified using Real-Time PCR analysis on mRNA from the distracted callus with specific TaqMan probes. This analysis was performed using a System 7500 Real-Time PCR instrument from Applied Biosystems. Real time PCR is used to quantify transcript levels using florescent dyes for reference genes and target genes.

The Assays-on-Demand TaqMan probes purchased from Roche diagnostics used in Real-Time PCR were to measure the expression of osteoblastic, vascular and chondrogenic factors during DO. The cDNA retrieved from tibial samples of the different stages of DO through reverse transcription was used to measure expression markers. cDNA is added to a universal master mix (Applied Biosystems; Branchburg, New Jersey) and loaded on to a 96-well plate, were the samples are duplicated in order to be equilibrated to a house-keeping gene.

The TaqMan reference assay markers used in our experiments are divided into three categories. Osteoblastic expression markers: Runx2, OSX (Osterix) and Ocn (Osteocalcin) identify the expression of osteoblastic factors during DO. Vascularization expression markers: VEGF (**V**ascular **E**ndothelial **G**rowth **F**actor), VEGFR1 (VEGF receptor 1), and VEGFR2 (VEGF receptor 2) detect the expression of vascularization factors. Chondrogenic expression markers: Sox9, Col2 (Collagen type II), and Col10 (Collagen type X) reveal the expression of chondrogenic factors during DO. The Reference genes are constitutively expressed (e.g. GAPDH) and are used to normalize the values of expression level between the different sample conditions.

3.7 Western Blotting and Protein Analysis:

3.7.1 Protein Extraction:

The tibial samples were snap frozen with liquid nitrogen and kept at -80°Celcius until processed. In order to extract the protein from the small callus, the tibia was crushed with a mortar and pestle. The crushed callus was suspended it 600 µL lysis buffer [20mM Tris-HCL pH 7.4, 150mM NaCl, 1.2% Triton X-100, 1mM EDTA, 1mM PMSF (10mg/ml Isopropanol), distilled dH20] that contains protease inhibitors (1µl/ml protease cocktail). After crushing, the samples were homogenized on ice using a Polytron at the highest setting for a maximum of 20 seconds. Homogenized samples were slowly mixed on a platform agitator at 4°C overnight. The next day, the samples were centrifuged and the supernatant collected in an eppendorf tube, and the pellet was processed as before by adding 600 µL lysis buffer with protease inhibitors and homogenized using a Polytron. Then the samples were centrifuged and the supernatant was added to the previously collected supernatant in an eppendorf tube. Tri-chloro acetic acid [100% (TCA), 500g TCA dissolved in 350 ml dH2O] was used to precipitate the protein. Three hundred µL of TCA were added to the supernatant and the tubes mixed by inverting twice, the samples were then incubated on ice for ten minutes. After incubation, the tubes were centrifuged and the pellet is washed with cold acetone. The remaining protein pellet was dried on a heating block and resuspended in 25 µL 5X loading buffer (1M Tris PH 6.8, 20% SDS, Glycerol, 5% bromophenol blue)

3.7.2 Western Blotting:

Isolated proteins were separated using SDS-PAGE electrophoresis on a 10% gel (10% SDS, 40% Acrylamide/Bisacrylamide, 10% ammonium persulfate, 1.5M Tris, TEMED). Migration was at 60 volts for 30 minutes, then at 140 volts for 1 hour. Separated proteins were transferred to a Nitro-Cellulose membrane in transfer buffer (1M Tris, Glycine, 10% SDS, methanol, water).

After transfer, the membrane was removed and placed in 25ml blocking solution for at least an hour. The blocking solution contained 5% skim milk or BSA and 2% horse serum dissolved in 1X TBS (3% Tris-base, 8% NaCl, PH 7.6). The membrane was washed 3 times with 1X TBS for 5 minutes each time. Anti-STAT1 antibody (1:1000) was added to the membrane diluted 10 ml TBS. The membrane was placed on a platform rotator overnight at 4˚C. The next day, the membrane was washed with 1X TBS, and the secondary antibody (2^{nd} ab) added diluted in 10 ml blocking solution in a 1:5000 concentration and rotated for at least an hour. The membrane is then washed with TBS for at least 20 minutes. The membrane was then placed on saran wrap and 2 ml ECL solution was added on the membrane and incubated for 1-3 minutes. Afterwards the membrane is blotted on whatman paper, wrapped in new saran wrap and placed in a cassette. The membrane was exposed to film for 1-15 minutes in the dark room. The film was developed and the size of the detected target antigen assessed by comparison with molecular mass markers. (MagicMark™, Invitrogen Carlsbad, Ca USA; Rainbow™ colored protein molecular weight markers, GE Baie d'Urfe, Qc Canada).

3.8 Statistical Analysis

Data was graphed with the PRISM software and analyzed using the statistical package included in the software suite. Sample size varied from 4 to 16 but was never less than 4 animals per group. For comparison of wild-type and mutant cohorts, Student's one-tailed *t* test was used. For analysis of data that included more than two groups, such as the data presented in Figures 20-23, analysis of variance (ANOVA) with the Dunnett's post test was used. A p value of <0.05 was accepted as significant.

4.0 Results:

4.1 Distraction Osteogenesis on a Mouse model:

We were able to successfully apply the operative protocol on a mouse model, and perform distraction osteogenesis on mouse tibia. We modified and perfected the surgery taking into consideration the smaller scale in which we had to maintain and fix the Ilizarov apparatus. We used custom-made rings designed especially to fit a mouse tibia and engineered out of light-weight stainless-steel with a thickness of 0.2cm. The threaded rods on which the rings slide on were also especially designed for the tiny Ilizarov apparatus; the rods measure 2.5 cm (Figure 4).

All the procedures were done under strict sterile environment, following the animal protocols set forth for this project. The mice that exhibited any kind of swelling, necrotic tissue or general discomfort were immediately euthanized.

Post-surgery the mice were monitored daily for a 5 day latency period, and we observed that they were able to move around the cage and eat normally and survive through the long process of DO and the consolidation phases until the tissue was collected.

4.2 Faxitron X-ray:

Samples collected from the three chosen time points, 17 days (end of distraction), 34 days (mid-consolidation), and 51 days (end of consolidation) were treated under the same conditions as Faxitron X-ray images were taken.

At 17 days, there was no visual difference between the FGFR3-/- mice and their wild-type littermates. The tibias looked very much the same; the tibias were collected

right at the end of distraction so no time had been given for bone formation and repair. Therefore there was no evidence of mineralization or new bone in either wild-type (Figure 5A) or mutant mice (Figure 5B) during DO at 17 days post surgery.

At 34 days, the results of the Faxitron X-ray revealed a large visual difference between the distracted tibias of the wild-type mice (Figure 6A) vs. FGFR3-/- mice (Figure 6B). The mutant mice revealed more mineralized bone, and the osteotomy is less visible in mutants, while in the wild-type mice the bone was not fully mineralized and the osteotomy as well as the gap between the two distracted extremities was still evident.

At 51 days, end of distraction, the Faxitron x-ray images resulted in a visual difference between the distracted tibias of the FGFR3-/- mice (Figure 7A) and those of the wild-type mice (Figure 7B). FGFR3-/- tibias had more mineralized bone and additional newly formed bone within the distraction zone as compared to the wild-type mice. However, at 51 days post surgery or the end of consolidation phase, the tibias for both wild-type and mutant mice were almost fully consolidated and their appearance started to be similar as the new bone commenced mineralization. The selected images are not highly representative of the entire sample population. The quantification of other parameters shows no significant differences between wild-types and mutant mice at end of consolidation.

The Faxitron X-ray data reveal that more bone was formed in mutant mice at mid-consolidation.

4.3 DEXA:

The results from the DEXA analysis further support the visual data obtained from the Faxitron X-ray images. DEXA analysis allows quantifying the following parameters: bone mineral density (BMD) and bone mineral content (BMC).

At 17 days post surgery or end of distraction, DEXA analysis was obtained from the distracted tibias. The BMD (Figure 8A) and BMC (Figure 8B) measurements revealed no significant differences in either parameter between the mutant and wild-type tibial samples.

At 34 days post surgery or mid-consolidation phase, the DEXA analysis revealed the most significant differences between the FGFR3-/- mutant mice and wild-type mice. BMD (Figure 9A) and BMC (Figure 9B) were significantly increased in FGFR3 mutant mice as compared to the age-matched, littermate wild-type controls.

At 51 days post surgery or end of distraction phase the DEXA analysis did not show any significant differences between the FGFR3-/- mice and the wild-type mice for either BMD (Figure 10A) or BMC (Figure 10C).

These data suggest that FGFR3 ablated mice have more bone formation during DO, however as more time is given for the bone to consolidate the wild-type mice appear to catch up with the mutant mice in terms of total amount of bone formed.

4.4 Micro Computed Tomography (μCT) Results:

μCT scans the bone samples and produces three-dimensional images of the tibia and the distracted callus that further support the results obtained from the Faxitron x-ray images. μCT Analysis also measures the following parameters, amongst others: Trabecular separation in mm, Trabecular number in 1/mm, Trabecular thickness in mm, and BV/TV (bone volume/tissue volume) in percentage %.

At 17 days post surgery or end of distraction there was very little newly formed bone to be measured within the distraction gap for either mutant mice (Figure 11A) or wild-type mice (Figure 11B). The parameters measured using micro computed tomography revealed a large variation in the statistical histomorphometry. This variation was due to the absence of bone within the distraction zone measured and a lack of precision using this technique on the small sample size collected at end of distraction phase.

At 34 days post-surgery, or mid-consolidation we were able to collect the most significant results using micro computed tomography, and we were able to visualize the difference between mutant mice (Figure 12A) and wild-type mice (Figure 12 B) in the images. The FGFR3-/- mutant mice had a higher trabecular number (Figure 13A) within the newly formed bone in the distraction gap, more so than that measured in the wild-type littermates. However, trabecular thickness (Figure 13B) remained unchanged. There was significant difference measured between the mutant and wild-type mice for the percentage of bone volume (Figure 13C) as compared to tissue volume, with the FGR3 knock-out mice having a significantly higher percentage of bone as compared to total tissue.

At 51 days post-surgery or end of consolidation, micro computed tomography revealed no difference between the tibias of the FGFR3-knock-out mice as compared to the wild-type littermates. At this phase of DO, the bones were almost fully consolidated and the parameters measured showed no significant differences between mutant and wild-type mice for either BV/TV (Figure 14A) or trabecular number (Figure 14B).

These results further confirm the data obtained with the other techniques, and show that the FGFR3 knock-out mice exhibited increased bone volume at mid-consolidation during DO as compared to their age-matched wild-type littermates. These results also confirm that as the wild-type mice catch-up with the mutant mice towards the end-of-consolidation phase, the bone properties and parameters obtained through μCT analysis confirms similarities in bone properties as well.

4.5 Biomechanical Properties:

We have observed that bone formation is significantly increased in the mutant mice as compared to the wild-type littermates. However, is the newly formed bone of sound quality? The quality of the newly formed bone was compared between the FGFR3 deficient mice and the wild-type littermates during DO.

The bone strength is measured using a three-point bending technique in which the force required to break the bone is measured in Newton (N).

At 17 days post-surgery or end of distraction there was very little bone formation within the distracted gap. The callus is made up of fibrous tissue that is very soft. At this phase we were unable to retrieve any data about the biomechanical properties of the new

bone because we were unable to perform the three-point bending technique on the samples, due to the softness of the forming callus.

AT 34 days post-surgery or mid-consolidation, we measured the biomechanical properties of the tibial samples using the three-point bending technique and we observed significant difference between the mutant and wild-type mice. The stiffness (Figure 15A) of the FGFR3-/- tibias was almost >3X higher than the tibia collected from the wild-type littermates. The force (Figure 15B) exerted on the tibial samples to achieve failure showed a significant increase in the mutant mice as compared to the wild-type mice. Thus, at mid-consolidation we can observe significant differences in biomechanical properties between the mutant and wild-type mouse tibias.

At 51 days post-surgery or end of consolidation, we recorded a small difference in biomechanical properties between the FGFR3 knock-out tibias and the wild-type tibias. At this phase the mutant tibias show a slight increase in stiffness (Figure 16) over their littermates, however both sets of samples exhibit almost fully consolidated bone and hence have more resistance during the three-point bending test.

These results confirm that the newly formed bone in the mutant mice is of good, sound quality and that the increased bone formation does not diminish the quality of the formed bone during DO.

4.6 Histology Results:

The tibia samples were embedded in MMA, sectioned and stained with Trichrome –Goldner in order to obtain histological analysis on the type of bone generated during DO. Trichrome-Goldner stains mineralized tissue green. At 17 days post-osteotomy the

sections from the FGFR3 knock-out (Figure17B) tibia revealed cartilage-like cells forming a growth-plate like structure were the cells are in columns and they have begun mineralizing. We also observed elongated spindle-like fibrous cells that are typical for intramembranous bone formation during DO. Hence, at end-of-distraction phase in the mutant mice we observed a simultaneous combination of both endochondral and intramembranous bone formation. However, in the wild-type litter mates (Figure 17A), the sections revealed the elongated spindle-like fibrous cells typical of intramembranous bone formation, however we were unable to observe the cartilage-like cells or the growth-plate like structure, and the amount of mineralized bone appears reduced in the wild-type as compared to the mutant samples.

At 34 days post-osteotomy, tissue from the FGFR3 knock-out mice (Figure 18B) exhibited increased mineralized bone tissue and we also observed osteiod being deposited by osteoblasts as well as osteocytes embedded within the newly mineralized bone. These samples also revealed the elongated spindle-like fibrous cells typical of intramembranous bone formation. In these FGFR3 kncok-out sections we did not observe the osteotomy because the distraction gap has been entirely filled with mineralized or newly developing bone tissue. However, in the wild-type sections (Figure 18A) there was significantly less mineralized bone and we observed the cells typical on intramembranous bone formation and the osteotomy site was still visible.

At 51 days post-osteotomy or end of consolidation phase, the samples were almost fully consolidated and the new bone was mineralized (Figure 19). (B),both wild-type (WT) and mutant (MT) sections showed elongated spindle-like fibrous cells which are typical of intramembranous bone formation.

Taken together, our results suggest that there is a difference in the type of bone formation during DO between normal and mutant mice. The FGFR3 ablated mice show a preference towards endochondral bone formation due to the highly organized chondrocyte structure which is similar to a growth plate in the middle of the distraction gap. While the wild-type mice show more evidence of intramembranous bone formation and much less chondrocyte-like structures.

4.7 Gene expression monitoring:

Samples for RNA were collected for the four phases of DO; 5 days (end of latency), 17 days (end of distraction), 34 days (mid-consolidation), and 51 days end of distraction. The expression of several differentiation markers was analyzed at the different DO phases and compared to an undistracted contralateral tibial control sample. TaqMan assays that detect osteoblastic, vascularization, and chondrogenic markers were analyzed by RT-q PCR. The osteoblastic markers consisted of Runx2, OSX (Osterix), and Ocn (Osteocalcin). The vascularization markers consisted of VEGF (**V**ascular **E**ndothelial **G**rowth **F**actor) and its two receptors VEGFR1 and VEGFR2, and they detect signals of vasculogenesis and angiogenesis which identify the formation of new vascular tissue (blood vessels, endothelium). The chondrogenic TaqMan assay markers were Sox9, Col2 (collagen type II) and Col10 (Collagen type 10), they are expressed in chondrocytes during endochondral bone formation.

There were no significant differences in the expression of osteoblastic markers between the FGFR3 knock-out mice and their wild-type littermates throughout the different phases of DO. We could not measure Ocn expression at 5 days post-osteotomy

or 51 days post-osteotomy (Figure 20C). The differences measured in Runx2 expression were not statistically significant due to low sample size (Figure 20A). OSX expression (Figure 20B) also revealed no significant difference between the wild-type and mutant mice during the different phases of DO.

Similarly, VEGF (Figure 21A) expression was not significantly different between the four phases of DO as compared to the undistracted tibia, as well as no significant difference measured between the mutant and wild-type mice. The VEGF receptors VEGFR1 (Figure 21B) and VEGFR2 (Figure 21C) recorded increased expression in the wild-type mice at 5 days post-osteotomy or end of latency phase. However, no difference was noted in the remaining DO time points between FGFR3 knock-out mice and their wild-type littermates.

The most significant difference between mutant and wild-type mice was measured for the expression of chondrogenic markers. Col2 (Figure 22B) and Col10 (Figure 22C) had significantly higher expression in the FGFR3 knock-out mice as compared to the wild-type littermates throughout the four phases of DO. Sox9 (Figure 22A) is a chondrogenic marker that is co-expressed with Col2 and is expressed in proliferating chondrocytes. Sox9 was expressed more in the FGFR3 knock-out mice at 17, 34 and 51 days post-osteotomy as compared to the wild-type littermates, although the increase did not reach statistical significance.

These data show that chondrogenic expression markers are up-regulated during DO in the mutant mice. The normal mice do not show a significant increase in any chondrogenic expression markers during DO. This evidence suggests that the mutant mice exhibit endochondral bone formation during DO within the distraction gap, while

wild-type mice show no specific preference for either bone formation types. The data obtained from the expression markers suggests that endochondral bone formation is the reason for the increased bone that we have observed thus far in our research.

4. 8 Western Blot Results:

Stat1 expression measured using Real-Time Q-PCR (Figure 23A) revealed significant differences between wild-type and mutant mice at each of the different phases of DO including the control group. Hence, we decided to follow up these results by studying the protein pattern of Stat1 during DO.

Tibia samples were collected for protein at 17 days post-osteotomy (end of distraction), 34 days post-osteotomy (mid-consolidation) and 51 days post osteotomy (end of distraction). The samples were snap frozen with liquid nitrogen and kept at -80°C until they were processed, and the protein collected and resuspended in loading buffer. The samples were run on a gel and then transferred to a membrane. The membrane was treated with antibodies that detect Stat1 and P-Stat1 (phosphorylated form of Stat1). We were unable to detect the presence of P-Stat1 in the collected samples; however Stat1 gave very clear bands on the western blot (Figure 23B). Measuring the band intensity from the western film (Figure 23C) revealed the highest signal obtained for Stat1 was at 17, and 34 days post-osteotomy in both mutant and wild-type mice, however the control samples which were undistracted contralateral tibia, only detected a signal in the FGFR3 knock-out samples. At 51 days post-osteotomy or end of consolidation phase we failed to detect any signal for Stat1 using the same antibodies.

5.0 Discussion:

The findings from our research have demonstrated that under similar conditions of mechanical stress during DO, mice lacking the FGFR3 gene produce more good quality bone than their wild-type littermate.

Ilizarov identified "the growth zone" of the distraction-regenerated region, a thin osteogenic layer which resembles a growth plate-like structure in the middle of the distraction gap (Ilizarov, 1989; Ilizarov, 1990). These observations in the dog model of DO are extremely similar to the histological results obtained from our own experiments. The sectioned tibial samples clearly show evidence of "the growth zone" identified by Ilizarov, where the FGFR3-ablated tibia exhibits cartilage-like cells forming a growth-plate like structure were the cells are organized into columns and are mineralizing.

The gene expression results obtained from Real-Time PCR suggest increased endochondral ossification over intramembranous ossification. Although the histological data reveals a mixture of both intramembranous and endochondral bone formation, the expression pattern of chondrogenic markers in the mutant mice is significantly increased as compared to the wild-type mice.

Histological evidence from a research in a canine model of DO revealed intramembranous ossification predominated in the distracted callus taking up most of the distraction zone between the dissected cortices (Kusec et al., 2002). In a rat model of DO, the histological characteristics revealed that cartilage was progressively resorbed from both ends of the distraction gap, and that the new bone was formed directly by intramembranous ossification (Sato et al., 1999) In these cases, intramembranous

ossification dominated the process of DO, and the histological results were very similar to those obtained from the wild-type mice in our research.

The evidence revealed by our experiments clearly shows that the mice lacking FGFR3 are at an advantage over their wild-type littermates during the process DO. The lack of expression of the FGFR3 gene accelerates the process of new bone formation during DO via endochondral ossification.

The tibial samples collected were analyzed using Faxitron (X-ray), DEXA, μ-CT scan, biomechanical testing, Real-Time PCR and Western blot. The results obtained from these analysis show that there is an increase in ossification among the FGFR3 knock-out mice as compared to wild-type mice during DO.

The parameters measured using DEXA and μ-CT scan reveal similar findings, with the most interesting set of data coming from the sample group at 34 days post surgery or mid-consolidation. At that particular time point during the process of DO, we observed a significant difference between the mutant mice and the wild-type mice within the parameters concerning bone mineral density and bone mineral content as measured by DEXA, and trabecular number and bone volume as measured by μ-CT scan. Testing the biomechanical properties of the samples as well, revealed that at the mid-consolidation phase the mutant mice exhibit a more rigid new bone formation than that seen in the wild-type mice.

The evidence revealed by these experiments show that there is a relevant and significant increase in bone formation in the mice lacking the FGFR3 gene. However, this significant observation is most prominently found at the mid-consolidation phase or 34 days post-surgery. In our research, it was observed that at some point during the

consolidation period, new bone formation in wild-type mice catches up with the accelerated new bone formation observed in the mutant mice. As we have seen, the results from the normal genotype samples collected at the mid-consolidation point of DO reveal less new bone formation in the distraction zone, with a decrease in bone volume, BMD, BMC, trabecular number and biomechanically weaker bone properties as compared to the mutant genotype sample population. In addition, the samples collected at the end of distraction point or 51 days post surgery from both genotypes, appear to have very similar characteristics. This reproducible observation indicates that if given enough time to heal and consolidate, the wild-type genotype catches up with the mutant. Hence, at some particular point during the consolidation period, both genotypes reach similar final results.

Therefore, in order to differentiate and identify the particular point during the process of DO where the genotypes catch up with each other, we need to define a finer time schedule for sample collection. An appropriate method needs to be established in order to better understand and monitor the process of consolidation during DO. The samples need to be collected more frequently, concentrating on the period of consolidation between the mid-consolidation and the end of consolidation phases. This focuses on the time zone between 34 days post surgery until 51 days post surgery, a time span of 17 days. The best method would be to add another time point in the middle of these existing time points, perhaps at 42 days post surgery. This new time point would give us a greater sample population to work with, as well as add extra insight into the process of bone remodeling and consolidation during DO. It will be easier to pin-point the specific time and environment in which the two genotypes catch-up in bone

formation, and possibly reveal more histological and molecular evidence relating to the difference we had already observed between the genotypes in previous experiments.

The preferred interpretation for the results in these experiments would be that inhibiting signaling through FGFR3 would lead to an increased bone formation rate during the middle phase of DO. This interpretation could be tested by measuring the difference between bone formation rates in both wild-type and mutant mice during DO. In order to be able to accurately measure the rate of bone formation during DO in mutant and wild-type mice, a new experiment has to be designed. This experiment must include dynamic histomorphometry analysis that measures rate of bone formation as the process of consolidation and new bone formation progress throughout the DO process. For dynamic histomorphometric analysis, the mice would be be injected with calcein subcutaneously (10 mg/kg) at 28 and 32 days post-surgery, and then the samples collected at day 34 or mid consolidation phase. The same would be done for all the time points we wish to collect samples from, the mice would be injected twice at 2 day intervals, 2 days prior to euthanasia (Oxlund et al., 2003). The samples will be collected and embedded in MMA and cut into ten-micrometer sections for dynamic analysis. The calcein labeled perimeters would then be determined using a digitizing image analysis system and a morphometric program, *Osteo* (BioQuant, Nashville, Tennessee), and then bone formation rate (BFR) would be calculated using the mineral apposition rate (MAR) measured (Iida-Klein et al., 2006).

Our results show that signaling through FGFR3 normally inhibits bone formation during DO. One way to interpret these results is by saying that signaling through FGFR3

would normally block endochondral bone formation, thus slowing down the process of bone formation during DO.

This interpretation is further supported by the results obtained from the gene expression analysis by Real-Time PCR. The only expression markers that revealed a significantly different trend within the FGFR3 deficient mice were the chondrogenic markers, Col2 (Collagen type II) and Col10 (Collagen type X). These genes are typically expressed during endochondral bone formation. Col2 and Col10 expression was not up-regulated in the wild-type mice through out the process of DO; however they showed significant increase in the mutant mice in all four time points chosen.

We could conduct several more experiments that would further establish our interpretation of endochondral ossification within the distracted tibia of the mutant mice. One such experiment would be immunohistochemistry in which sections of the tibia embedded in paraffin or a special type of MMA could be stained for Col2 and Col10. Immunohistochemistry is a qualitative technique used for the anatomical identification of different cellular and extracellular components. The samples are frozen, embedded in paraffin wax, carefully sectioned and then blocked using immunoblotting antibodies that would stain tissue expressing Col2 and Col10 protein.

Another experiment that would detect the presence of Col2 and Col10 within the distracted tibia of the mice would be performing in situ hybridization on sections of the bone sample. In situ hybridization uses a labeled complementary RNA strand (probe) to localize specific mRNA sequences in a section of tissue; in this case we are trying to locate Col2 and Col10 mRNA expression in a bone section. There are numerous commercially available kits that utilize radioactive or non-radioactive labels for the

probes (GreenStar™ Biotin). The in situ sections are carefully prepared so as to eliminate unspecific binding and accurately detect mRNA expression where specific hybridization has occurred.

Our results suggest that inhibition of FGFR3 signaling would be beneficial in DO. Consequently, these findings imply that inhibiting FGFR3 is beneficial for the clinical aspects of DO, and would help accelerate the process of bone consolidation and decrease the length of time in which the external fixator has to remain on the patient.

The first step to bring us closer to a clinical therapy is trying to inhibit FGFR3 expression in normal wild-type mice and comparing the results with those already seen in the knock-out mice. One technique we can use to achieve this is by injecting antibodies that block ligand binding to FGFR3. These anti-bodies could be found commercially, however they need to be monoclonal blocking antibodies that are mouse specific and could be diluted into convenient injections that are not harmful to mice. These doses are based on a published treatment regimen for intra-peritoneal injection in rodents (Bauer et al. 2003; Mordenti et al., 1999; Straight et al., 2005). The injection solution is diluted in PBS, and the placebo injections are PBS only. The injections would be performed by skilled animal care personnel in our animal facilities.

Another possible technique is the use of small molecule inhibitors to block FGFR3 expression. Recently, these types of inhibitors have shown tremendous potential in blocking several receptors including FGFR3 using very low concentrations. In a recent study the activity of the small molecule inhibitor PD173074 was confirmed against FGFR3 by demonstrating inhibition of autophosphorylation in cultured cells (Trudel et al., 2004).

A third possible technique that has been recently discovered is using small interfering RNA (siRNA) strategies to knockdown FGFR3. A siRNA sequence targeting vector has to be chosen using the Oligoengine siRNA design tool (www.oligoengine.com) and screened for efficacy before it is used to develop the parent shRNA expression vector targeting FGFR3. The shRNA expression vector is expressed in Lentiviral duplexes or plasmids; however it is easier to transfect cells with lentiviruses. We can then transduce bone marrow cells with the lentiviruses containing the FGFR3 target sequence, and then transplant the bone marrow into wild-type mice prior to DO. The siRNA should knockdown the translation of FGFR3 RNA and hence inhibit FGFR3 expression (Estes II et al., 2006; Ohmori et al. 2007)

Additional experiments could involve molecular genetics to target upstream regulators of FGFR3. One such experiment would be the inactivation of FGF18 which has been already identified as being the physiological ligand for FGFR3. We can create a chondrocyte-specific FGF18 knock-out mouse, where we inactivate the FGF18 gene in chondrocytes using the Cre/loxP methodology (Meyers et al., 1998). One could engineer the targeting vector using recombineering techniques (Copeland et al., 2001; Court et al., 2002), followed by homologous recombination in embryonic stem cells. We predict that the conditional FGF18 knock-out mice would have a similar phenotype to the FGFR3 deficient mice, and would yield similar results within the process of DO.

The research and experiments carried out have furthered our understanding of the molecular mechanisms involved in bone formation during DO. The demonstration of the role of FGFR3 signaling in DO using molecular genetics-based approaches will allow for

future development of targeted therapeutic intervention strategies that could have a major clinical impact.

If in fact the experiments we have discussed are carried out in the near future in context with the evidence already observed, and yield positive results then we can start work on a therapeutic regimen that would benefit DO patients.

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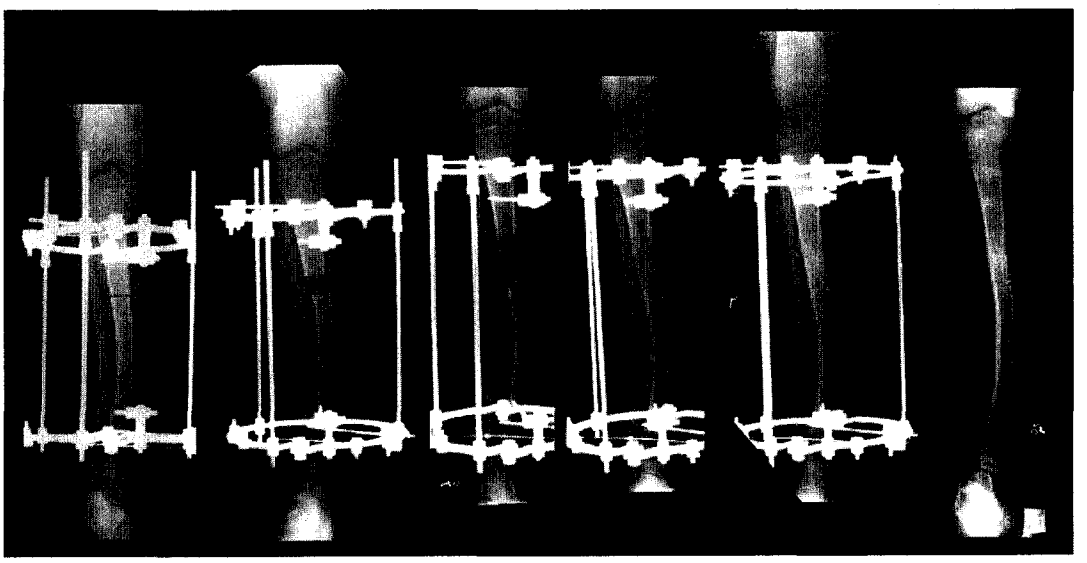

Figure 1: Radiological analysis in a patient with DO
The osteotomy and distraction gap are clearly visible at the beginning of the procedure (left panels). Following distraction, the newly formed bone becomes apparent as radio opaque material within the gap (middle panels). The Ilizarov apparatus is kept in place for several months post-distraction to allow the newly formed bone to fully consolidate (right panels).

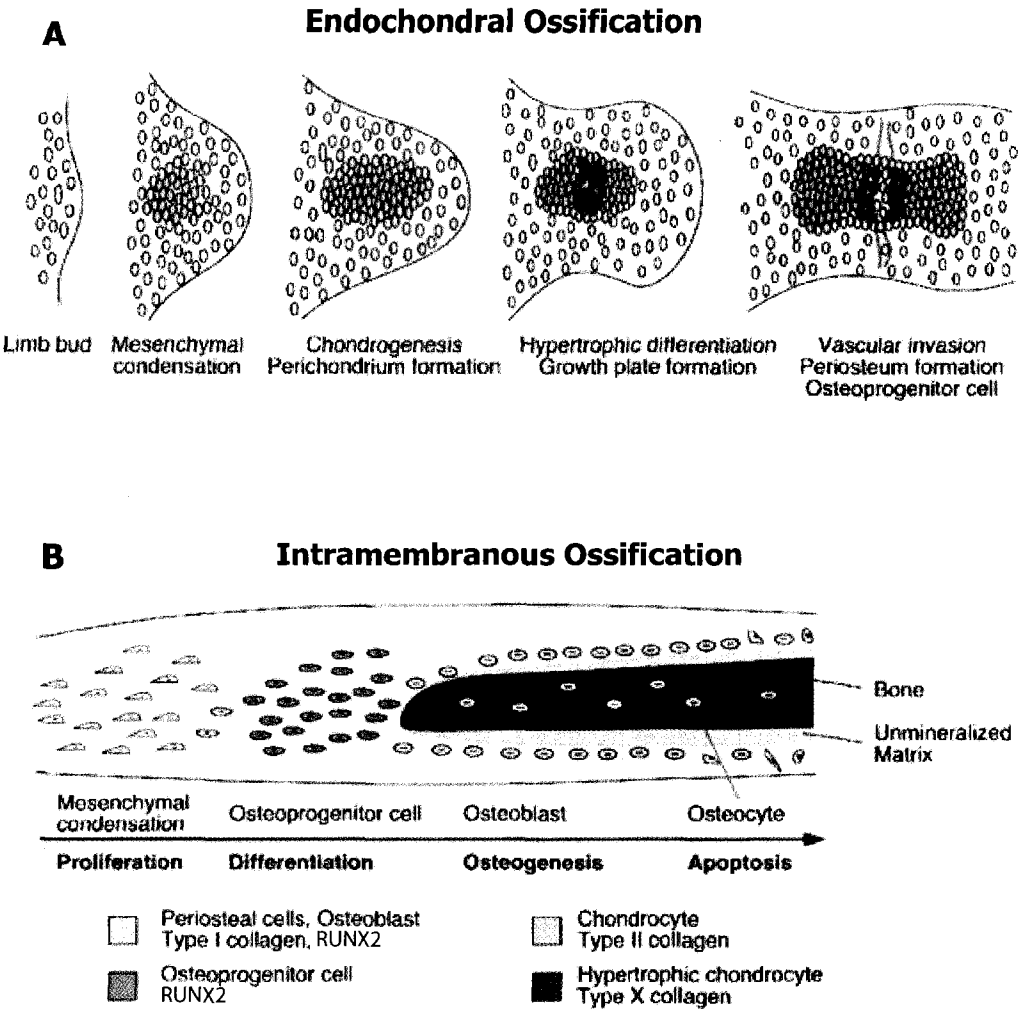

Figure 2: Endochondral and intramembranous ossification
(**A**) Schematic illustration of endochondral ossification. Endochondral skeletal growth commences with the appearance of a limb bud, the formation of a mesenchymal condensation, expressing type II collagen (blue). In the center, cells undergo hypertrophic differentiation to form chondrocytes that express type X collagen (purple). Development into the mature growth plate coincides with the expansion of the perichondrium (yellow), vascular invasion, and the creation of a center of ossification containing type I collagen-expressing osteoblasts (yellow). (**B**) Schematic illustration of developing intramembranous ossification. Undifferentiated mesenchymal cells differentiate into osteoprogenitor cells that express RUNX2 (pink). The osteoprogenitor cells then develop into mature osteoblasts expressing both RUNX2 and type I collagen (yellow). Osteoblasts deposit and mineralize bone matrix. Eventually, osteoblasts either die by apoptosis or are embedded in the matrix, and are then called osteocytes. (Modified from Ornitz, Marie, 2002)

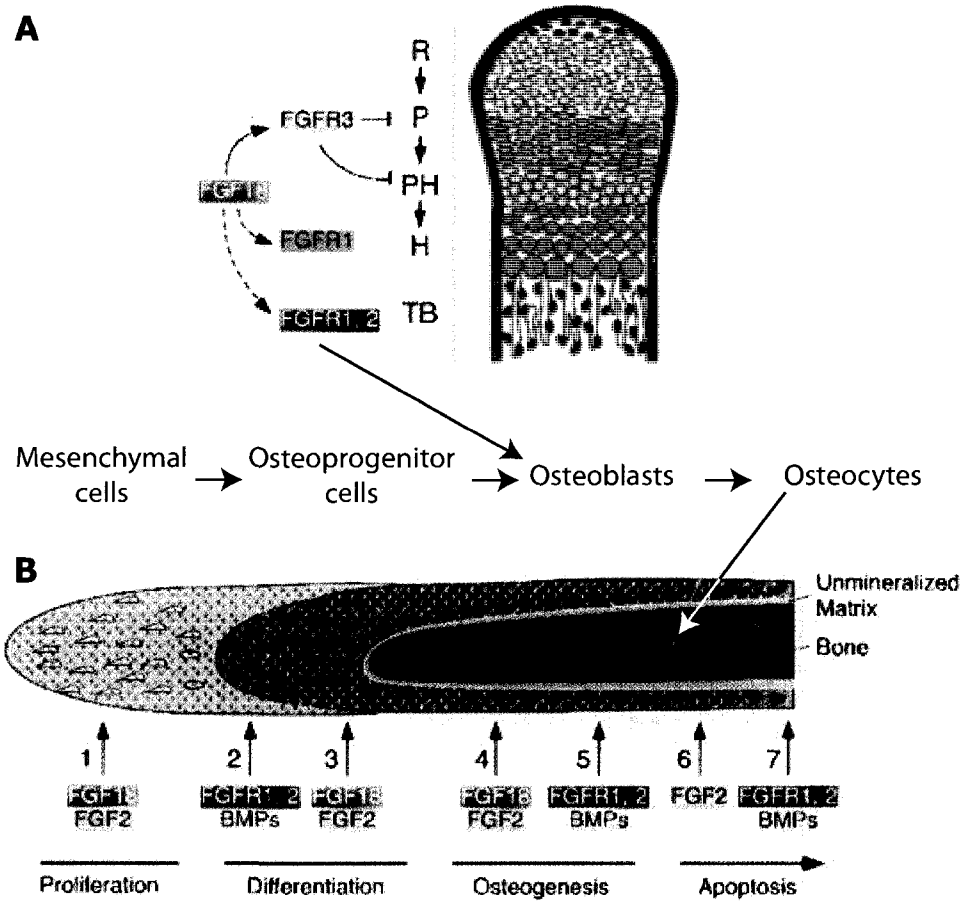

Figure 3: Regulatory pathways in endochondral and intramembranous bone

(**A**) Chondrocytes progression through reserve (R), proliferating (P), prehypertrophic (PH), and hypertrophic (H) stages of bone development. The expression patterns and interactions of molecules that regulate these events are color coded and diagramed. FGFR1, 2, 3, Fibroblast growth factor receptor 1, 2, 3; FGF18, Fibroblast growth factor 18. Signaling through FGFR3 inhibits chondrocyte proliferation and differentiation (P and PH stages). (**B**) During intramembranous ossification, mesenchymal cells proliferate and differentiate to become osteoprogenitor cells. Osteoprogenitor cells differentiate into pre-osteoblasts and then into mature osteoblasts which form the bone matrix. The stimulatory effects of FGFs and BMPs during this differentiation pathway are shown by arrows in the illustration: FGFs, FGFRs, and BMPs effects on cell proliferation (1), differentiation (2, 3), osteogenesis (4, 5) and apoptosis (6, 7). (Modified from Ornitz, Marie, 2002)

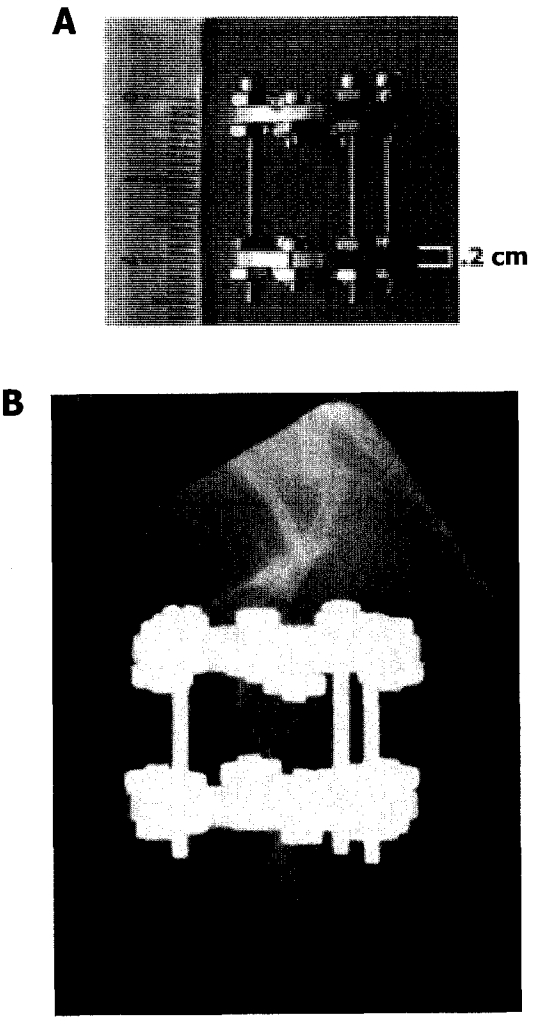

Figure 4: Distraction osteogenesis in a mouse model
(**A**) Circular external fixator (Miniaturized version of the Ilizarov Apparatus) adapted for mice. (**B**) X-ray image showing the osteotomy performed after installation of the external fixator on the tibia of a mouse. Distraction is applied at a rate of 0.2 mm every 12 hours for 12 days, followed by 34 days consolidation phase.

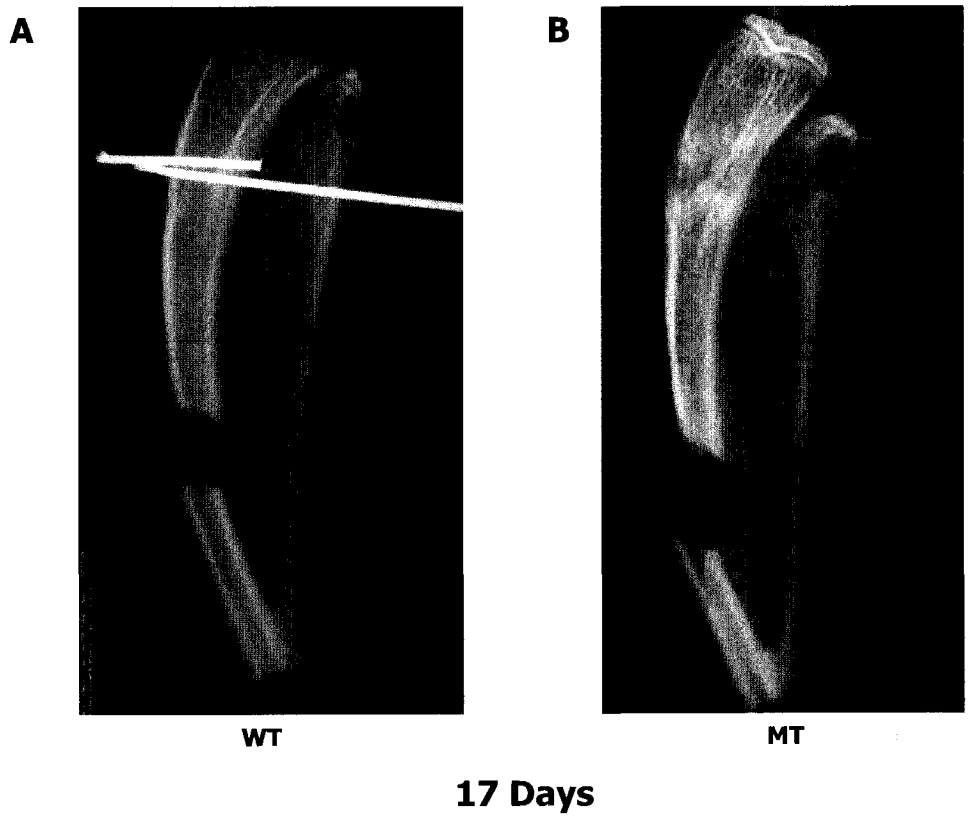

Figure 5: No evidence of new bone formation in WT or FGFR3-deficient mice at end-of-distraction phase of DO

(**A**) Faxitron X-ray image of a WT (wild-type) sample collected at 17 days post surgery or end of distraction phase. (**B**) Faxitron X-ray image of FGFR3-deficient sample collected at the same time point. The distraction gap is clearly visible as a radiolucent space. The images reveal no difference between wild-type and mutant tibial samples at end-of-distraction phase of DO.

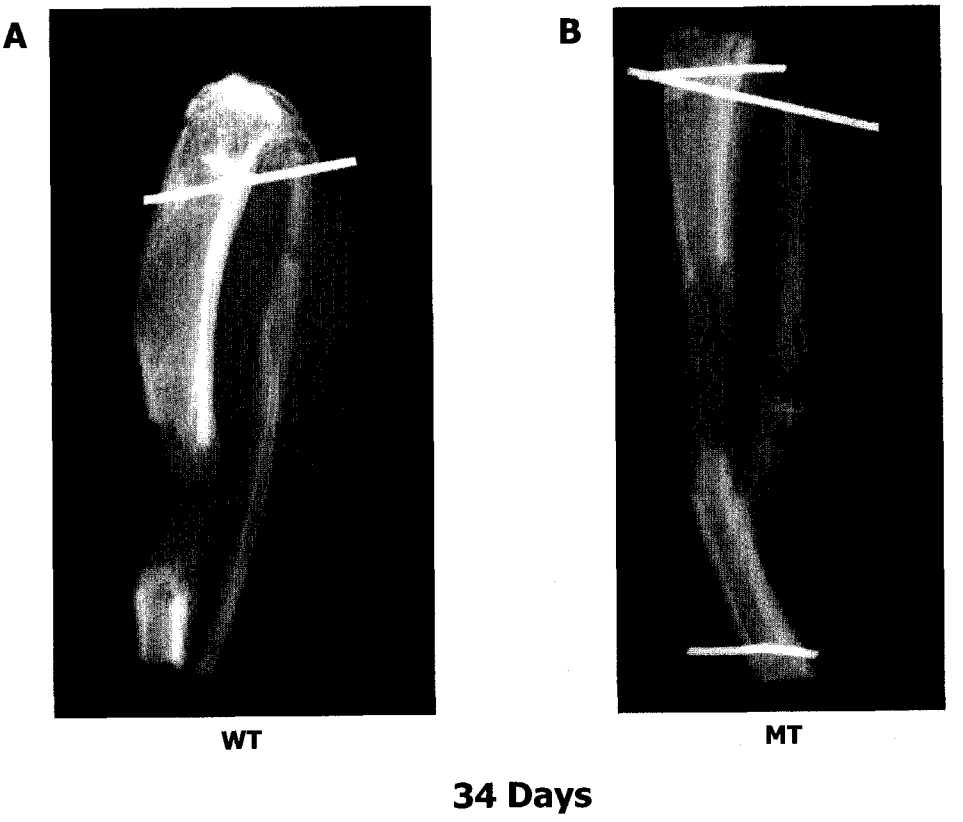

Figure 6: Increased bone formation in FGFR3-deficient mice at the mid-consolidation stage of DO

(**A**) Faxitron X-ray image of WT (wild-type) sample collected at 34 days post surgery or mid-consolidation phase. (**B**) Faxitron X-ray image of FGFR3-deficient collected at the same time point. The advancing edges of the mineralization front are visible in the WT sample, while the interzone is fully mineralized in the MT (mutant) animals.

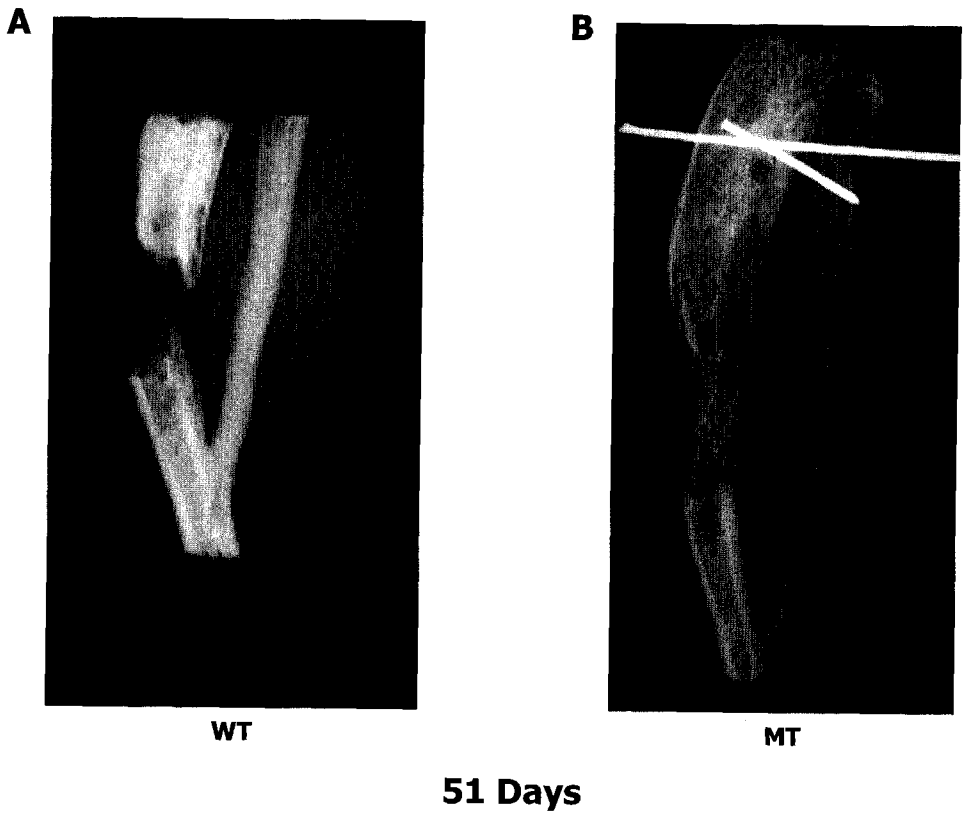

Figure 7: Increased bone formation in FGFR3-deficient mice at the end of consolidation stage of DO

(**A**) Faxitron X-ray image of wild type (WT) sample collected at 51 days post surgery or end-of-consolidation phase. (**B**) Faxitron X-ray image of FGFR3-deficient tibia collected at the same time point. The images reveal increased bone formation in the mutant (MT) tibial samples at the end-of-consolidation phase of DO.

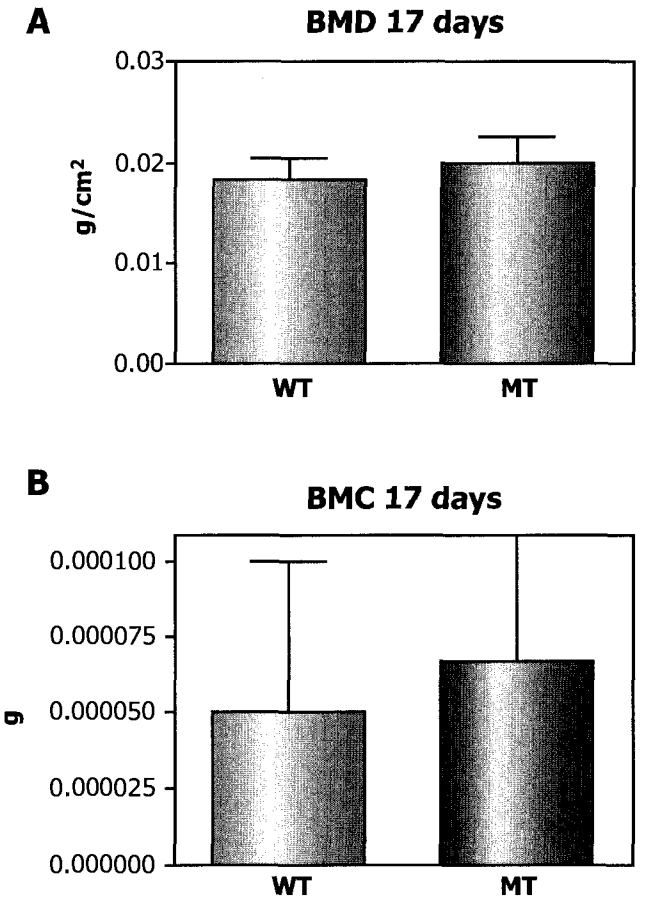

Figure 8: DEXA analysis results from samples collected at 17 days post surgery or end of distraction phase

Bone Mineral density, BMD (**A**) and Bone Mineral Content, BMC (**B**) calculated from DEXA analysis of wild type (WT) and mutant (MT) samples collected at 17 days post-osteotomy or end of distraction phase. No significant differences were measured.

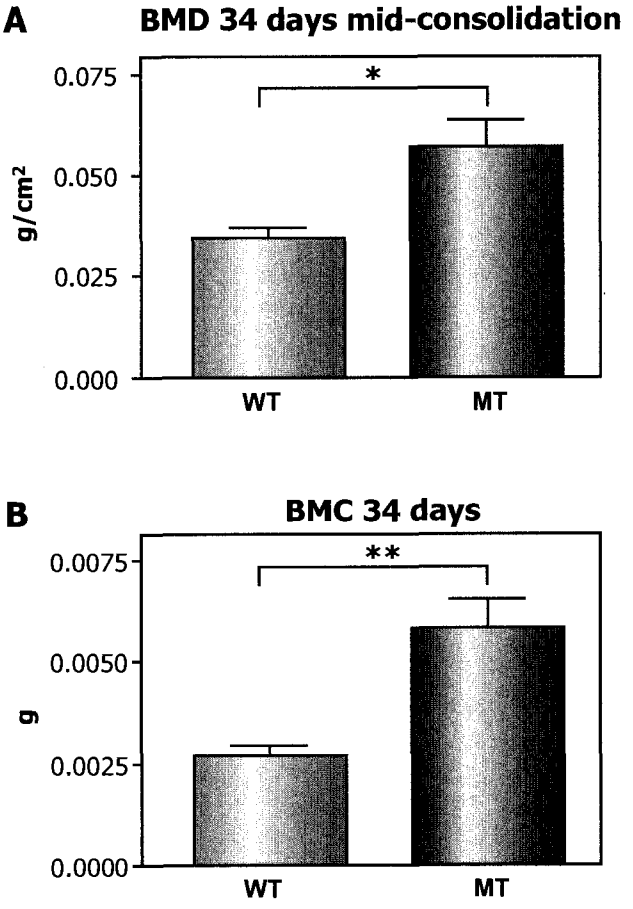

Figure 9: DEXA analysis results from samples collected at 34 days post surgery

Bone Mineral density, BMD (**A**) and Bone Mineral Content, BMC (**B**) were calculated from DEXA analysis of wild-type (WT) and mutant (MT) samples collected at 34 days post-surgery. The results reveal increased amounts of mineralized tissue in the FGFR3 mutant mice at mid consolidation, *, $p<0.05$; **, $p<0.01$.

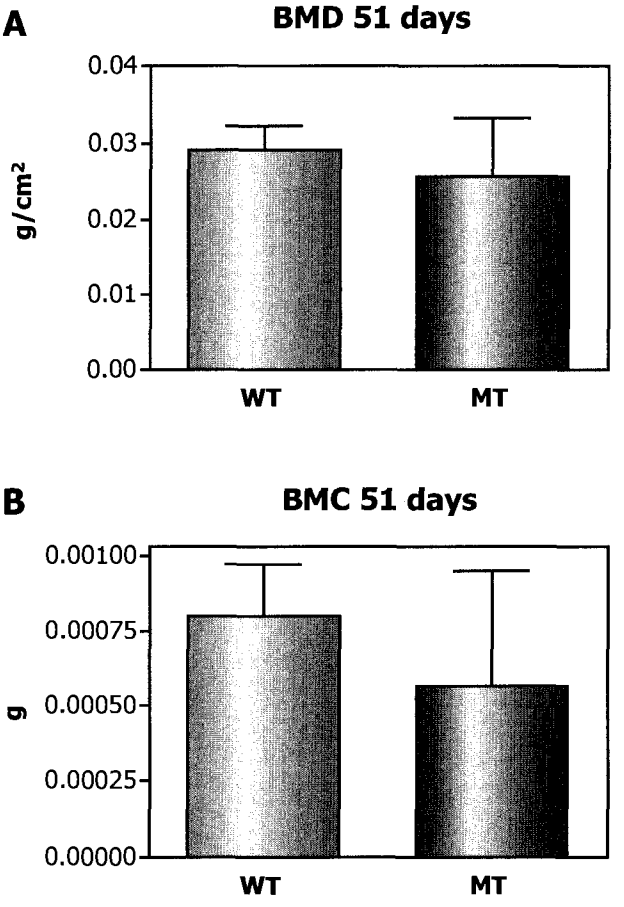

Figure 10: DEXA analysis results from samples collected at 51 days post surgery

Bone Mineral density, BMD (**A**) and Bone Mineral Content, BMC (**B**) calculated from DEXA analysis of wild-type (WT) and mutant (MT) samples collected at 51 days post-osteotomy or end of distraction phase. No significant differences were measured.

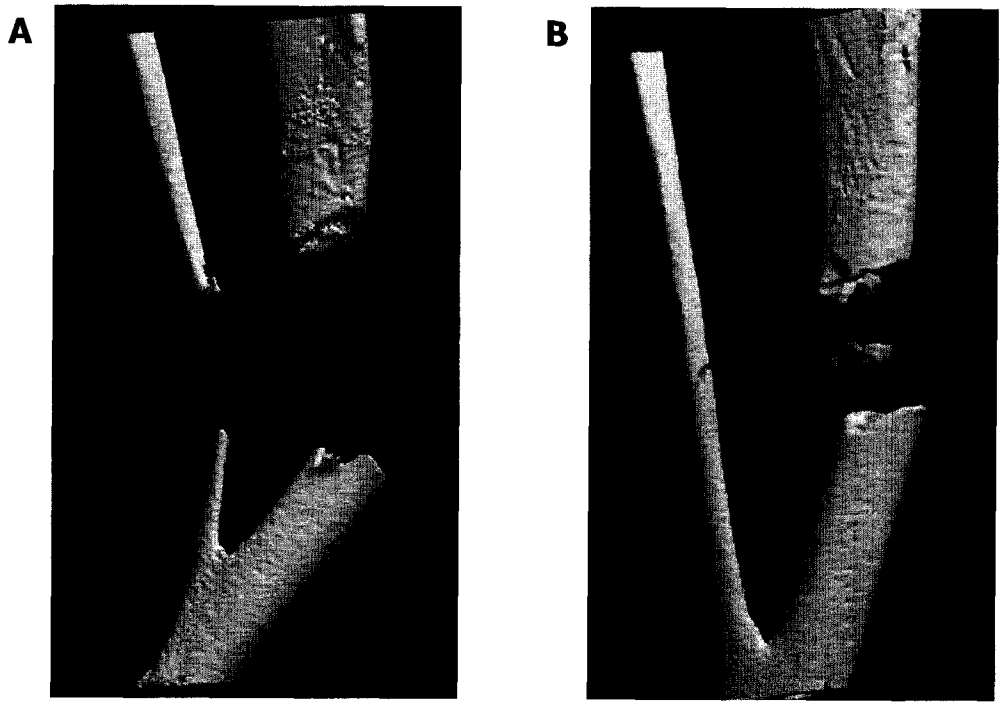

Figure 11: Micro-CT images reveal no difference between FGFR3-deficient mice and WT mice at end of distraction phase of DO

(**A**) Micro-CT image of wild-type (WT) mouse tibia taken at 17 days post surgery or end of distraction phase, reveals no new bone formation within the distraction zone.(**B**) Micro-CT image of FGFR3 deficient (MT, mutant) mouse tibia collected at the same time point, shows minimal new bone formation.

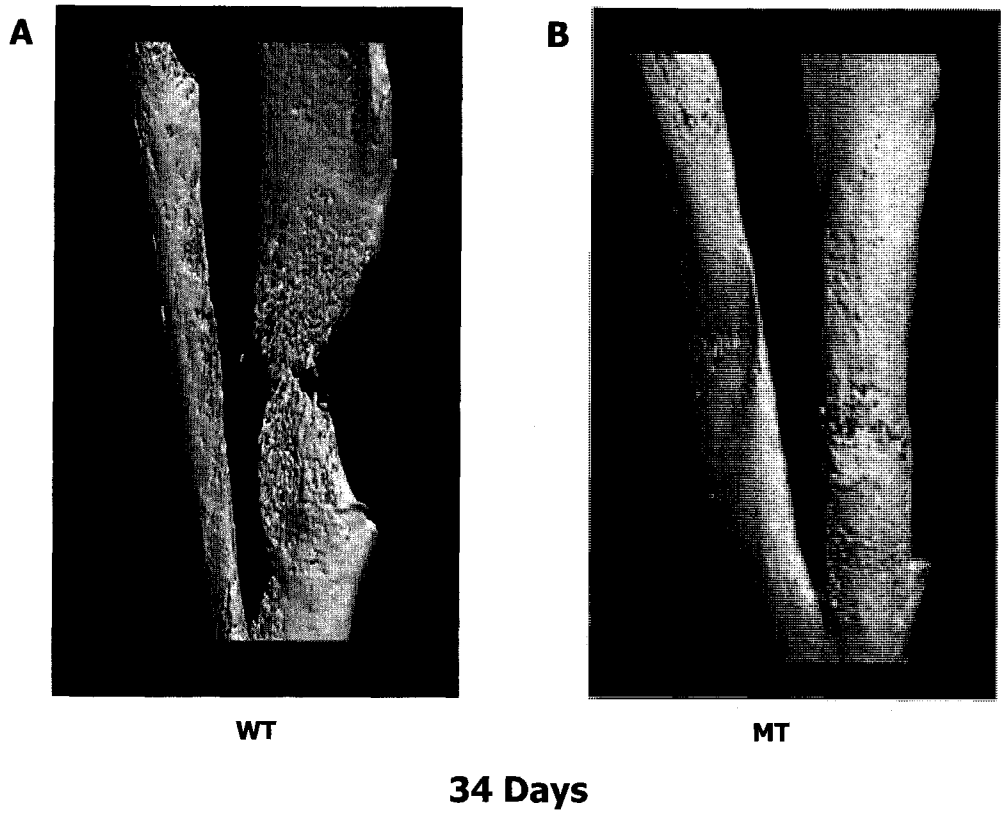

Figure 12: Micro-CT images reveal increased bone formation in FGFR3-defeceint mice at the mid-consolidation phase of DO

Micro-CT images of mouse tibia taken at 34 days post surgery or mid-consolidation phase, reveals an increase in new bone formation in the FGFR3 deficient mice (**B**; MT, mutant) as compared to the wild-type (WT) littermates (**A**). The advancing edges of the mineralization front are still visible in the WT image (**A**), while the MT (**B**) image shows a fully mineralized tibia with no evidence of the osteotomy.

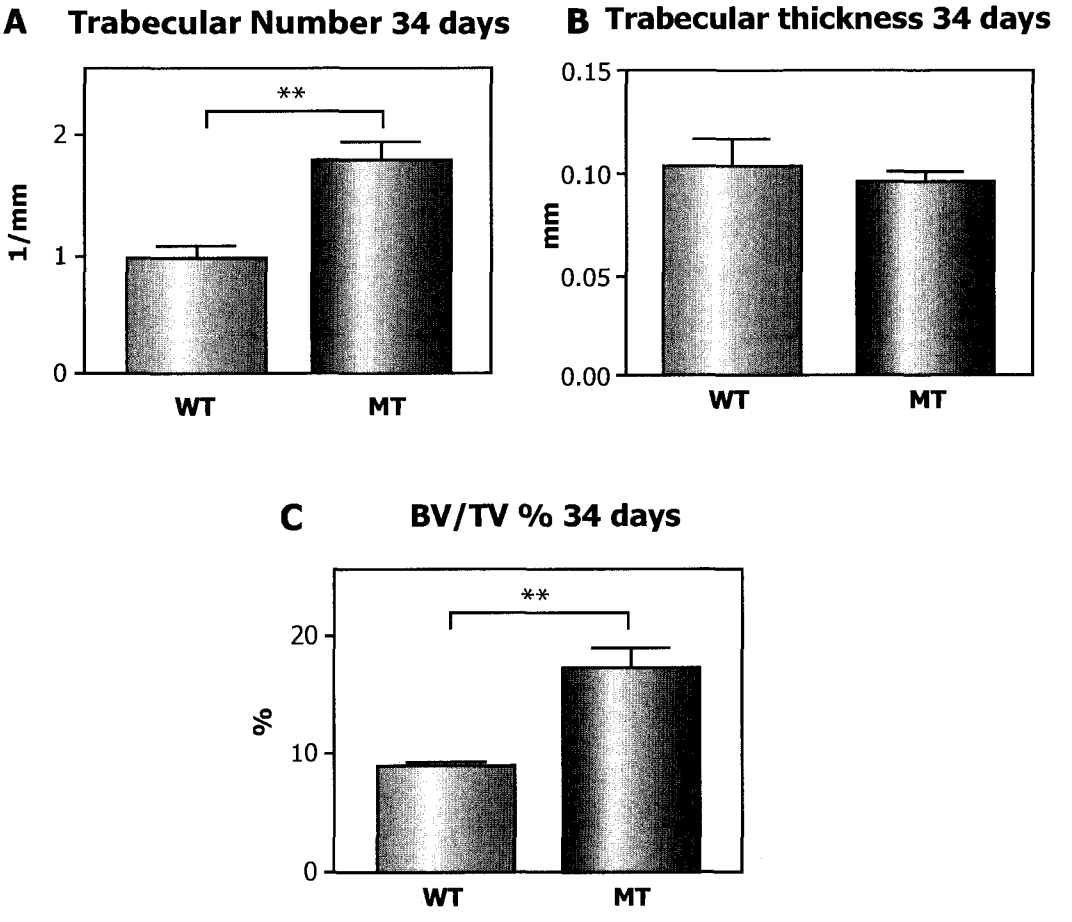

Figure 13: Parameters measured by Micro-CT analysis reveal significant difference between WT and MT mice at 34 days post surgery

Micro-CT parameters analyzed for samples collected at 34 days post surgery or end of distraction phase reveal significant difference between wild-type (WT) and FGFR3 deficient (MT, mutant) tibial samples within (**A**) trabecular number, (**C**) bone volume/tissue volume, but not in (**B**) trabecular thickness,**, $p<0.01$.

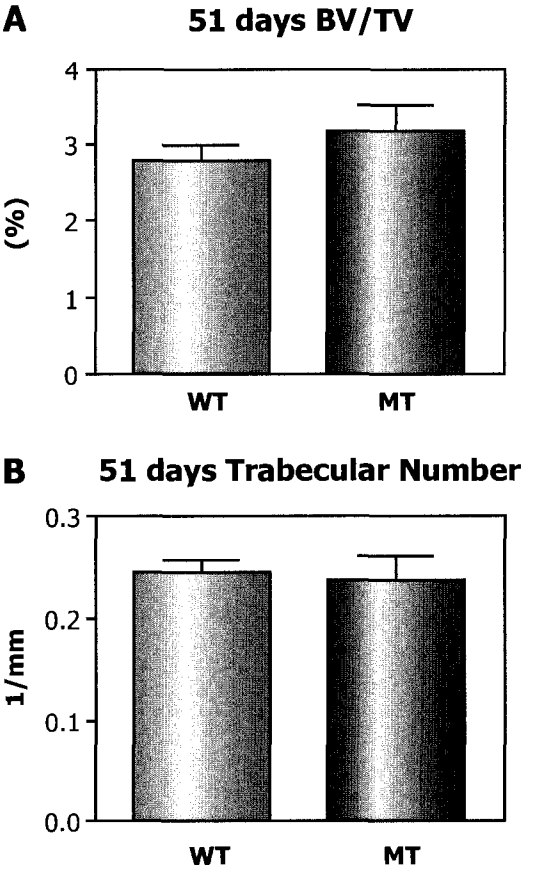

Figure 14: Parameters measured by Micro-CT analysis reveal no change between FGFR3-null and wild-type mice at 51 days post surgery

At 51 days post-surgery or end of consolidation phase, the Micro-CT analysis reveals no difference between the distracted tibias of the wild-type (WT) mice or the FGFR3 deficient mice (MT) in the (**A**) percent bone volume/tissue volume and the (**B**) trabecular number parameters.

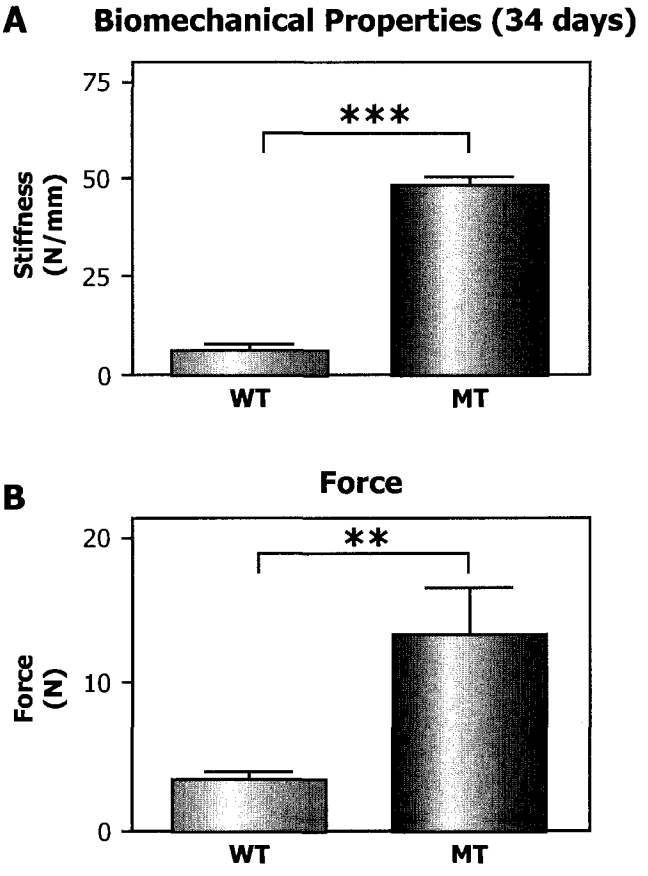

Figure 15: Biomechanical property of distracted tibia collected at 34 days post surgery

The biomechanical property of the newly formed bone within the distraction zone was estimated by measuring the following parameters using the three-point bending technique: (**A**) stiffness of the new bone was significantly increased in the mutant (MT) mice as compared to the wild-type (WT) mice. (**B**) The force required to break the bone was also significantly increased in the FGFR3-ablated mice as compared to the wild-type mice, indicating good biomechanical properties for the new bone in the FGFR3 deficient mice, **, $p<0.01$,***, $p<0.001$.

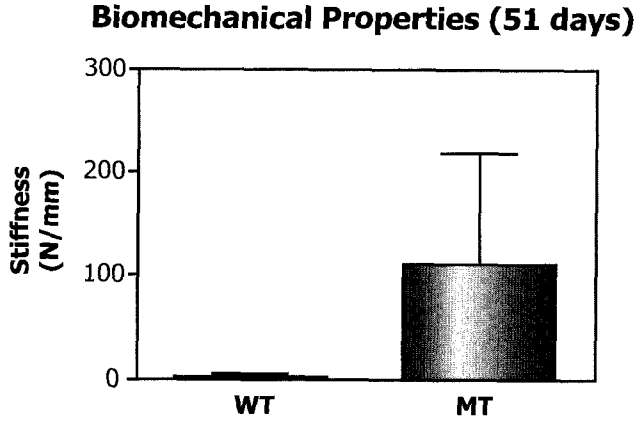

Figure 16: Biomechanical property of distracted tibia collected at 51 days post surgery

The biomechanical property of the newly formed bone within the distraction zone was estimated using the three-point bending technique. An increase in stiffness in the mutant (MT) mice as compared to the wild-type (WT) mice was observed. The results did not reach statistical significance due to low sample size.

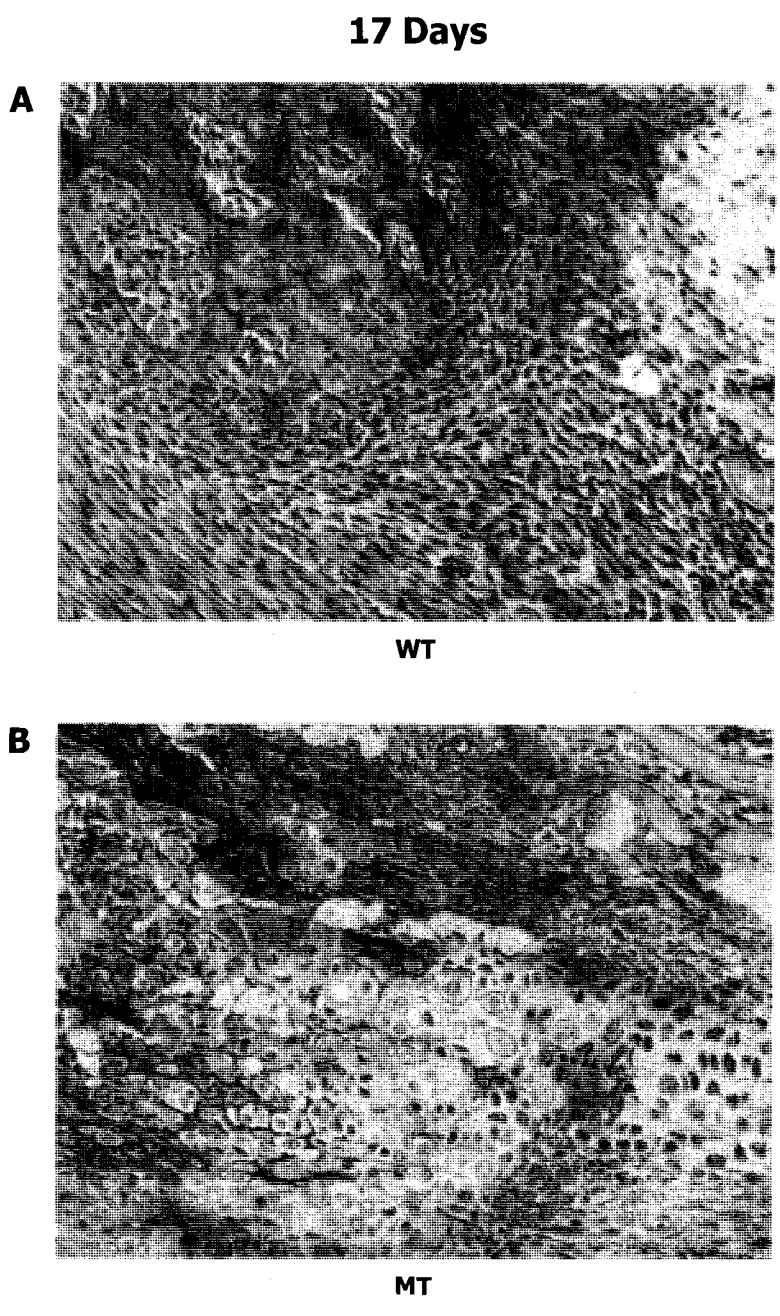

Figure 17: Histology images captured at 17 days post surgery
Samples were embedded in methyl methacrylate, sectioned, and stained with Trichrome Goldner (mineral stain in green). Histology images captured at 40x magnification shows considerably increased mineralized tissue in the FGFR3-/- samples as compared to WT samples (**A**). The FGFR3 deficient sections (**B**) also revealed cartilage-like cells forming a growth-plate like structure were the cells are in columns alongside elongated spindle-like fibrous cells that are typical for intramembranous bone formation, hence a simultaneous combination of both endochondral and intramembranous bone formation at the end-of-distraction phase. WT, wild-type; MT, mutant.

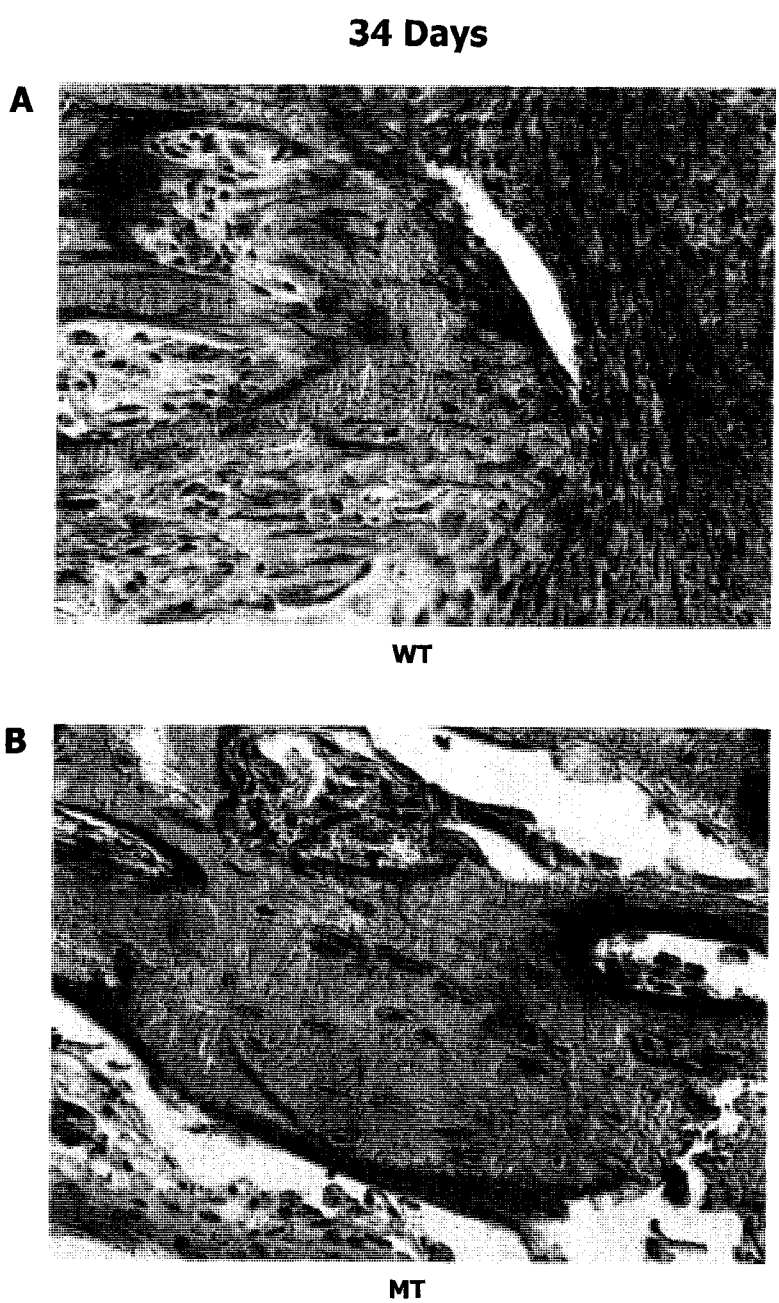

Figure 18: Histology images captured at 34 days post surgery
Samples were embedded in methyl methacrylate, sectioned, and stained with Trichrome Goldner (mineral stain in green). Histology images captured at 40x magnification shows considerably increased mineralized tissue in the FGFR3-/- samples (MT, mutant) as compared to wild-type (WT) samples. The FGFR3 deficient sections (**B**) also revealed osteocytes embedded within the newly mineralized bone, alongside elongated spindle-like fibrous cells that are typical for intramembranous bone formation. However, in the wild-type sections (**A**) there was significantly less mineralized bone and we observed cells typical of intramembranous bone formation.

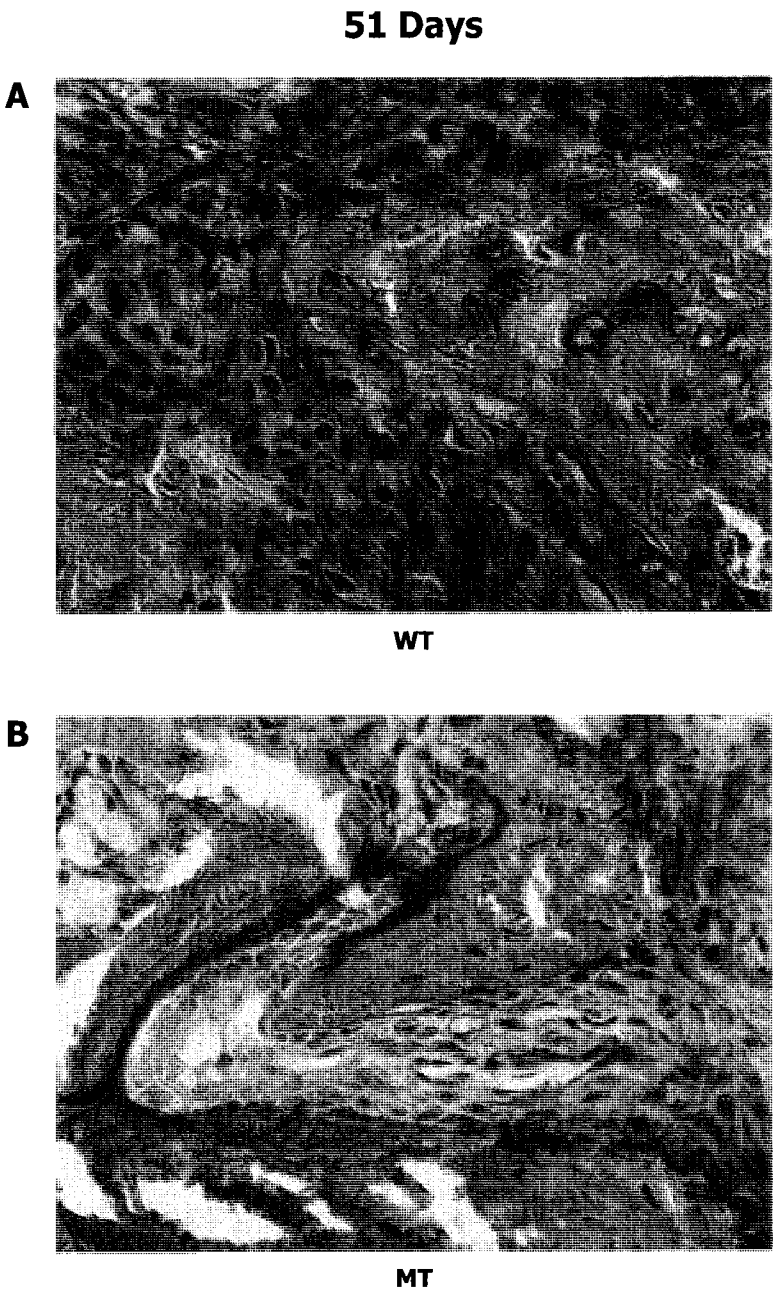

Figure 19: Histology images captured at 51 days post surgery
Samples were embedded in methyl methacrylate, sectioned, and stained with Trichrome Goldner (mineral stain in green). Histology images captured at 40x magnification at the end of consolidation phase when the samples were almost fully consolidated and the new bone was mineralized nevertheless revealed increased mineralized tissue in the FGFR3-/- samples (**B**) as compared to wild-type samples (**A**), and both wild-type and mutant sections showed elongated spindle-like fibrous cells which are typical of intramembranous bone formation. WT, wild-type; MT, mutant.

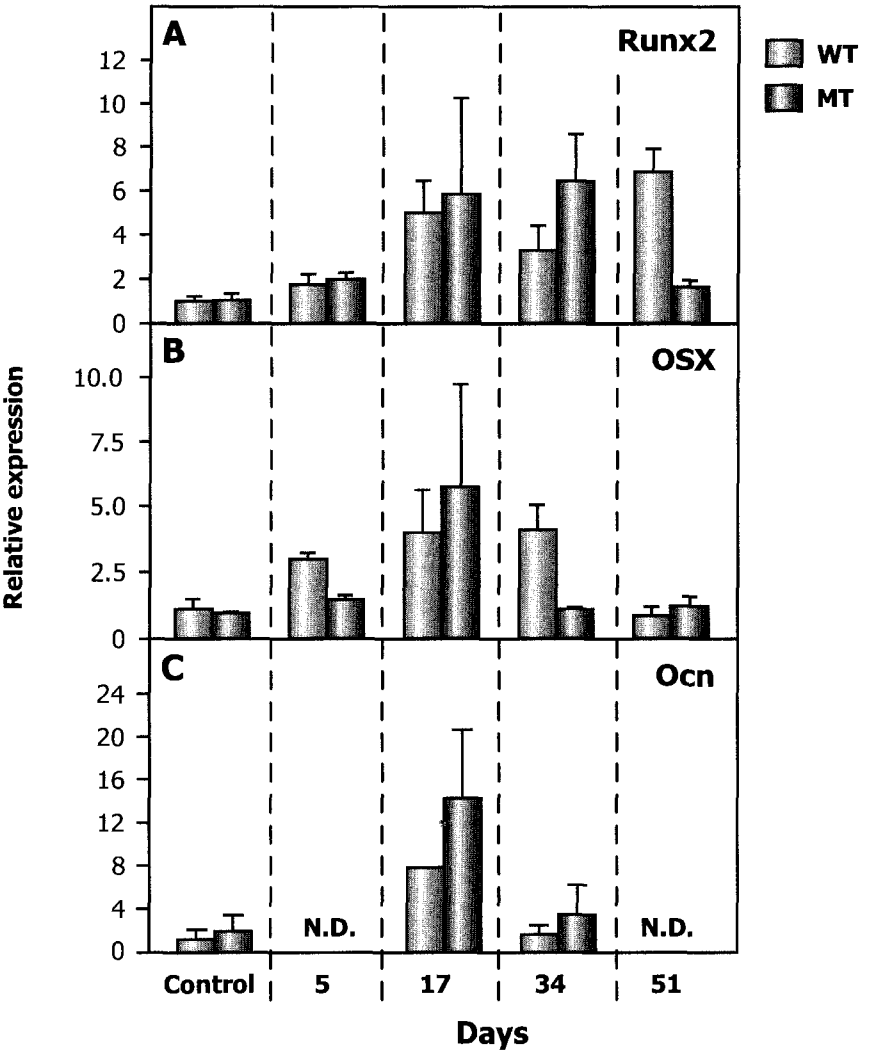

Figure 20: Expression of osteoblastic differentiation markers in FGFR3-null and wild-type mice during DO

RNA collected from tibia at 5 days (end of latency), 17 days (end of distraction), 34 days (mid-consolidation), and 51 days (end of consolidation) post-osteotomy was analyzed by RT-q PCR. Undistracted contralateral bone served as control. Osteoblastic markers were analyzed. Osteocalcin, Ocn (**C**) was non-detectable, N.D. at 5 days and 51 days post-osteotomy,Runx2 (**A**) showed a definite trend but statistically not significant due to a low sample size, and Osterix, OSX (**B**) revealed no significant difference between the wild-type and mutant mice during the phases of DO. WT, wild-type; MT, mutant.

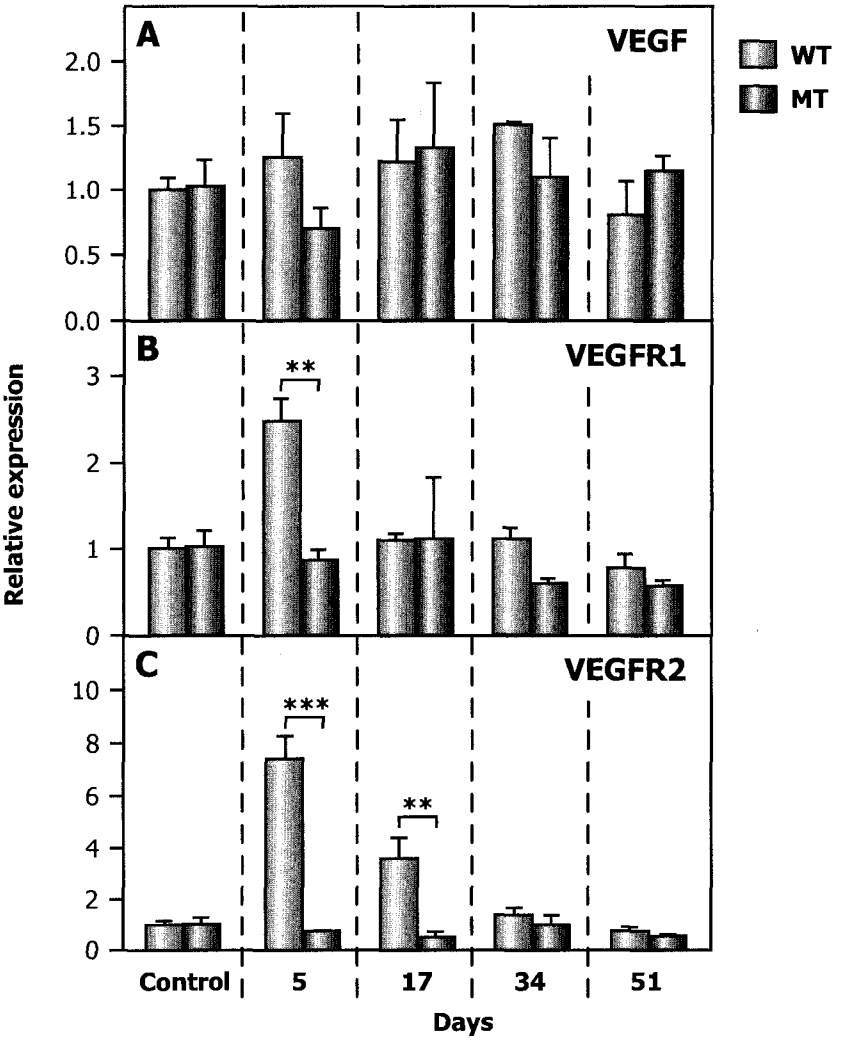

Figure 21: Expression of vascularization differentiation markers in FGFR3-null and wild-type mice during DO

RNA collected from tibia at 5 days (end of latency), 17 days (end of distraction), 34 days (mid-consolidation), and 51 days (end of consolidation) post-osteotomy was analyzed by RT-q PCR. Undistracted contralateral bone served as control. Vascularization markers were analyzed. There was no recorded difference in Vascular Endothelial Growth Factor, VEGF (**A**) expression throughout the phases of DO, between the mutant and wild-type mice. Vascular Endothelial Growth Factor receptors, VEGFR1 (**B**) and VEGFR2 (**C**) recorded increased expression in the wild-type mice at 5 days post-osteotomy or end of latency phase, and at the 17 days post surgery or end of distraction phase for VEGFR2. WT, wild-type; MT, mutant. **, $p<0.01$, ***, $p<0.001$.

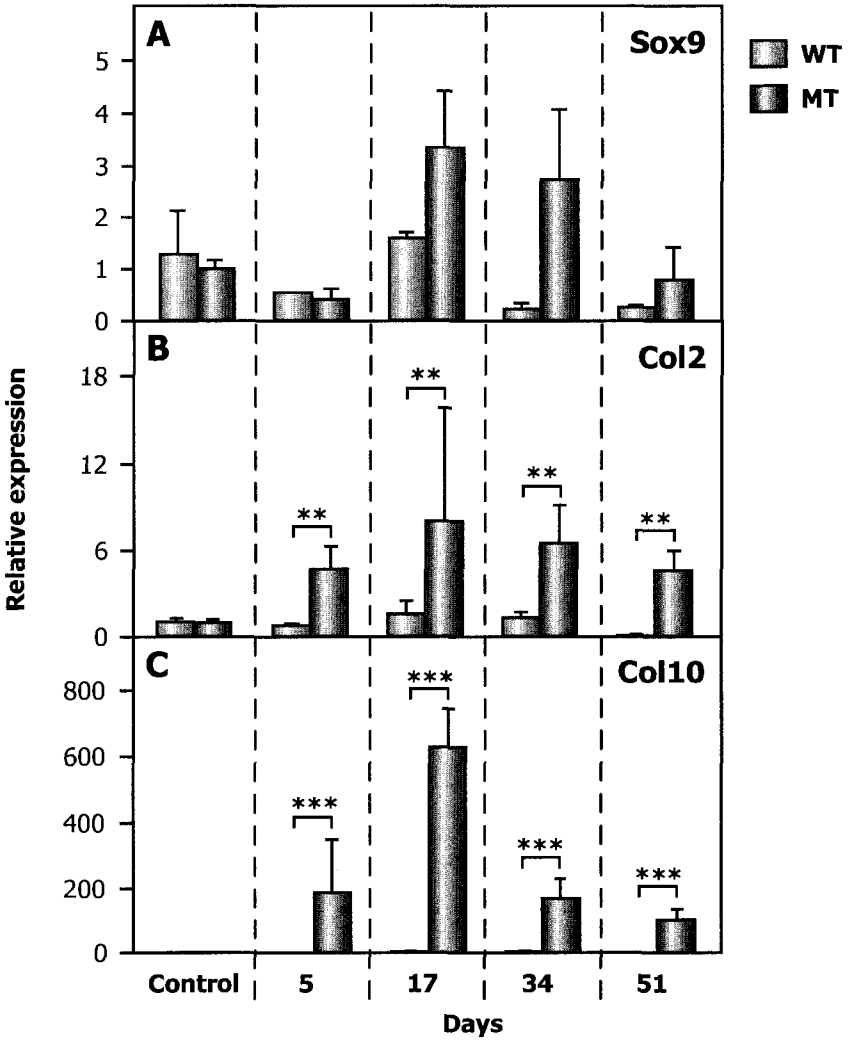

Figure 22: Increased expression of chondrogenic differentiation markers in FGFR3-null mice during DO

RNA collected from tibia at 5 days (end of latency), 17 days (end of distraction), 34 days (mid-consolidation), and 51 days (end of consolidation) post-osteotomy was analyzed by RT-q PCR. Undistracted contralateral bone served as control. Chondrogenic markers were analyzed. Collagen type II, Col2 (**B**) and Collagen type X, Col10 (**C**) had significantly higher expression in the FGFR3-/- mice (MT) as compared to the wild-type (WT) littermates throughout the four phases of DO. Although, Sox9 (**A**) was expressed more in the FGFR3-/- mice at 17, 34 and 51 days post-osteotomy as compared to the wild-type mice, it did not reach statistical significance, **, $p<0.01$, ***, $p<0.001$.

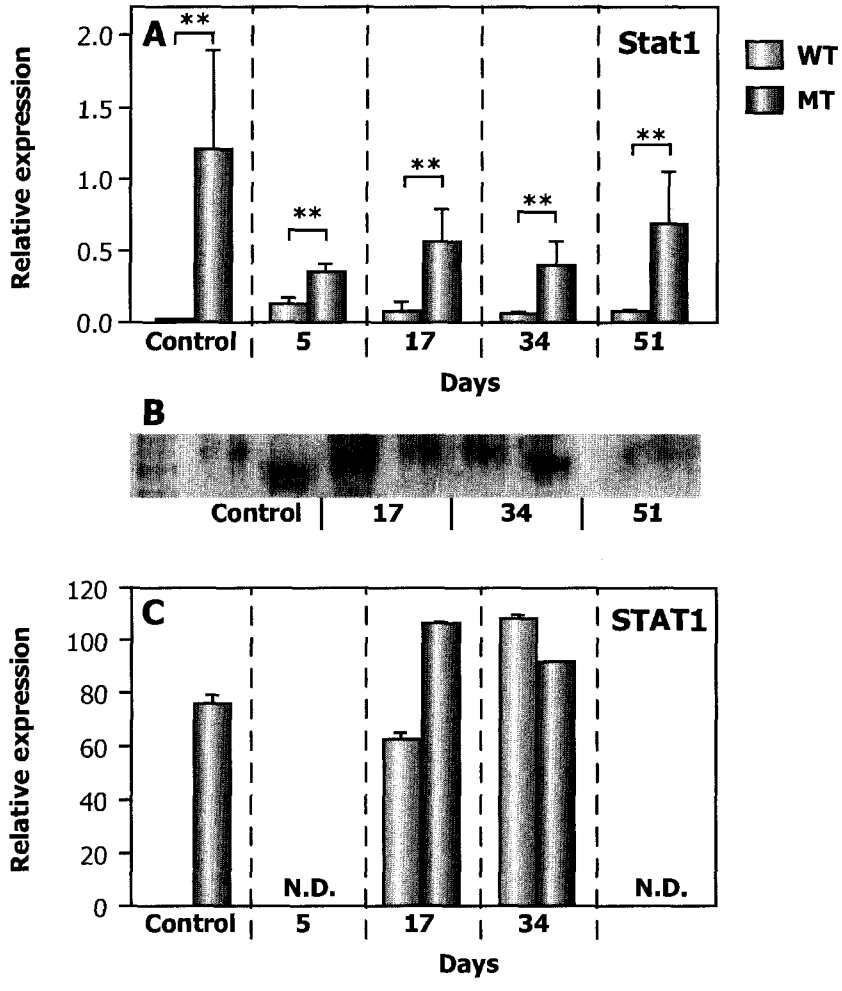

Figure 23: Increased Stat1 mRNA expression but no difference in Stat1 protein expression patterns during DO

Signal Transducer and Activator of Transcription1, Stat 1 expression measured using Real-Time qPCR (**A**) revealed significant difference between wild-type and mutant mice at each of the different phases of DO including the control group.

Tibia samples were collected for protein at 17 days post-osteotomy (end of distraction), 34 days post-osteotomy (mid-consolidation) and 51 days post osteotomy (end of distraction). Stat 1 gave very clear bands on the western blot (**B**), at 86 kDa. Measuring the band intensity (**C**) revealed the highest signal obtained for STAT1 at 17, and 34 days post-osteotomy in both mutant and wild-type mice, however the control samples which were undistracted contralateral tibia, only detected a signal in the FGFR3-/- sample. At 5 and 51 days post-osteotomy we failed to detect any signal for STAT1. WT, wild-type; MT, mutant; N.D., not detected. **, $p<0.01$.