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Bone Formation Around Implants In Adult Transgenic Mice With Selective Runx2-Ii Deficiency

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BONE FORMATION AROUND IMPLANTS IN ADULT TRANSGENIC MICE WITH SELECTIVE RUNX2-II DEFICIENCY

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CLINICAL DENTISTRY

ABSTRACT

Titanium-aluminum-vanadium (Ti-6Al-4V) tacks were placed in the tibiae of 10 female wild type (Runx2 $^{flox/+}$ or Runx2 $^{flox/flox}$) and heterozygous (Runx2$^{+/-, ColII\ Cre}$) mice littermates to examine healing and bone response. Animals were divided into control and test groups of 5 each. A novel generation of Runx2 knock-out mice where the gene is only deleted in chondrocytes was used. Ten days after implant surgery, specimens were recovered and prepared for histological, histomorphometric, and micro-CT morphometric analysis. Histological examination revealed that wild type animals presented larger regions of bone formation and endochondral ossification when compared to heterozygous mice. Micro-CT morphometric analysis of the same bones demonstrated that tissue response area (TRA) was larger in the wild type ($1.06mm^2 \pm 0.25$) than in the heterozygous mice ($0.60mm^2 \pm 0.28$). This result was statistically different when groups were compared ($P < 0.05$). In addition, bone formation area (BFA) for the wild type was 0.25mm2 (±0.06) while BFA for heterozygous mice was $0.12mm^2$ (± 0.05). This represented a significantly different ($P < 0.05$). Bone formation around implant sites in wild type animals was statistically greater than the heterozygous group. Histological and micro-structural findings were supported and quantified by histomorphometric analysis, which established that titanium implants placed in the wild type group showed greater bone-to-implant contact percent (76.9 ± 6.5) than devices placed at the heterozygous group (57.5 ± 11.6). Once more, the results were significantly different ($P < 0.05$).

Results of the experiments revealed that Runx2 activity specifically in chondrocytes is required for a normal healing response to implants and that loss of one copy can alter the process of bone formation in a mouse model.

DEDICATION

To my parents (Patricia and Jaime) and to my fiancée, Andrea.

ACKNOWLEDGMENTS

I would like to express my sincere appreciation and thanks to all persons involved in this investigation. In first place, to my mentor Dr. Michael McCracken, for his patience, guidance, and dedication throughout the course of the master's program. To the members of my committee, thanks. Especially, to Dr. Amjad Javed and Dr. Jack Lemons. My appreciation goes to my colleagues (Dr. Xia, Dr. Chen, Dr. Li and Annie) who gave me technical, laboratory and clinical support to successfully complete this research project. My gratitude goes to my family, my fiancée and my dear friends at both, the Biomaterials and Prosthodontics programs, who with support and care, made this academic experience unforgettable.

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INTRODUCTION

The long-term success of osseointegrated implants in the treatment of completely and partially edentulous patients with a sufficient amount and quality of bone has been well documented in the literature.[1, 37] Initial stability of the implant is, in effect, one of the fundamental criteria for obtaining long-term osseointegration.[3] Achieving implant stability depends on the implant-bone relation, the surgical technique and on the microscopic and macroscopic morphology of the implant used.

The osseointegration mode of implants is influenced by the features of the implant system. Important aspects of a more rapid implant osseointegration include the need to achieve a primary congruence between the implant and the bone directly after insertion, the need to insert the implant with minimal surgical trauma and the capability of the implant surface to integrate with the adjacent bone. It has generally been thought in implant dentistry that osseointegration requires a healing period of at least 3 months in the mandible and 5 to 6 months in the maxilla depending on local bone density and other patient based considerations.[8]

Dental implants continue to provide a predictable and viable treatment option for patient's missing teeth.[28] However, there are some clinical situations where bone formation around the implants is not achieved. The reasons can be related to different systemic diseases, infections, smoking, healing problems, genetic disorders and other factors. Sometimes these factors are difficult to identify. One area of research is the genes which are related to failure and a deficient healing process. A gene that plays an

important role in the bone formation process is *Runx*2. This gene transcribes *Runx*2-II and *Runx*2-I isoforms with distinct N-terminal. Deletion of both isoforms results in complete arrest of bone development, whereas selective loss of *Runx*2-II is sufficient to form a grossly intact skeleton with impaired endochondral bone development.[36]

Implant Healing

Long term clinical success of implant supported restorations is critically related with a biological process called "osseointegration". This process originally called for healing periods of several months and was aimed at the establishment of a direct bone-to-implant contact that, according to definition, must be documented by means of histology. The prerequisites for osseointegration included: minimal trauma during surgery, establishment of primary implant stability and avoidance of infection and micromotion during healing.[7]

The initial host response after implantation is characterized by an inflammatory reaction elicited mainly by the inevitable surgical trauma and modified by the presence of the implant. The osteotomy for the implant is comparable to a bone wound. In the early bone response to the implant, first contact with the implant surface is the blood clot, with associated platelets and fibrin. Peri-implant tissue healing starts with an inflammatory response as the implant is inserted in the bone cavity, but an early afibrillar calcified layer comparable to the lamina limitans or incremental lines in bone is just observable at the implant surface both in vitro than in vivo conditions.[27] Inflammatory cells, initially polymorphonuclear granulocytes, and later monocytes, migrate from post-capillary venules into the tissue surrounding the implant. After the blood comes into contact with

the implant surface, proteins are adsorbed from blood and tissue fluids. At the implant side an oxidation of metallic implants has been described both in vitro and in vivo.[4, 34]

Within the first day of implantation, mesenchymal cells, pre-osteoblasts and osteoblasts cover the implant surface as an afibrillar calcified layer that results in collagen fibrils of osteoid tissue. In this way, less differentiated cells in the osteogenic lineage, or perhaps mesenchymal cells, migrate to colonize the implant surface.[15] Recent studies have shown that the interaction of red blood cells, fibrin and platelets with the implant surface may modulate the migration, differentiation and activity of osteogenic cells during peri-implant healing.[16]

Within a few days of implantation, a woven bone is identified. Then a reparative trabecular bone delimiting large marrow spaces rich in blood vessels and mesenchymal cells is present at the gap between the implant and the host bone. The peri-implant osteogenesis can proceed from the host bone to the implant surface (*distant osteogenesis*) and from the implant surface to the host bone (*contact osteogenesis*) in the so called *de novo* bone formation. This early bone response to the implant gradually develops into a biological fixation of the device and consists in an early deposition of a newly formed reparative bone just in direct contact with the implant surface. To successfully complete this process the vascularization at the surgical site is of critical importance.[12]

An early good biological fixation is also known as ‘primary stability” which consists of a rigid fixation of the implant within the host bone cavity together with a lack of micromotion of the implant. In some situations this fixation may allow shortening the time, before loading the implant, favoring the clinical procedure of early or immediate implant loading. There have been many retrospective investigators that have concluded

that primary stability is the first clinical outcome of success in implant therapy.[8, 21, 29] However, this condition is not always achieved clinically. Some studies have described fibrous tissue membrane formation around the implant that may be caused by the excessive mobility of the device.[10, 32] This formation causes displacement at the bone-implant interface inhibiting osseointegration.

In case osseointegration is achieved, the remodeling capacity of the bone is tested. Like normal and physiological bone adaptation in the skeleton, wounds maturate around implants via modeling and remodelling mechanisms. These are the two primary mechanisms by which bone at the interface can adapt to mechanical loading, and are thought to be responsible for reshaping or consolidation of bone at the implant site.[9] Mature adult bone is continuously being turned over, so that the net activity of bone resorbing cells equals the net activity of bone forming cells. Remodeling includes the process of bone resorption followed by bone formation and provides a mechanism for self repair and adaptation to stress. Bone remodelling is a complex process involving not only interactions between cells of the osteoblastic lineage and bone matrix proteins, but also a variety of systemic and local regulatory factors.[18]

During the past decade, important advances have been made in the understanding of the endogenous basic regenerative potential of bone.[26] Right now special attention is given to the different type of cells, growth factors and genes, cytokines and tissues involved during the inflammatory, formation and remodeling phases of bone healing and not just in the reaction of the host to a specific implant material. Improving our knowledge in this specific field will give to the scientific community tools to better comprehend the physiopathology of implant failure in some clinical situations.

Genetics of Bone Formation

Formation of bone during development and skeletal remodeling requires the temporal and interdependent expression of osteoblastic cell growth and phenotypic genes. Many laboratories have contributed to the definition of the sequence of events that result in the maturation of osteoblast, on the basis of stages that are characterized by the expression of genes and functional properties. Parameters of osteogenesis have been established using in vitro cell culture and in vivo rodent models, and by determining modifications in gene expression in normal and affected tissue. Profiles of gene expression in vivo further define the substages of osteoblast maturation, and these substages are altered as a result of genetic mutations [25]

There are three major stages of osteoblastogenesis: proliferation, matrix maturation, and mineralization. These are characterized by sequentially expressed genes that support the progression of osteoblast differentiation through developmental transition points. The first transition requires exit from the cell cycle and commitment to osteoblast phenotype; and the second transition requires signals for extracellular matrix mineralization, which promotes completion of osteoblast differentiation to the mature osteocyte phenotype. Normal bone development is initiated by the coordinated expression of developmental regulatory proteins that dictate the temporal and spatial organization of the osteogenic cells.[25]

Runx2 is a master gene regulating bone development. The Runx2 gene produces two major transcripts driven by alternative promoters designated P1 and P2, respectively. Runx2 Type I (Runx2-I) is derived from the P2 proximal promoter and begins with the sequence MRIPV encoded by Exon 2. Runx2 Type II (Runx2-II, formerly called Osf2) is

controlled by the P1 distal promoter and begins with MASNS encoded by exon1.[20] Thus, there are 4 alleles that generate two distinct gene products, Runx2-I and Runx2-II, which differ only by the amino acids at their N-terminal region.[5] Homozygous Runx2 null mice (derived from inactivating mutations affecting all four alleles) results in arrested bone formation and the complete lack of all mineralized skeletal elements due to the absence of osteoblastic differentiation.[24]

Different Roles of Runx2

Several investigators have studied the roles of Runx2 in different physiological and pathological processes. Runx 2 was identified as a genetic cause of the cleidocraneal dysplasia disorder (CCD), an autosomal dominant disease characterized by a hypoplastic clavicle, open fontanelles, supernumerary teeth, short stature, and changes in skeletal patterning.[24] Hypomorphic mutations of Runx2 also have been described that result in mild CCD or dental phenotype characterized by delayed eruption of permanent teeth.[38] Genetic ablation of Runx2 results in mice that die at birth without a mineralized skeleton. The homozygous Runx2 -/- mice completely lack both intramembranous and endochondal bone ossification, whereas the heterozygote lacks clavicles but otherwise has a normal skeleton. Thus, a full gene dosage is required for clavicle development in the early stages of embryogenesis, consistent with the CCD phenotypes in humans caused by haploinsufficiency of Runx2.[13]

Using in vitro pluripotent cell models, it has been documented that Runx 2 can activate genes characteristic of the mature osteoblast phenotype but lacks the ability to produce a mineralizing bone matrix, in contrast to BMPs, Runx2 remains the earliest

transcription factor essential for bone formation.Runx2 also functions in cellular differentiation of non-osseous tissues. Runx2 is induced in endothelial cells by IGF-1, where its expression appears to contribute to the ability of these cells to form tubular vessels. Runx2 also regulates cell migration and vascular invasion in bone.[23]

Notably, angiogenesis is essential for bone formation, and Runx 2 regulation of these processes may reflect a regulatory network of signals for the timing of these events, as required by tissue formation and turnover. Runx2 expression in endothelial cells has been linked to vascular calcification. Pathologic expression of Runx2 in breast and prostate cancer cells appears to be related to metastasis. The activation of Runx2 and osteoblastic genes in the cancer cell in the bone microenvironment also contributes to the resulting osteolytic lesions that form the bone tumor.[6]

The association of Runx2 to a very specific subnuclear domain mediated by a signal that is present only in the Runx family of proteins may provide a mechanism for specificity in recruiting coregulatory proteins and organizing transcription factor complexes that control tissue-specific gene expression.[25] Thus Runx2 is an integral component of a mechanism for the convergence of osteogenic signaling pathways on gene promoters.

One function of Runx factors in supporting organogenesis appears to involve their function in cell growth control. Some authors have discovered that Runx2 has a growth suppressive role in osteoblasts and may support the evolution stage from proliferation to exit from the cell cycle for phenotype commitment.[33]

Examination of the growth properties of Runx2-deficient cells proposes that the Runt-related transcription factor Runx2 is essential for stringent control of osteoblast

proliferation. Calvarial-derived osteoprogenitors cells from Runx2–/– and Runx2ΔC/ΔC (deleted in both C terminal regions) mice display increased cell growth upon inactivation of Runx2 compared to wild-type. Additionally, the reintroduction of Runx2 into Runx2-deficient cells by adenoviral delivery restores stringent control of proliferation in osteoblasts. Consistent with the cell growth regulatory role of Runx2, other studies in normal osteoblasts established that the levels of Runx2 are strongly regulated upon entry and exit from the cell cycle in osteoblasts.[25]

Runx2 executes several biological roles in the osteogenic lineage. Runx2 supports osteoblast maturation at a key developmental transition by attenuation of osteoblast growth and functionally supporting exit from the cell cycle, as well as promoting lineage commitment and bone cell phenotype development by activating genes specific for mature osteoblasts. The central role of Runx2 in the development of the osteoblast phenotype is feasible through its structural and functional properties, which facilitate these mechanisms of transcriptional control.[25]

Genes and Implant Failure

During the past two decades, osseointegrated implant dental treatment has become a safe, predictable and successful prosthetic alternative for many patients around the world. However, in some clinical situations, failures may occasionally occur. Many studies have been conducted in this area to determinate possible reasons for this clinical complication.

Usually these studies are more related with the inherent characteristics of the implant used (design, surface, material, occlusion, etc.) than with the specific host

response to the treatment. This tendency may be changing and more immunological, microbiological and genetic research is been done in this area.

Genetic research is usually divided into two different approaches: the normal (bone healing process) and the disease (Implant failure). In the first one, some authors have isolated genes that are differentially expressed in bone healing with implants; providing evidence that selected gene transcripts are induced by titanium implants under regulatory control strongly associated with the nature of osseointegration.[31]

The disease approach is more complex. Different specific host characteristics may disturb the healing process; genetic disorders are one of them. Unfortunately, there is not much information about the relationship of this condition and dental implant failure. There have been some previous investigators that have conducted research in this area trying to determinate the association between gene polymorphism and this clinical condition.[11, 19] These authors investigated whether the individual's capacity to produce higher or lower quantities of interlukin-I could influence implant failure mechanism and also the association of the transforming growth factor-ß1 with early implant failure. It is possible that functional polymorphisms in other inflammatory mediator or growth factor genes could have a more determinant role in early osseointegration failure. However, these investigations failed to present a genetic association in implant loss. The role of this gene polymorphism remains to be clarified.

The determination of genetic disorders related with implant failure could be of clinical value to a precise and early identification of patients at high risk for implant loss. In general, factors associated with the patient appear more critical in determining risk for implant failure than those associated with the implant itself. Several risk factors can be

modified. In identifying these implications and making appropriate interventions, clinicians can enhance success rates while improving oral function, esthetics, and patient well-being.

RATIONALE AND OBJECTIVE

The rationale for this study is based on results from previous studies regarding the important role of Runx2 during the bone formation process in the rodent model. These results have stimulated the interest of the scientific community to understand the relationship of this specific gene with some pathological human conditions. The majority of these animal studies have described the functions of this gene in normal physiological scenarios. However, there is no information available in the literature about the role of the haploinsufficiency of this gene at more complex processes, such as bone healing subsequent to the implantation of a prosthetic device. The overall purpose of this study was to establish the role of Runx2 in the bone formation process around titanium implants placed in the tibia of mice.

We tested the following hypothesis:

There will be a measurable difference in the bone formation quantity associated with titanium implants placed in mice with Runx2 haplo insufficiency (+/-) compared to wild type mice (+/+).

MATERIALS AND METHODS

Experimental Animals

The study protocol was approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham. Animals were maintained in accordance with the standards of the Guide of Care and Use of Laboratory Animals (NCR 1996).

Runx2 floxed mice were generated by Dr. Amjad Javed (unpublished). In this mice line, exon 8 of Runx2 is floxed (Runx2 $^{flox/flox}$). To generate cartilage-specific Runx2 knockouts, we crossed the transgenic line that express Cre recombinase under the control of the Collagen type II promoter (kindly provided by Dr. Rosa Serra) with Runx2 $^{flox/flox}$ mice to generate heterozygous mice with one allele Runx2 exon 8 deleted (Runx2 $^{+/-, ColII\ Cre}$). Runx2 $^{+/-, ColII\ Cre}$ male mice were crossed to Runx2 $^{flox/flox}$ female mice. The offspring thus has following genotypes: (a) Runx2 $^{-/-,ColII\ Cre}$, (b) Runx2 $^{+/-,ColII\ Cre}$ (c) Runx2 $^{flox/+}$ and (d) Runx2 $^{flox/flox}$. Homozygous Runx2 $^{-/-, ColII\ Cre}$ died immediately after birth. Then, ten female wild type (Runx2 $^{flox/+}$ or Runx2 $^{flox/flox}$) and heterozygous (Runx2$^{+/-, ColII\ Cre}$) mice littermates were selected and divided into control and test groups of 5 each. Consequently, five pairs of wild type and heterozygous received implants surgery. Age of animals ranged from 3 to 14 months. Skeletal differences between wild type and heterozygous mice are displayed in Fig 1. All the animals in both groups received one titanium-tack at the flat surface of the tibia. The sample size was 10 animals, 5 wild type and 5 heterozygous (Runx2-II mutant) mice.

Surgical Protocol

All procedures were performed using sterile surgical technique. Preoperatively, the animals received acetaminophen diluted in water (300ml/kg) for 2 days. Instruments were sterilized using steam autoclaves. Mice were anesthetized with volatile gas (Isoflurane), and shaved and scrubbed to present a surgical field, which was draped using sterile surgical towels. A 0.5 mm incision was made along the medial proximal aspect of the tibia to expose the bone (Fig 2). Periosteum was reflected from the bone exposing the flat surface of the tibia below the knee. A slow surgical handpiece (Nobel Biocare, Yorba Linda, CA) was used to create a perpendicular osteotomy, traveling through the medullary canal and the opposite cortical plate using carbide ¼ round burs and copious saline irrigation. Subsequently, a 0.7 mm diameter titanium bone tack (BioHorizons, Birmingham, AL) was placed with a delivery instrument into the osteotomy. The surgical site was closed using resorbable sutures. Skin was approximated using Vicril 6.0 suture. All mice recovered from the surgery and displayed normal mobility and activity after 30 minutes or 1 hour. Acetaminophen diluted in water (300ml/kg) was administered for 3 days following the procedure. Implants were allowed to heal for 10 days. Mice were euthanized with carbon dioxide inhalation followed by cervical dislocation.

Specimen Preparation

Ten tibias were retrieved and soft tissue was removed. Specimens were fixed in phosphatebuffered paraformaldehyde for 24 hours and then transferred to 70% ethanol. An acrylic square guide was fixed at one end of every specimen to guarantee the correct orientation of the sample during the micro-CT procedures (Fig 3). Tacks were removed

manually carefully using a needle holder. The metallic surfaces were then examined for any remnants of tissues.

Micro-CT Analysis

The micro-CT device, a μCT 40 microfocus X-ray source (SCANCO Medical AG, Brüttisellen, Switzerland), equipped with a complete imaging and evaluation software for image acquisition and slice reconstruction, was used (Fig 4).

A total of 10 micro-CT scans were taken for control and experimental specimens. Longitudinal orientation of the sample was carefully supervised. High resolution (8 μ slices) was used for all the scans. After scanning, images were made parallel to the longitudinal direction of the tibia and to the long axis of the titanium tack. Subsequently, a micro-CT section at the middle of the tack site was selected for bone mass comparisons.[17] Supracortical tissue response area (TRA) corresponding to the proximal dorsal area of healing and bone formation area (BFA) corresponding to proximal dorsal area of new bone were quantified in square millimeters. TRA was defined by corresponding histological section of same bone. After micro-CTs were complete, three-dimensional reconstructions were obtained; selecting 120 scans corresponding to the implant site (0.7mm) and surrounding bone (0.3mm). Final approximated dimension of each 3D reconstruction was 1mm x 3mm.

Histological Preparation and Analysis

After micro-CT evaluation, samples were decalcified for 24 hours in 5% formic acid solution. X-rays were taken before and after the procedure to determine the endpoint

of the decalcification process.

Acrylic guides were removed with a surgical blade. Dehydration, clearing and infiltration processes were automatically achived using a tissue processor (Sakura Finetek Company, Tokyo, Japan). Dehydration was performed in serial steps of graded ethanol, followed by a clearing reagent (xylene). Paraffin infiltration was achieved. Special care was taken during this step to ensure that the specimens were in the correct orientation to make sections directly comparable with the micro-CT scans.

Five microns sections were produced from each specimen with the Leica RM 2135 manual rotary microtome (Leica Microsystems Inc, Bannockburn, IL, USA). The sections were stained with hematoxylin & eosin (H&E).

Histological analysis was performed using a Nikon Eclipse TE-2000 E microscope equipped with an image system (NIS-Elements Advanced Research, Nikon Instruments Inc, Melville, NY, USA). Digital micrographs were obtained using a digital camera (Nikon Digit Sight DS-Fi1) connected to the microscope (Fig 5).

Histomorphometry

A middle section representing the 50% level of each specimen was selected for histomorphometric analysis. Each section was examined and photographed by light microscopy and then analyzed using BioQuant Image Analysis System (R&M Biometrics, Nashville, Tenn, USA). The high-resolution micrographs (final magnification ×20) permitted the quantification of the percentage of bone integration of the titanium tack as described below. Because the tacks were removed before the micro-CT analysis, a special digital template was constructed to facilitate the calculation of the percentage of

bone-to implant contact (Fig 6). In this way, the following histomorphometric parameters were determined:

1. Length of titanium surface (L1): corresponding to the total length of the outer-most titanium tack surface in contact with the tissues.

2. Length of bone integrated tissue (L2): corresponding to the total length of bone integrated tissue in contact with the outer-most titanium tack surface.

3. Bone to Implant Contact (BIC) = (L2/L1) × 100.

Statistical Analysis

Comparisons of mean BIC (Bone to Implant Contact), TRA (Tissue Response Area) and BFA (Bone Formation Area) between the control and experimental groups were made using analysis of variance (ANOVA). Age was included as a blocking variable, in order to remove possible effects of age differences from the treatment group comparisons. Alpha was set at 0.05 for all comparisons. Data was analyzed using SAS statistical software, release 9.1.3. (SAS Institute, Cary, NC, USA).

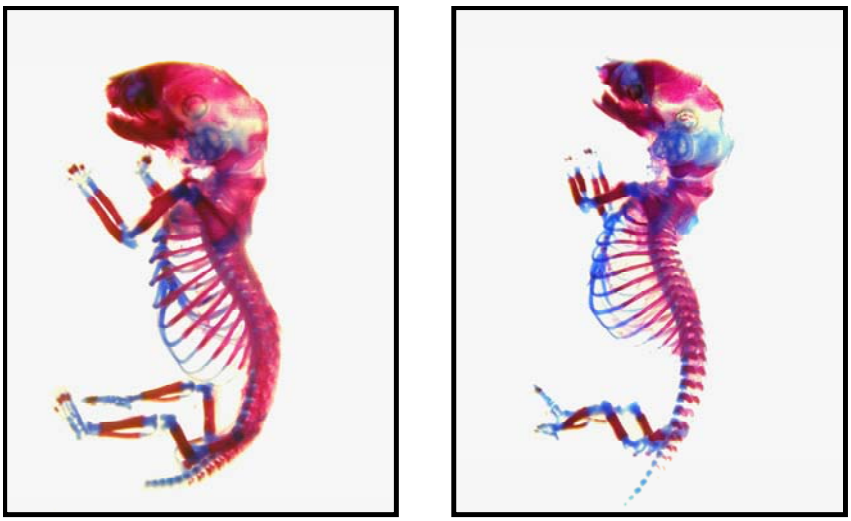

Fig 1 Skeletal differences between wild type and heterozygous mice. Wild type (left) animal develop a normal skeleton while heterozygous (right) animal (genetic ablation of half of the gene) is characterized by the absence of clavicles but otherwise a normal skeleton. Red color corresponds to the bone and blue color corresponds to the cartilage.

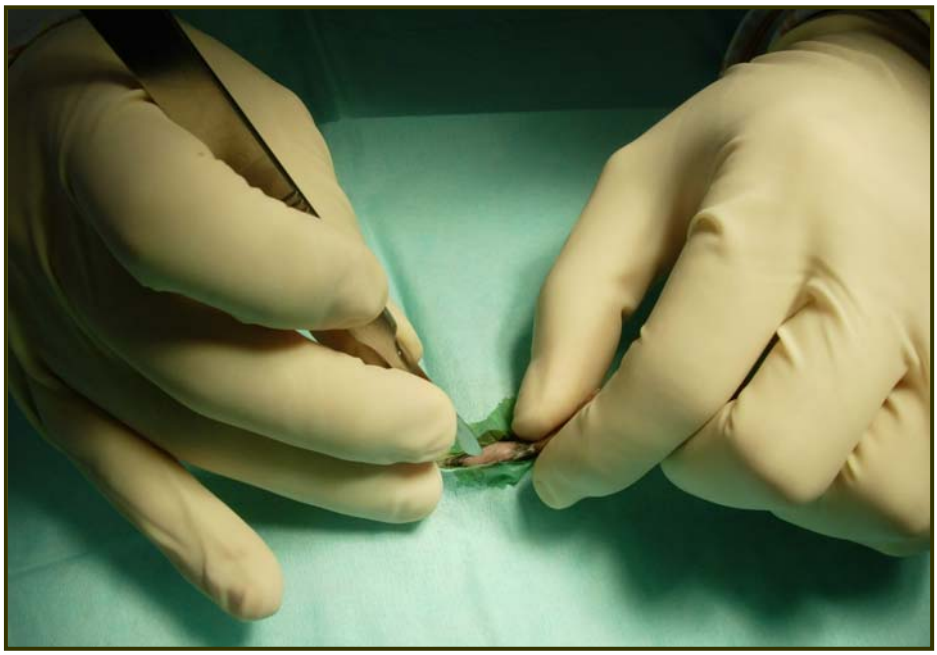

Fig 2 View of the surgical sterile field during the incision at the medial proximal aspect of the mouse tibia.

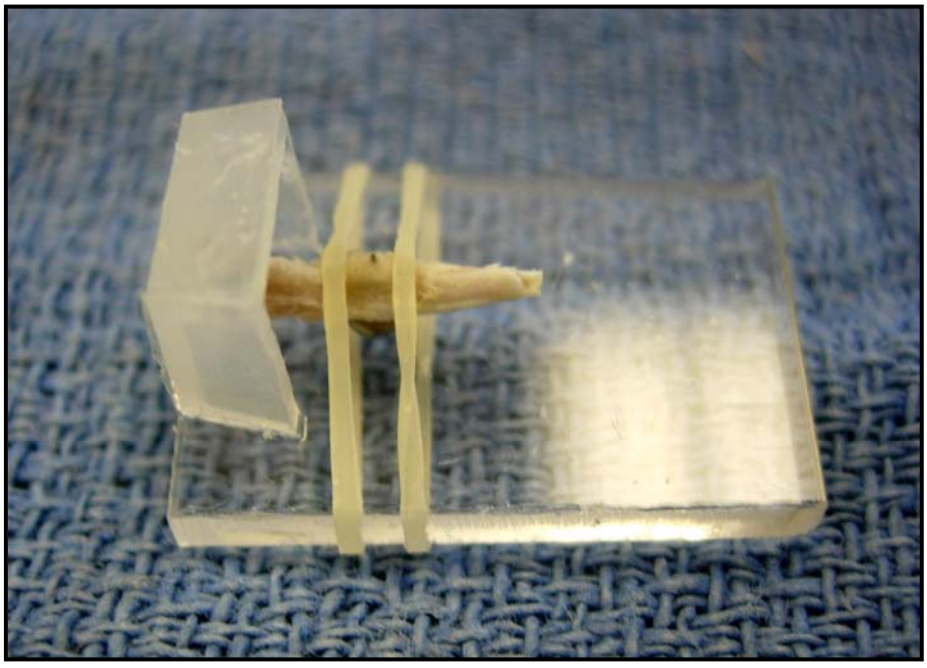

Fig 3 The acrylic positional guide. This guide was used to keep the orientation of the sample during the micro-CT analysis.

Fig 4 Micro-CT device utilized in this study (SCANCO μCT 40) to analyze bone formation adjacent to the implants

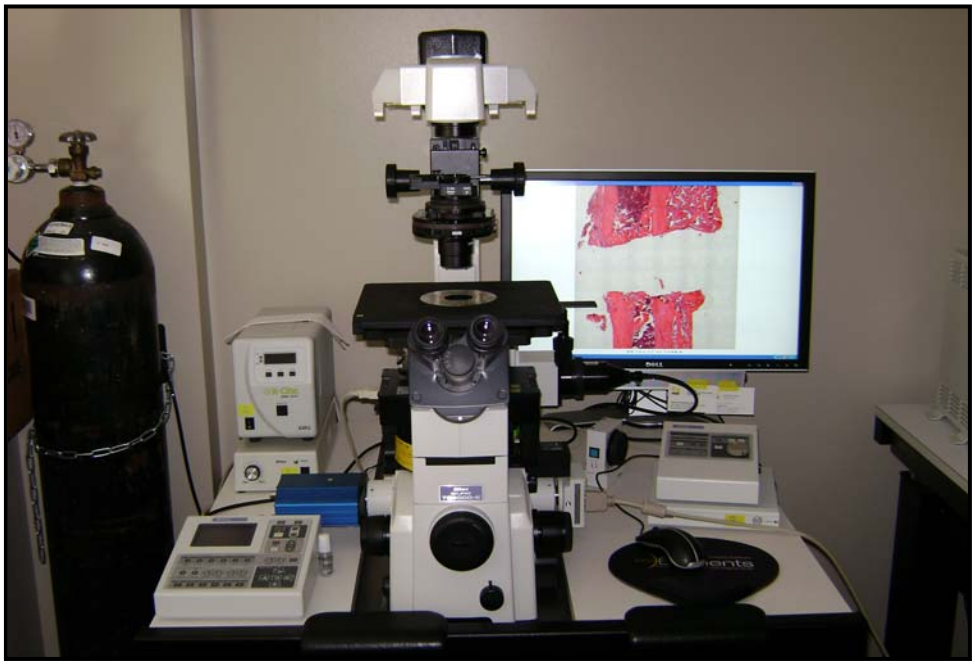

Fig 5 Digital microscope and software system used for the histological analysis.

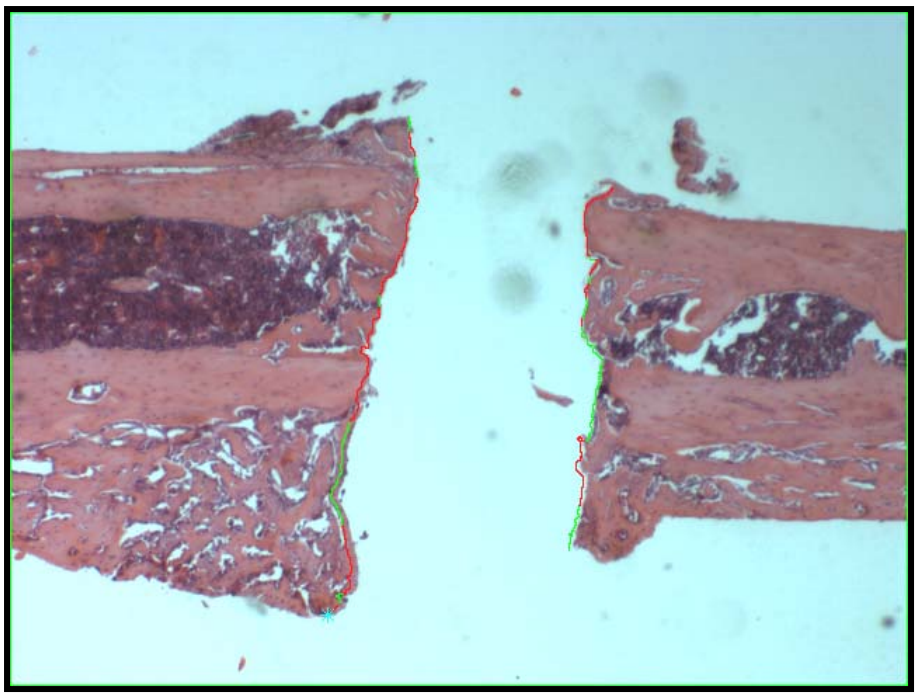

Fig 6 Digital template constructed for the histomorphometric analysis. Red line corresponds to bone integrated tissue and green to unmineralized or uncalcified tissue (×10).

RESULTS

Under general anesthesia, perpendicular tibial osteotomies were created and titanium tacks implants were delivered. Healing was uneventful following the tack placement in all 10 mice and for all surgical sites. The tissues presented only minor signs of inflammation during the first three days of healing, and no infection was observed during the 10 days following the surgeries. All animals were ambulatory following surgery, no distress was observed at any mice.

When the specimens were retrieved, it was noted that 2 heterozygous animals had non-stable implants. The other 8 implants were stable.

Histological Findings

Ten mice (5 wild type and 5 Runx2 heterozygous mutant mice) were used for titanium tack implantation. The tack sites with surrounding tissues are shown in Fig 7 (decalcified sections). Cortical bone and medullary bone, and the healing response around the device site were identified clearly in the wild type and experimental samples (Fig 8). However, areas of tissue healing and cellular activity appeared to be different when sections are compared using higher magnification (×40); showing a more endochondral ossification in the wild type mice (Fig 9).

Different types of tissues and cells were observed at the matrixes formed in response to the tack implantation; these provisional matrixes were rich in trabecular bone, collagen tissue, osteoblastic cells and chondrocytes. Cellular activity in wild type mice was greater when compared to heterozygous mice (Fig 10). However, the number of chondrocytes appeared to be greater when the wild type was compared with the

heterozygous animal (Fig 11). The bone tissue next to the device surface was lined with osteoblast-like cells indicating that bone formation in this area was in progress (Fig 12).

Micro-CT Imaging

To define the ultra-structure and micro-architecture of the healing bone around the titanium tack, micro-CT analysis was performed. Multiple CT sections of tibia were obtained at 8μ resolution and assemble for 2- and 3-dimensional reconstructions. Central sections were used to calculate tissue response area-TRA and bone formation area-BFA (Fig 13).

Two-dimensional reconstructed bone from wild type and heterozygous mice showed differences in total area of new tissue and new osteotomies ten days after the implantation (Fig 14A-B). Three-dimensional reconstructions demonstrated consistent micro-structural differences between the wild type and heterozygous bones around the implanted devices. The amount and the size of the new bone formation were identified in the 3D reconstructions (Fig 14C-D). We further confirmed that wild type animals develop a larger region of new bone formation when compared to the heterozygous animals (Fig 15) and also when the 3-D reconstructions were analyzed using a different orientation (Fig 16).

Micro-CT Morphometric Analysis

Tissue response area (TRA) was larger in the wild type ($1.06mm^2 \pm 0.25$) than in the heterozygous mice ($0.60mm^2 \pm 0.28$). This result was statistically different when

groups were compared ($P < 0.05$). In addition, bone formation area (BFA) for the wild type was 0.25mm2 (±0.06) while BFA for heterozygous mice was 0.12mm^2 (± 0.05). This represented a significantly different ($P < 0.05$). Bone formation around implant sites in wild type animals was statistically greater than the heterozygous group. Total tissue healing and bone formation areas are displayed graphically in Fig 17 and 18.

Histomorphometric Analysis

Histomorphometric analysis was performed to quantify the findings observed during the histological and micro-structural analyses. High resolution micrographs permitted the quantification of the percentage of bone integration (BIC) along the zone representing the outer titanium surface of the device. Titanium implants placed in the wild type group showed greater bone-to-implant contact percent (76.9 ± 6.5) than devices placed at the heterozygous group (57.5 ± 11.6). Once more, the results were significantly different ($P < 0.05$). Bone-to-implant contact percent is displayed graphically in Fig 19.

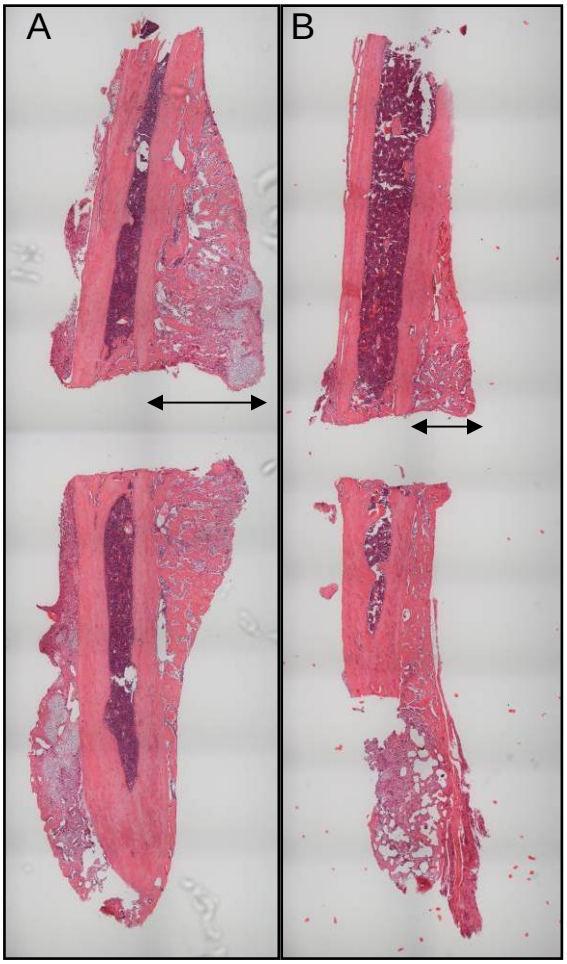

Fig 7 Longitudinal tibia sections of implant site (implant removed) for wild type (A) and heterozygous (B) mice. The cortical and medullary pre-existing bones and supracortical tissue healing response (double arrow head line) were clearly identified. Wild type response appears greater in volume within canals in the supracortical regions. *(×10)*

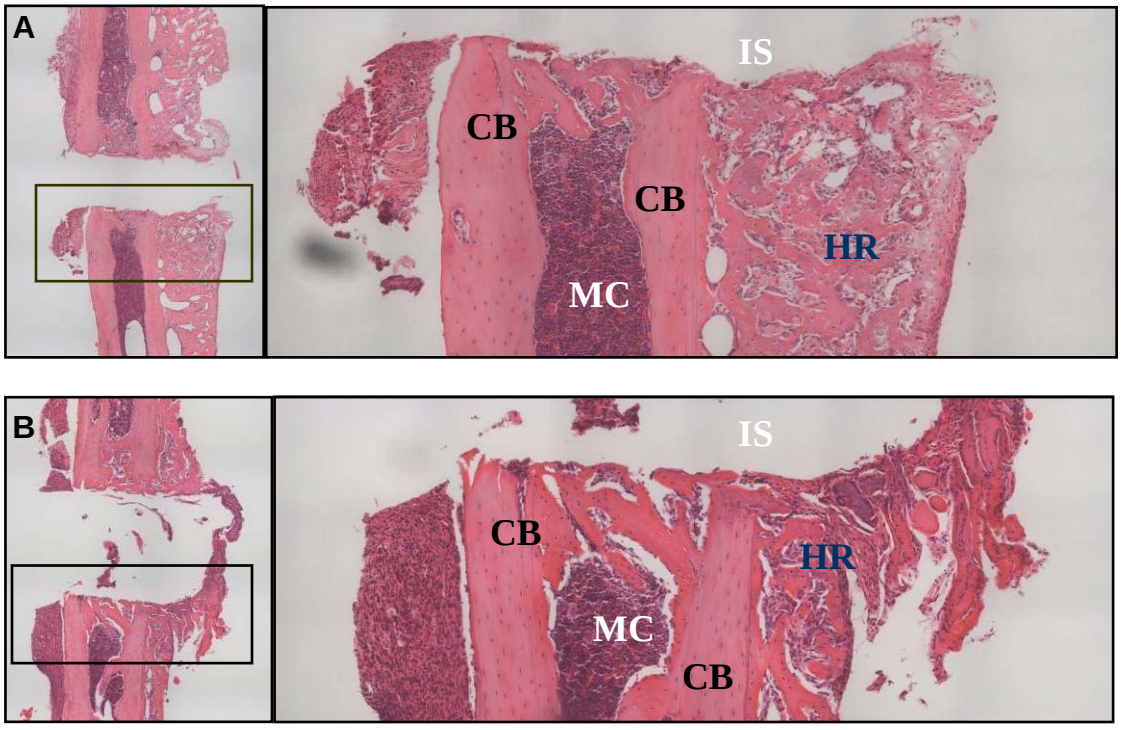

Fig 8 Images of titanium implant sites showing surrounding tissues; middle tibial sections of wild type (A) and Heterozygous (B) mice. The boxed areas show higher magnification *(×40)*. The anatomical structures are clearly identified: cortical bone (CB), medullary canal (MC) healing response (HR) and the implant sites (IS).

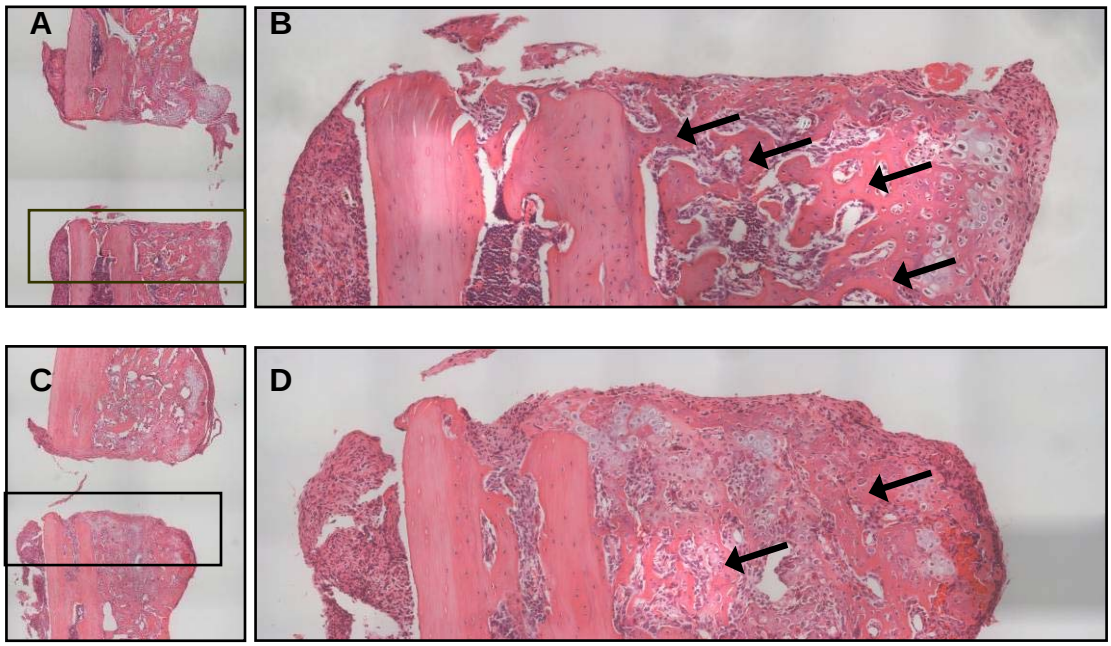

Fig 9 Images of titanium tacks sites showing the endochondral ossification; middle tibial section (A) of wild type animal. *Mag. ×10.* The boxed area shows higher magnification (B) ×*40*. Differences in the size of newly formed tissue immediately lateral (right) to the cortical bone between wild type and heterozygous (C, D) mice were identified. More endochondral ossification (arrows) could be observed in the wild type (A, B) animal when compared to the heterozygous type (C, D).

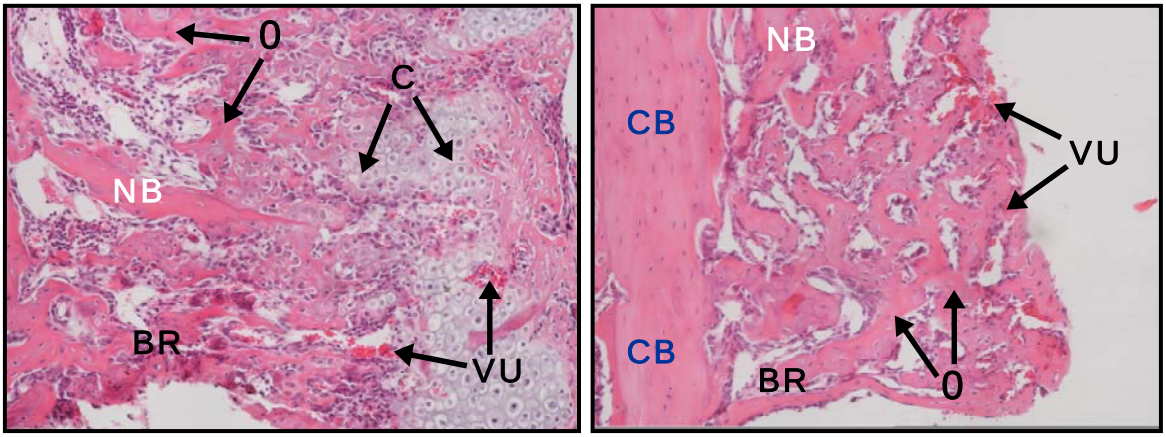

Fig 10 Images show different structures present in the wild type and heterozugous animals. New formed bone (NB), bone remodeling (BR), connective tissue (CT), osteocytes (O), chondrocytes (C) and vascular units (VU) can be identified histologically. Cellular activity was greater in the wild type mice when compared to the heterozygous mice.

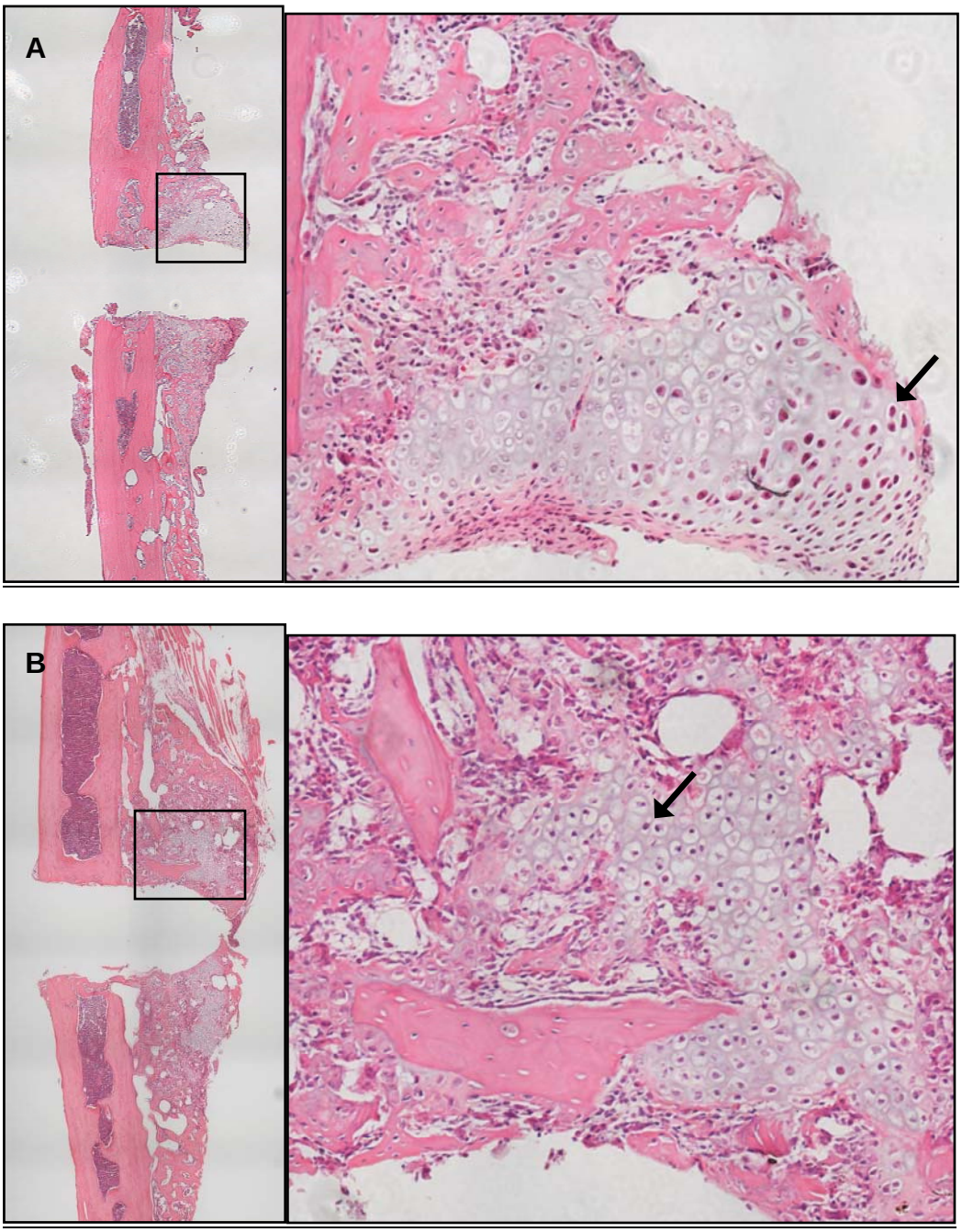

Fig 11 Wild type (A) and Heterozygous (B) tibial middle sections stained with hematoxylin & eosin (H&E). *Original mag. ×10 and ×40 (boxed areas)*. Different type of cells and tissues were observed at the area of tissue response. Chondrocytes (arrows) are predominantly present at the healing matrixes.

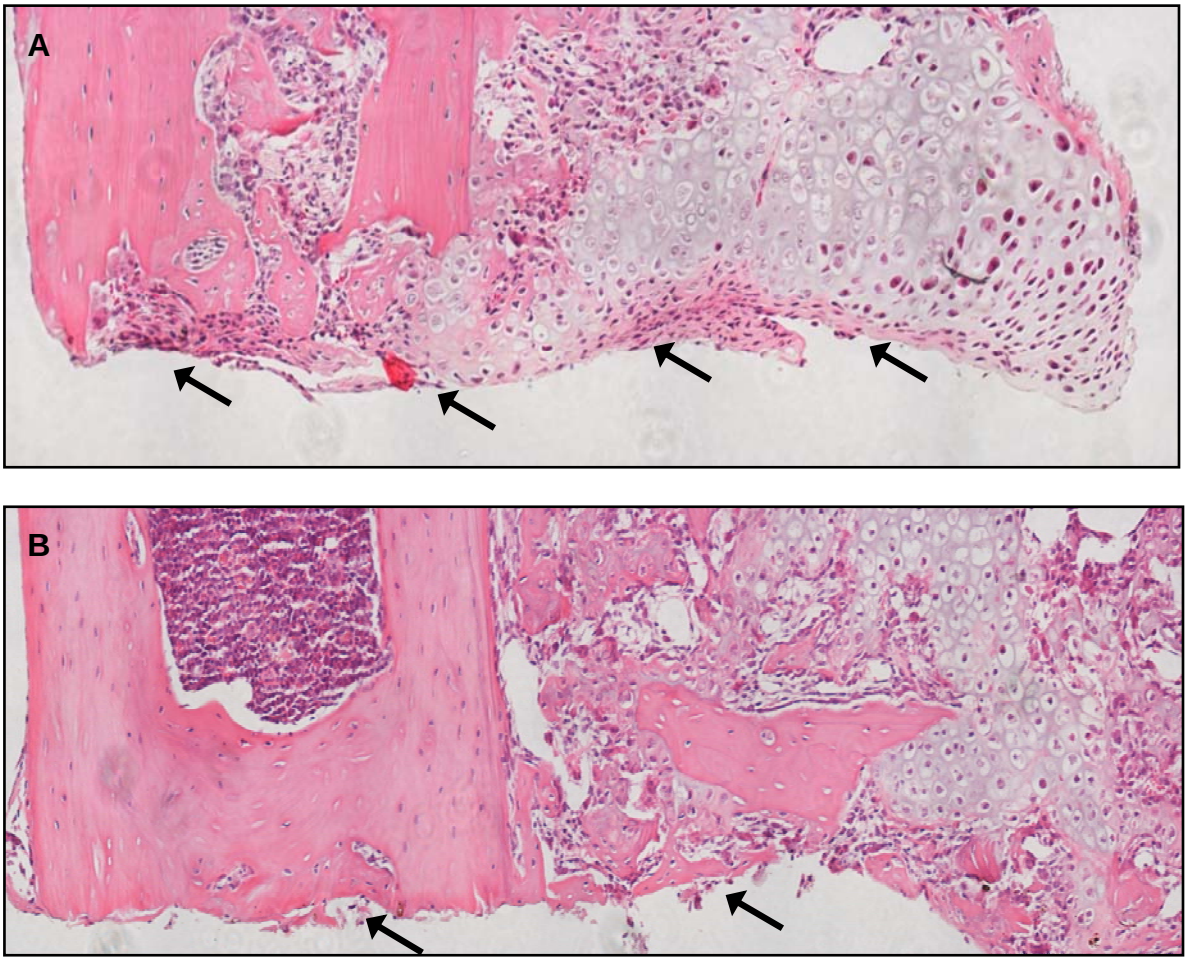

Fig 12 Images show osteoblast-like cells in close contact with the titanium surface of the implant. Wild type (A) and Heterozygous (B) mice. *Mag. ×40*. The presence of this type of cells appears to be more frequent in the wild type animals.

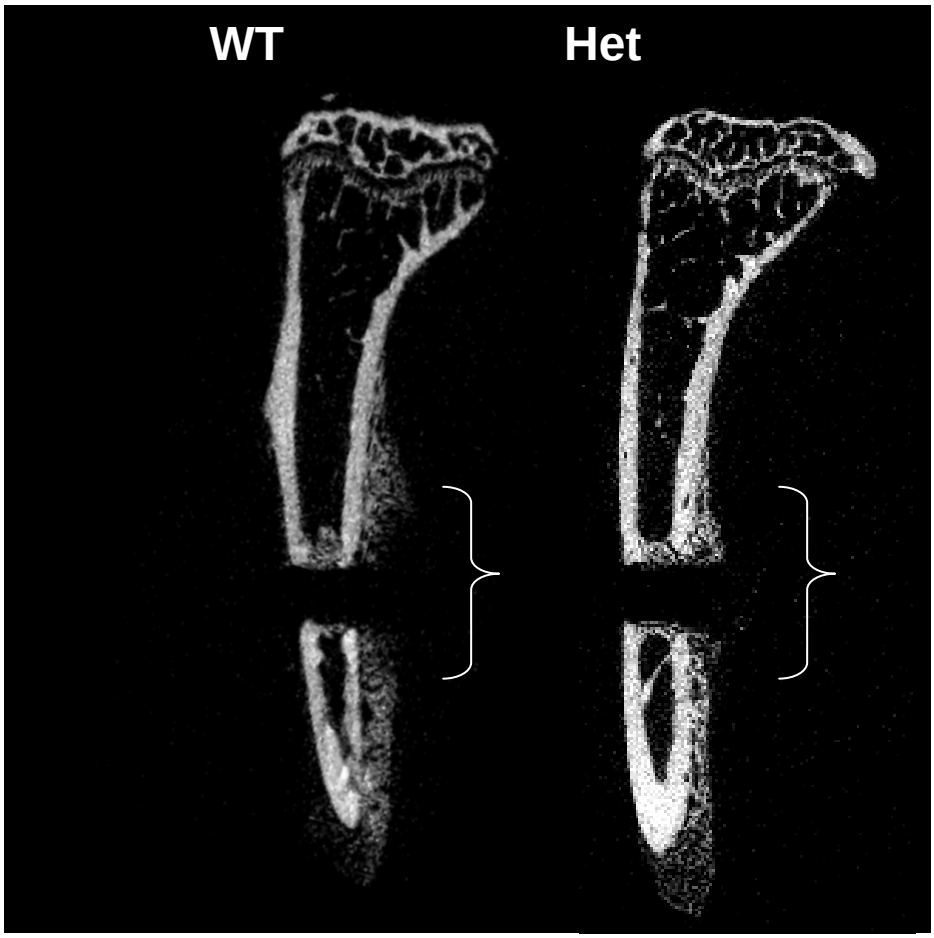

Fig 13 Images showing two dimensional micro-CT scans at the middle of the tack sites of the different sizes of the bone response. Wild type (left) and Heterozygous (right) mice. Areas of tissue growth were identified around the implant sites (brackets) showing aggressive supracortical mineralized tissue response. Wild type response appeared greater than heterozygous response.

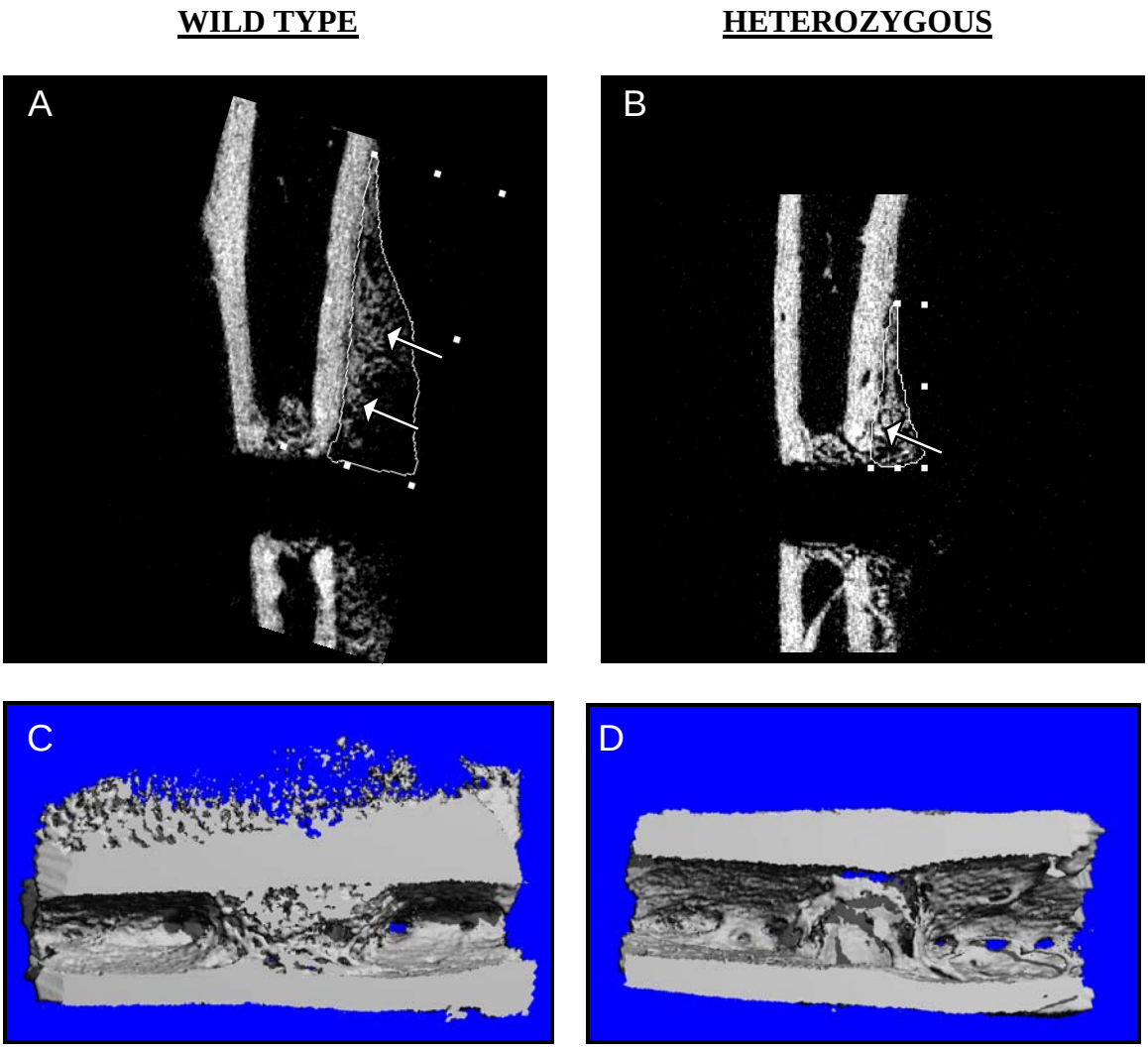

Fig 14 Images of two-dimensional (A,B) and three-dimensional (C,D) reconstructions of the titanium tack site. Wild type (left) and Heterozygous (right) mice. The proximal dorsal areas of supracortical tissue response (boxed) and new bone (arrows) were quantified in square millimeters (A,B). Amount and size of the new bone formation were significantly greater in wild type animals compared to heterozygous (C,D).

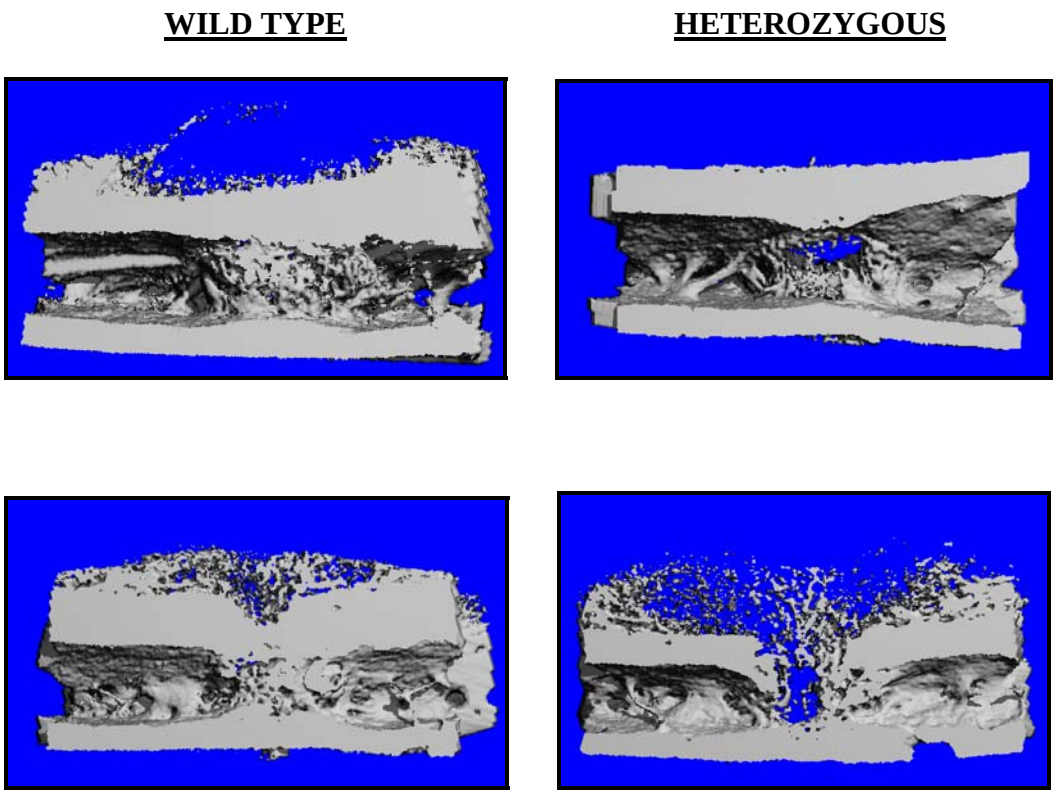

Fig 15 Images of three-dimensional reconstructions (diameter 1mm x 3mm) of the tack sites showing different amounts of bone formation. Wild type (left) and Heterozygous (right) mice. In general, the degree of bone response associated with wild type animals in the region of the implant, appeared to be greater than heterozygous animals.

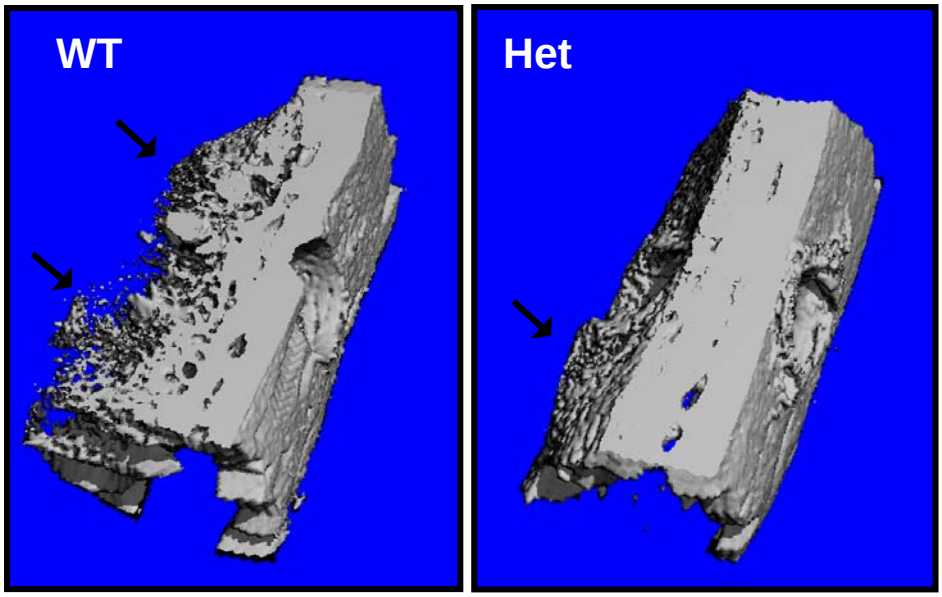

Fig 16 Lateral view of the 3-D reconstructions of the implant sites at the mice tibia. Wild type (left) and Heterozygous (right) animals. A larger area of new bone formation (arrows) was found at the wild type when compared to the heterozygous mice.

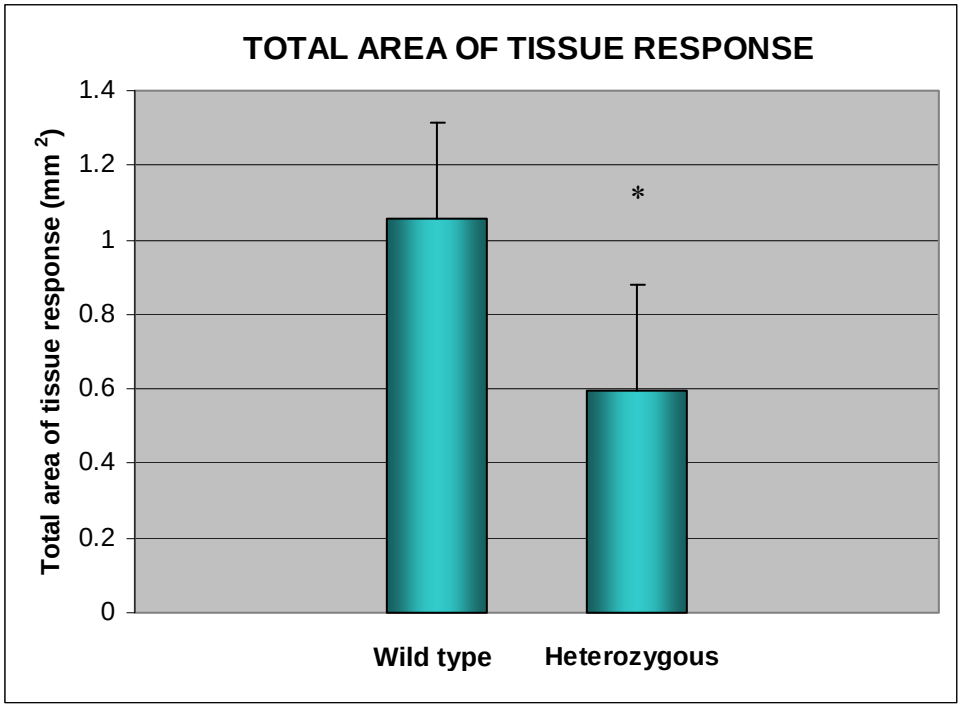

Fig 17 Area of tissue response associated with the titanium tack device in the supracortical proximal region (±SD). Wild type animals demonstrated significantly greater areas of tissue response than the heterozygous type ($P < 0.05$).

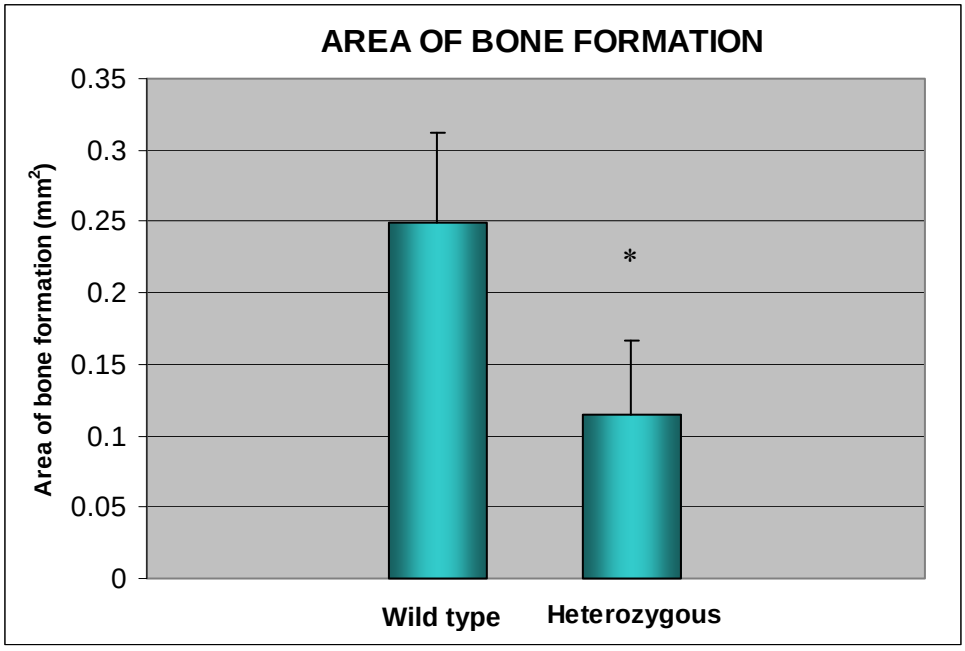

Fig 18 Area of bone formation associated with the titanium implant in the supracortical proximal region (±SD). Wild type animals demonstrated significantly greater areas of new bone formation than the heterozygous type ($P<0.01$).

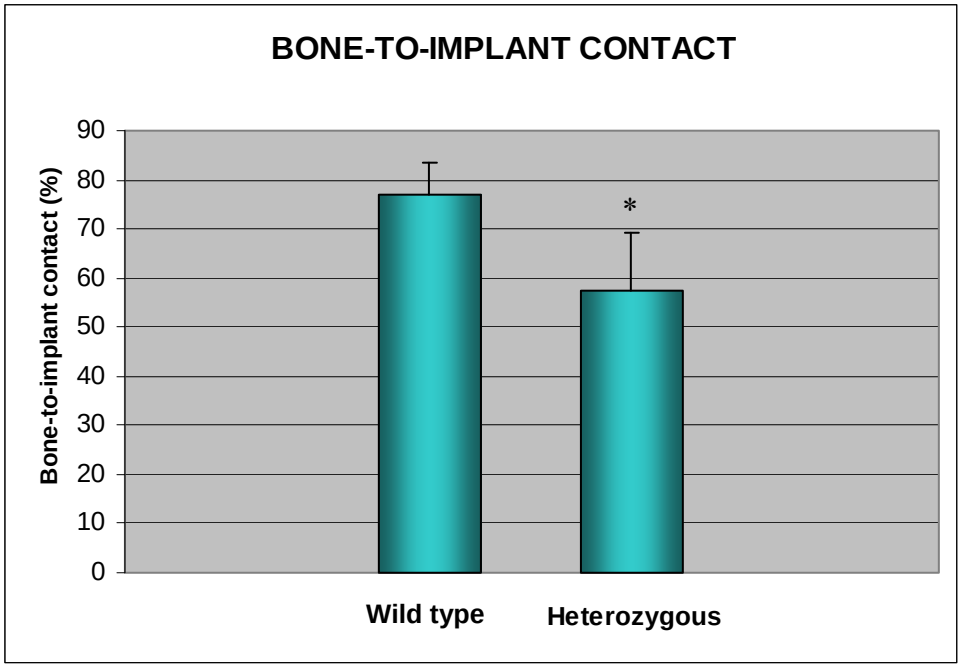

Fig 19 Bone-to-implant contact associated with the titanium tack device (±SD). Wild type animals demonstrated significantly greater bone-to-implant contact than the heterozygous type ($P<0.01$).

DISCUSSION

The Runx2 gene has been shown to be fundamental for bone formation in mice. Haploinsufficiency (+/-) of this gene causes physiological alterations of the healing process, while complete deletion (-/-) generates mice that die at birth.[5, 25] In order to test if the Runx2 gene deficiency has an effect in the bone healing associated with implants, we utilized osteotomies followed by titanium tack implantation in wild type and heterozygous mice.

A number of Runx2 gene knock-out and knock-in mouse models have established an essential role of this gene in skeletogenesis. In all cases the homozygous mutant animals exhibit embryonic or perinatal lethality due the complete failure of bone formation. It is important to note that in these models Runx2 gene is mutated or deleted in every cell type, tissue and organ of the animal. Therefore, the phenotype of these animals reflects loss of Runx2 function in the entire body. Previous studies have used the Runx2 haploinsufficient mice from these models. [35]

Skeleton formation in higher vertebrates involves both intramembranous and endochondral ossification. Endochondral ossification consists of two steps: a) formation of cartilage template and b) replacement of the cartilage skeleton. Two cell types (chondrocyte and osteoblast) that arise from the same mesenchymal precursors are central for this process. Chondrocytes are responsible for formation of the cartilage which is later replace by mineralized matrix, synthesized by osteoblasts.

Traditional Runx2 knock-out models can not distinguish the individual contribution of chondrocytes or osteoblasts in bone formation, bone remodeling and bone repair. In our studies we used a novel generation of Runx2 knock-out where the gene is

normal in every cell type of the body, except it is deleted in chondrocytes. To achieve a chondrocyte and cartilage tissue selective ablation of Runx2, we used collagen type II cre-recombinase. A selective Runx2 deletion in chondrocytes results in perinatal lethality due the failure of endochondral ossification. Only the heterozygous animal survives. For this reason, we used the haploinsufficient model in chondrocytes to examine the role of Runx2 gene in wound healing after implant installation.

Different techniques are available to study the histological characteristics of wound healing. In this study, we examined decalcified specimens. First, this provided sections with excellent cellular detail and facilitates the study of the events involved in bone tissue modeling and remodeling. Second, micro-CT analysis is complicated by the presence of metallic implants. Several articles have quantified implant histology where the actual implant has been removed, for example frozen sections and paraffin sections. [2, 7, 30]

A 0.7 mm diameter, Ti-6AI-4V implant (titanium tack) was selected for this study. The selection was strongly influenced by the anatomical architecture and the dimensions of the bone; especially by the wide flat surface of the mouse tibia (2mm to 3mm). The diameter of commercially available mini-implants usually exceeds 1.5 millimeters and these were considered too large for the mouse tibia. The barbed tip of the tack used in this study is designed to retain the tack in clinical applications. However, in our study the presence of the barbed tip of the implant made the removal more difficult and in some cases produced the displacement of the tissues surrounding the implant. For future studies, this type of undercut should be avoided if a decalcified technique is utilized.

An important clinically outcome was observed for two heterozygous animals. The titanium tacks were not stable at the surgical site after the healing period, suggesting that a lower degree of bone integration was present in these mice. Later this suggestion was confirmed by histomorphometry (BIC).

Some authors have described the histological healing process of wild type and Runx2 haploinsufficiency mutant mice.[35] These authors reported newly formed bone at the osteotomy sites in the wild type mice and a dramatically delayed wound healing in the mutant after 3 weeks. However, the authors did not place an implant. In our study, similar histological findings were observed after 10 days of healing in the presence of implants. In both studies, more new bone was observed in the wild type than in the heterozygous mice regardless of the presence or absence of an implant. These data suggest that the loss of one copy of Runx2 gene generates a significant alteration of bone healing.

Other authors have compared healing around implants (titanium, polymeric and stainless steel) with healing of osteotomies.[14] However, animals used were wild type mice with no haploinsufficiency. Our study compares the bone formation around titanium implants in wild type mice with heterozygous mice, in a Runx2 haploinsufficient model.

Commercially pure titanium has been used in implant dentistry for several years. Recent studies have demonstrated that osteoblast attachment to titanium discs enhanced Runx2 activity and bone formation.[22] In our study the titanium surface of the implant used was probably an important factor during the healing process. Although bone formation was greater in the wild type mice, bone formation at the heterozygous animals was also achieved. This result suggests that the presence of a biocompatible material may

improve bone formation regardless of the Runx2 gene mutation.

In order to quantify two-dimensional and three- dimensional bone formation after implant placement, we followed a similar micro-CT testing protocol utilized by other authors.[17] Two-dimensional and three-dimensional reconstructions were obtained from the bones and micro-CT morphometric analysis was performed. New bone formation around the tack was observed in both animals using a 2D approach. A greater bone response was observed in the wild type group when compared to the heterozygous animal. Three-dimensional reconstructions showed differences in bone formation between the wild type and the heterozygous animals around the surgical sites. In all cases the amount of new bone around the implant surface was larger in the wild type group. The morphometric analysis of the supracortical bone response quantified the total area of tissue response and the amount of bone formation within this area. The tissue response area was not totally filled by new bone; probably because another tissues (unmineralized or uncalcified) were present or perhaps because it was too early in the healing process for the skeletal progenitor cell differentiation. This analysis gave us the opportunity to support our histological findings and confirm that the haploinsufficiency of Runx2 gene produces physiological alterations in the bone formation process.

Taken together, our study reveals that Runx2 activity specifically in chondrocytes is required for a normal healing response and that loss of one copy can alter the process of bone formation. A better understanding of the role of Runx2 haploinsufficiency in chondrocytes in mice may allow the scientific community to develop dental interventions for patients with similar genetic deficiencies in the bone formation process.

FUTURE RESEARCH

The animal model was successfully used to study healing and bone formation around titanium implants. This model is strongly recommended for future studies in the same field. However, a different implant design is suggested to avoid tissue displacement and clinical retention of the barbed tip design.

Longer healing periods can be used to determinate if the haploinsufficient animal response is the same after 20 or 30 days. Implant loading must also be considered in future studies due its importance in osseointegration. If possible, prospective studies should be conducted using large sample sizes.

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APPENDIX

IACUC NOTICE OF APPROVAL

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL

DATE: April 24, 2007

TO: Michael McCracken, D.D.S., Ph.D.
SDB 603 0007
FAX: 975-6108

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: Title: Bone Formation Around Implants in Adult Transgenic Mice with Selective RunX2- II Deficiency
Sponsor: Internal
Animal Project Number: 070408120

On April 16, 2007, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the use of the following species and numbers of animals:

Species	Use Category	Number in Category
Mice	B	20

Animal use is scheduled for review one year from April 2007. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 070408120 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7692.

Institutional Animal Care and Use Committee
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