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Deficiency in immunoadaptor protein DAP12 leads to altered fracture repair

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by

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Deficiency in immunoadaptor protein DAP12 leads to altered fracture repair

Brett Martin, DDS

Abstract

Purpose: To determine if genetically deficient DAP12 mice demonstrate impaired fracture healing responses.

Methods: All studies were performed under IACUC approval. Genetically deficient DAP12 and age-matched C57BL/(6) (B6) control mice were used. The right tibia was cleaned and prepped for surgery and a closed, mid-diaphysis fracture was created. The fractures remained unstabilized to promote healing through the formation of a cartilage intermediate. Mice were sacrificed at 7, 10, 14, 21, and 28 days post fracture and processed for histology by decalcifying the tibia and embedding the samples into paraffin wax. Serial sections were collected through the callus and every tenth slide was stained with Milligan's Trichrome, which stains for bone tissue. Using stereology, the volume of the fracture callus, cartilage, bone, bone marrow, and fibrous tissue was determined.

Results: Histological and stereological analysis demonstrated less trabecular bone percentage and volume, increased and delayed cartilage resorption, decreased bone marrow volume, and decreased overall callus volume in the $DAP12^{-/-}$. Furthermore, $DAP12^{-/-}$ mice displayed elevated levels of fibrous tissue within the callus. Statistical significance was not observed in the callus volume between the $DAP12^{-/-}$ and control B6 mice.

Conclusion: Our data indicates that DAP12 deficiency disrupts the fracture healing process. Enhancing the understanding behind the mechanism of fracture repair can lead to improve healing strategies and potential therapeutics.

TABLE OF CONTENTS

Page

List of Figures

Page

1. INTRODUCTION

Bone-related accidents and injuries pose a substantial threat to the health and lives of children, adolescents, and adults. The burdens felt by the parties involved can be overwhelming and/or detrimental to living a healthy life. It has been estimated that roughly 6.3 million fractures occur in the United states each year, leading to half a million hospitalizations, over 800,000 emergency room visits, and more than 2,600,000 physician office visits.[1] Furthermore, the expense involved in caring for patients with fractures ranges from $12-$18 billion per year, which could double or triple in the coming decades.[1] Thus understanding the fracture healing process may lead to improved therapeutics that may decrease this economic strain.

Bone fractures account for 10-25% of all injuries suffered in the pediatric population, with the risk twice as high for boys as for girls.[2] The most common skeletal fractures in children involve the distal end of the forearm followed by phalanges of the hand.[2] Furthermore, within the maxillofacial region, the nasal bone is the most common fracture site.[3,4] Concomitant dental injury occurs in 10% of maxillofacial trauma, requiring the use of pediatric dentists and oral and maxillofacial surgeons.[5] Therefore, all bone injuries are serious and require medical attention.

Fracture healing involves complex cell and tissue communication, differentiation, and proliferation. This repair process involves three overlapping phases that is completed roughly 6-8 weeks post fracture: inflammatory, reparative, and remodeling.[6] The inflammatory reaction peaks 48 hours after injury and lasts

for 1 week. This response involves activation of the complement cascade, platelet aggregation, and release of alpha-granule contents.[6] Polymorphonuclear leukocytes (PMNs), lymphocytes, blood monocytes, and tissue macrophages are attracted to the wound site and release cytokines to stimulate angiogenesis. Eventually a hematoma forms within the medullary canal between the fracture ends. Before the inflammatory phase resides, the reparative phase begins and lasts for several weeks. This phase results in formation of a fracture callus that bridges the two bone ends.[6] The callus, which begins as cartilage, acts to enhance mechanical stability at the fracture site, and will eventually be replaced by bone. To initiate formation of the fracture callus, mesenchymal cells begin to differentiate into fibroblasts, chondrocytes, and osteoblasts at the injury site.[6] The potential sources of these mesenchymal cells include the periosteum, endosteum and bone marrow and mechanical disruption of these sites results in altered healing.[7] Furthermore, mesenchymal cell fate is determined by intrinsic and environmental signals within these three tissues types.[7] The periosteum supports both chondrogenesis and osteogenesis, whereas bone marrow and endosteum supports osteogenesis during fracture repair.[7]

As healing continues, chondrogenesis generates a large cartilage callus comprised of an avascular basophilic matrix.[6] By the middle of the second week, bone formation begins by endochondral ossification. Soon the callus, composed of woven bone, becomes more rigid and the fracture site is considered internally immobilized.[6] The final remodeling phase begins with the replacement of woven bone by lamellar bone and resorption of the excess callus. The woven bone is

resorbed by osteoclasts, which are large multinucleated cells formed by the differentiation of haematopoietic precursors. The osteoclasts become polarized and adhere to the mineralized surface and begin bone resorption. Soon osteoblasts enter the fracture site and are able to lay down new bone.[8] Gradual modification of the fracture region will occur under the influence of mechanical loads until optimal stability is achieved.[6] Thus, fracture healing requires the body to regenerate naturally and without interruptions.

During the three phases of fracture repair, multiple cell interactions occur via growth factors, cytokines, receptors, and intermediate signaling. Transforming growth factor-beta (TGF-β) is expressed at the fracture site. It is a chemoattractant for macrophages and also promotes angiogenesis. Furthermore, TGF-β induces differentiation of MSCs into chondroblasts and osteoblasts.[6] Fibroblasts growth factor-I and II (FGF-I; FGF-II) promote blood vessel formation, stimulate collagenase, and regulates growth plate chondrocytes.[6] Bone morphogenetic proteins (BMP-2; BMP-4; BMP-7) are involved in multiple processes, including vasculogenesis and angiogenesis along with acting as a transcription factor for osteoblast differentiation, activation of monocytes.[6] Finally, interleukins (IL-1; IL6) stimulate proteases, collagen production by fibroblasts, and subsequent collagen crosslinking, and bone resorption.[6]

The role of macrophages during inflammation and repair has been extensively studied. Macrophages possess the ability to perform both injury-inducing and repair-promoting tasks during an inflammatory response.[9] Depending on which signaling mediators are present, macrophages can follow either classical activation

or alternative activation.[10] Classical macrophage activation is mainly induced by lipopolysaccharide (LPS) or TH1-related cytokines such as interferon-gamma (IFN-gamma) and interleukin-12 (IL-12).[10] Once activated, classical macrophages synthesize and release pro-inflammatory mediators, such as IL-1, IL-6, and TNF-α, leading to cytotoxicity and tissue injury.[11,12,13] Conversely, alternative macrophage activation is mediated by glucocorticoids or Th2-related cytokines, such as IL-4, IL10, and IL-13.[14,15] Once activated, an anti-inflammatory and tissue repair response occurs with the release of IL-10, transforming growth factor beta 1 (TGF-B1), vascular endothelial growth factor (VEGF), and epithelial growth factor (EGF).[16,17] Control of macrophage activation to either classical to alternative pathways is critical for normal hemostasis. The balance in mediator production that controls pathway activation ultimately determines the healing outcome of the injured tissue.

A recent study demonstrated the importance of a novel type of macrophage, osteomacs, which reside within bone-lining tissues.[18] Osteomacs localize to areas of active bone matric deposition and remodeling.[18] During physiologic bone formation, osteomacs are in direct contact with osteoblasts and form a cellular canopy structure that supports mineralization.[18] In vivo suppression of osteomacs have demonstrated significantly less mineralized bone deposition within a fracture site.[18] Thus, osteomacs plays a pivotal role in normal bone development and remodeling.

Many physiological and pathological conditions can significantly alter the normal fracture healing process. Generally speaking, as people age, the capacity to

heal declines.[19] Medical professionals are very aware of bone structure, bone capabilities, and bone deterioration which have been verified through scientific research. Previous studies have demonstrated that middle-aged mice have delayed bone fracture repair compared to juveniles, which continues to decline in elderly mice.[20] Furthermore, the potential for angiogenesis during fracture repair decreases in elderly mice.[21] Studies have also shown that changes in gene expression required for physiological bone and cartilage formation were altered in elderly mice, resulting in a delay in healing.[22] For example, prolonged expression of osteoclastic, cartilaginous, and fibroblasts markers were shown in aged rats. This persistent expression suggests prolonged remodeling of bone.[23] Moreover, various acute and chronic systemic and inflammatory diseases can severely alter the healing capacity of bone. Examples include diabetes, AIDS, cancer, jaundice, and obesity.[24] Certain medications, such as glucocorticoid steroids, non-steroidal anti-inflammatory drugs (NSAIDs), and chemotherapy agents may further disrupt bone healing.[24] Understanding the effects of systemic diseases on fracture repair may lead to therapeutics that improves wound healing.

In addition to well-characterized signaling molecules mentioned previously, other, less well-studied proteins are also likely to be important for bone healing. One of these proteins that may be involved in fracture repair is DNAX activation protein of 12kDa (DAP12). DAP12 is an evolutionally conserved, transmembrane disulfide-bonded homeodimer that transduces activating signals to various hematopoietic cells, including dendritic cells, neutrophils, basophils, eosinophils, mast cells, monocytes, macrophages, natural killer cells (NK), and some B and T

cells.[25] DAP12 is also expressed on osteoclasts in the bone marrow and on microglial cells in the brain.[26] One of the signaling partners of the DAP12 receptor is triggering receptor expressed on myeloid cells-2 (TREM-2). TREM2 is a transmembrane protein receptor belonging to the immunoglobulin and lectin-like superfamily.[27] TREM 2, similar to DAP12, is expressed in cells of the myeloid lineages, including macrophages, osteoclasts, and dendritic cells. Upon ligand activation, TREM2 binds to DAP12 on the cell membrane which activates intracellular signaling cascades.[27] Studies have shown that DAP12 and/or TREM2 deficiencies lead to impaired osteoclast development and formation of mononuclear osteoclasts.[27,28] Thus, understanding the relationship between DAP12 and bone repair is a key underlying component to this current study.

The structure of DAP12 involves an extracellular region mainly consisting of cysteine residues. Embedded within the transmembrane region of DAP12 lies the acidic amino acid aspartic acid (**Figure 1**).[25] DAP12 is activated indirectly through the binding of ligands to its associated receptor. The signaling of DAP12 is through an immunoreceptor tyrosine-based activation motifs (ITAMs), which is present within the cytoplasmic domain.[28,29] Upon DAP12 ligand activation, phosphorylation of the tyrosine residues occurs by Src family tyrosine kinases. Once phosphorylated, a signaling cascade is triggered to activate phospholipase C-gamma and extracellular signal-regulated kinase (ERK).[29] The result of activation of DAP12 includes both activation and inhibition of cellular functions. Previous studies have demonstrated a down-regulation of Toll-like receptors (TLR) resulting in the suppression of macrophages and dendritic cells and pro-inflammatory cytokines

production.[30] However, the exact function is still unknown and more research is required.

In the current study, the role of DAP12 in fracture healing was investigated. We hypothesized that DAP12 deficiency resulted in altered fracture repair by disrupting soft and hard callus formation. To test this, we created reproducible bone fractures followed by Milligan's Trichrome staining and histomorphometry of DAP12$^{-/-}$ and control C57BL/(6) (B6) mice. The data demonstrated that DAP12 deficient mice displayed impairments in callus formation, fracture remodeling and overall repair.

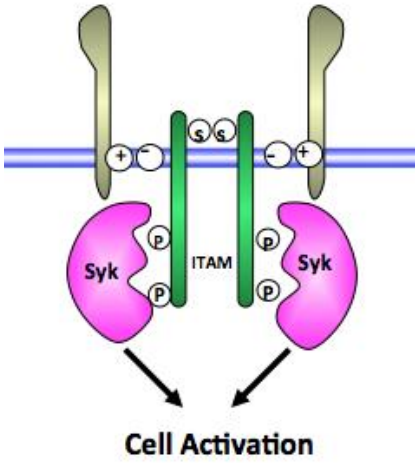


Figure 1. Structure of DAP12. DAP12 is a transmembrane receptor protein present at the cell surface as a disulfide-bonded homodimer. The protein bears an immunoreceptor tyroisine-based activation motif (ITAM) that mediates signal propagation by activation of Syk tyrosine kinases.

2. MATERIALS AND METHODS

Animals

DAP12$^{-/-}$ and C57BL/6 (B6) control mice were bred and all procedures were conducted according to the University of California at San Francisco Institutional Animal Care Use Committee (IACUC). Three- to 5-month old; 30-35 grams (g) males and aged-matched wild-type B6 mice were used to conduct all experiments.

Non-stabilized fractures

DAP12$^{-/-}$ mice and their B6 littermates were anesthetized with an intraperitoneal injection of a 1:1 solution of 50mg/mL ketamine and 0.5mg/mL dexmedetomidine (50mg/kg body weight). Anesthetized mice were placed pronate under a fracture apparatus specifically designed to deliver a highly reproducible fracture injury (**Figure 2**). The mechanical components of the apparatus consist of a blunt two-pronged base to frame the tibia and a 2mm-thick blunt arm connected to a guided, movable 500g weight. The right tibia was centered on the base and under the arm before the weight is lifted to a distance 3.5 cm above the tibia and then dropped to create a closed mid-diaphysis fracture via three-point bending previously described.[30,31] The fracture was then allowed to heal without stabilization. In this model, healing occurs through endochondral ossification during which a cartilage intermediate is replaced by bone.[30] Mice were revived with an intraperitoneal injection of 5mg/mL antipamezole (50mg/kg body weight) and were then monitored for signs of physical discomfort. The mice were allowed to move freely after recovery from anesthesia within their cage. Subcutaneous injections of 0.03mg/mL buprenoprine (0.05 mg/kg body weight) were given for

pain control per approved protocol. Buprenoprine injections were given subcutaneously immediately, 4-8 hours, 24 hours, and 32 post-operation. If any signs of pain were noticed, additional subcutaneous injections were administered. Mice were then sacrificed by cervical dislocation following deep inhalation anesthesia (Metofane) at 7, 10, 14, 21, and 28 days.

Histological and Histomorphometric Analysis

Following euthanasia, the tibia was collected from each animal by cutting mid-diaphysis of the femur and distal to the ankle joint. Tibia were fixed in 4% paraformaldehyde (PFA) at 4^{o}C overnight, then decalcified in 19% EDTA (pH 7.4) for 14 days at 4^{o}C. Samples were dehydrated through a progressive ethanol wash and then embedded in paraffin.

To visualize the tissue within the callus, serial sagittal sections (10 μm) were collected through the entire tibia and every tenth slide was stained with Milligan's Trichrome. Histomorphometric analysis using the Olympus CAST system was completed to determine the volumes of the fracture callus (TV), cartilage (CV), trabecular bone (BV), bone marrow (MV), and fibrous tissue (FV).

The volume of the callus, cartilage, bone, marrow, and fibrous tissue (TV, CV, BV, MV, or FV) was calculated using Cavalieri's Principle with the equation for conical frustum: TV, CV, BV, MV, FV = $\frac{1}{3}h\sum_{i-1}^{n-1}(A_i + A_{i+1} + \sqrt{A_i A_{i+1}})$, h was the distance between sections and equal to 300μm, n was the total number of sections analyzed for each callus sample, A_i and A_{i+1} were the areas of callus, cartilage, bone, or blood vessels in sequential sections.

Statistics

Statistical analyses were performed using the JMP software from SAS. All data were expressed as the means ± 95% confidence intervals. The statistical significance of the differences between mean values was evaluated using the Wilcoxon test. Differences were considered significant at $p<0.05$.

Figure 2. Fracture apparatus. Apparatus used to deliver a highly reproducible fracture injury of DAP12$^{-/-}$ and B6 mice. The right tibia was centered on the base and a 500g weight was lifted to a distance 3.5 cm. The weight was then dropped to create a closed mid-diaphysis fracture via three-point bending

3. RESULTS

Histological Analysis

Milligan's Trichrome staining demonstrated cartilage tissue formation within the fracture callus followed by cartilage resorption and woven bone deposition. Compared with B6 mice, DAP12$^{-/-}$ mice showed more residual cartilage tissue and delayed woven bone formation (**figure 3**). Furthermore, DAP12$^{-/-}$ showed elevated levels of fibrous tissue within the fracture callus (**figure 3**).

Quantitative histomorphometric analysis was performed (**figure 4**). At day 7, DAP12$^{-/-}$ mice showed significantly less woven bone formation (14.97% ± 4.89% vs. 29.09% ± 4.52, receptivity; $p<0.003$) and significantly more fibrous tissue formation (59.96% ± 9.27% vs. 36.96 ± 8.72%, respectively; $p<0.01$). Ten days after fracture, no significant difference was observed between DAP12$^{-/-}$ and B6 mice. Fourteen days after fracture, DAP12$^{-/-}$ showed significantly more fibrous tissue within the fracture callus (11.76% ± 3.28% vs. 5.38% ± 2.23%, respectively; $p<0.01$). Twenty-one days after fracture, DAP12$^{-/-}$ showed significantly higher levels of cartilage tissue (2.84% ± 0.64% vs. 0.04% ± 0.06%, respectively; $p<0.001$) and significantly less marrow tissue (58.61% ± 6.43% vs. 67.45% ± 1.80%, respectively; $p<0.01$). Twenty-eight days after fracture, DAP12$^{-/-}$ showed significantly higher levels of cartilage (1.71% ± 0.62% vs. 0.14% ± 0.19%, respectively; $p<0.01$). Finally, no significant difference in callus volume between DAP12$^{-/-}$ and B6 mice was observed at any time point.

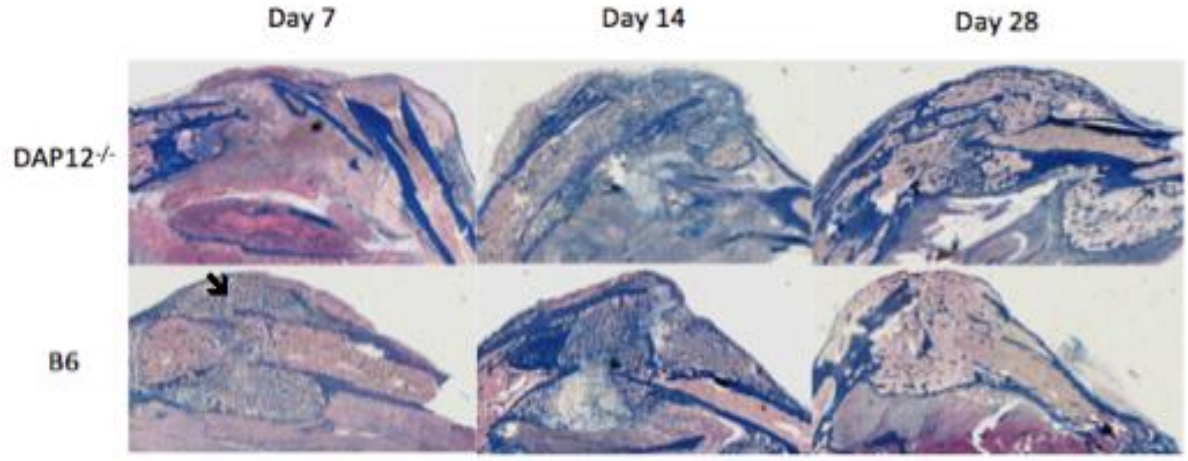
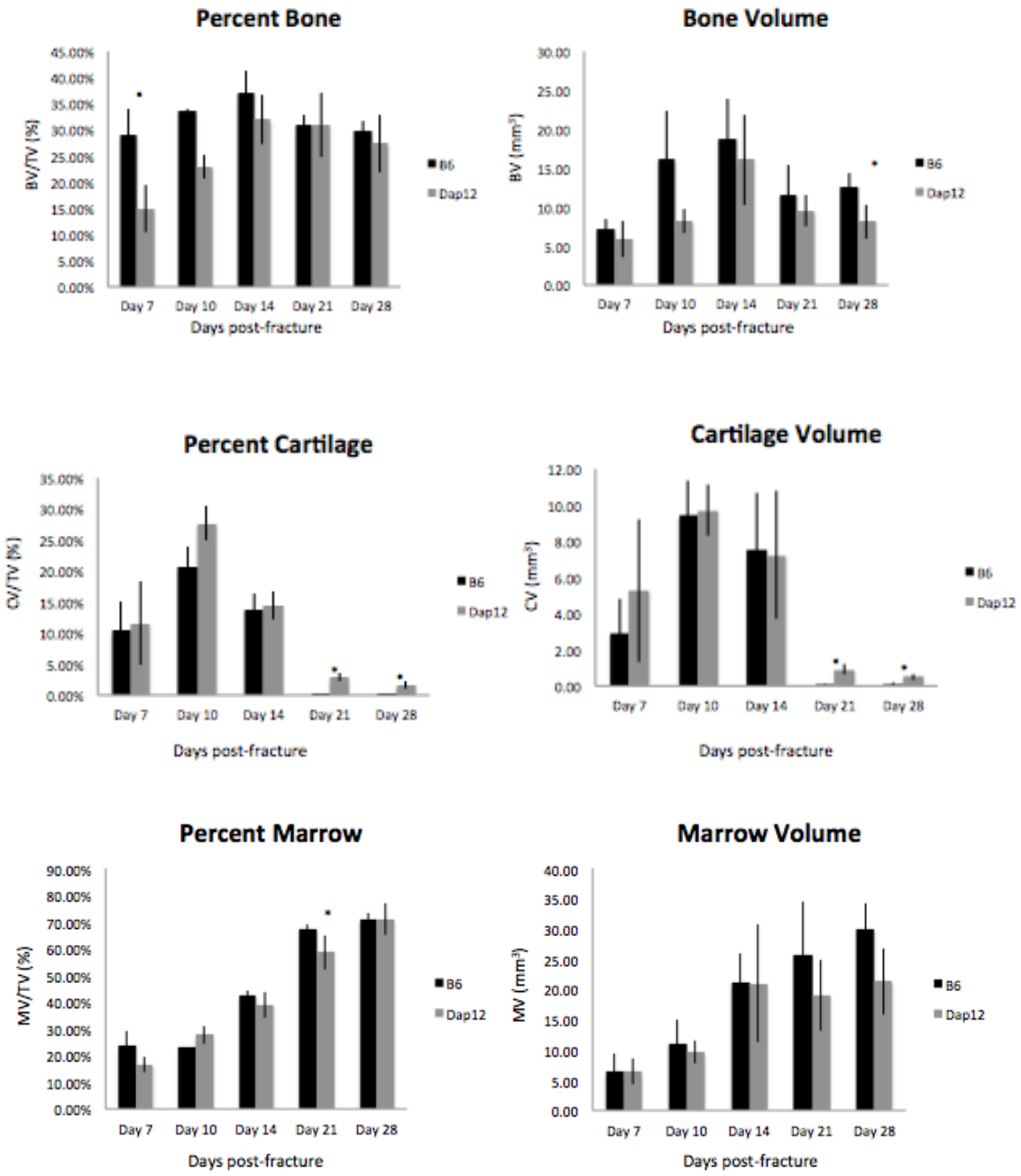


Figure 3. Milligan's Trichrome staining in B6 and DAP12$^{-/-}$ mice.
Representative sagittal images of fracture callus in B6 and DAP12$^{-/-}$ mice 7, 14, and 28 days post-fracture. B6 mice showed elevated levels of woven bone at 7 days after fracture (arrow). Fourteen days after fracture, DAP12$^{-/-}$ mice had significantly

higher fibrous tissue. Twenty-eight days after fracture, DAP12$^{-/-}$ had significantly higher levels of cartilage tissue



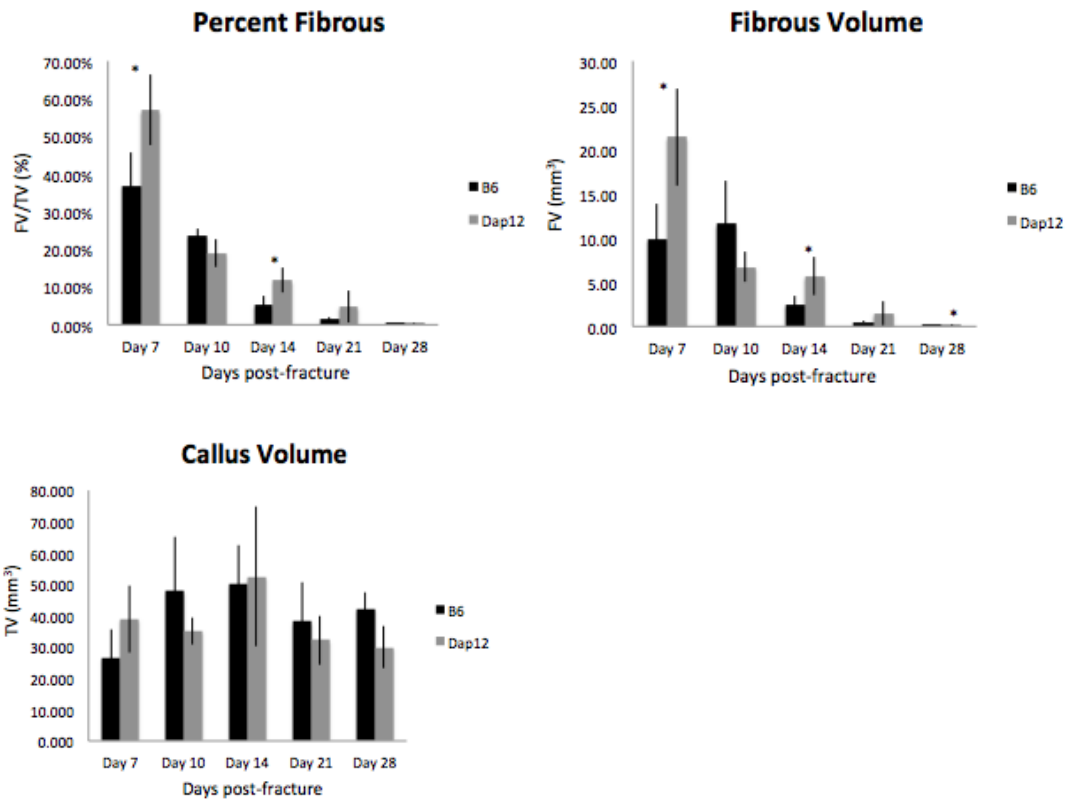


Figure 4. Healing curves of fracture callus in B6 and DAP12$^{-/-}$ mice. Quantitative histomorphometric analysis of the fracture callus of B6 and DAP12$^{-/-}$ mice. DAP12$^{-/-}$ mice had significantly less newly formed woven bone formation at day 7. DAP12$^{-/-}$ mice had significantly more cartilage tissue and fibrous tissue at day 21 and 28 and day 7 and day 14 respectively. DAP12$^{-/-}$ had significantly less bone marrow at day 21. No significant difference was observed in callus volume in the DAP12$^{-/-}$ and B6 mice. *$p<0.05$.

4. DISCUSSION

Bone fracture healing is a complex process involving multiple cells lineages, integral cell communication and signaling, and remodeling of tissue. Here we demonstrate DAP12 as an important signaling protein in in normal fracture repair. DAP12$^{-/-}$ showed statistically significant elevated levels of cartilaginous tissue at days 21 and 28, along with significantly less bone at day 7. These finding

demonstrated a decrease in woven bone formation and remodeling in DAP12$^{-/-}$ mice. Similar results were observed in Kaminura et. al.[32]

Micro-CT and micro-architectural analysis by Kaminurar et al.[32] further showed an increase in trabecular thickness and decreased percentage of new woven bone in the DAP12$^{-/-}$ mice compared to B6. Different osteoclast activity at the fracture site may cause this changed phenotype. During normal bone repair, osteoclasts are involved in removal of cartilaginous soft callus and remodeling of bony hard callus.[33] DAP12 is one of the primary signaling adaptors in osteoclast lineage cells.[34] It has been reported that DAP12$^{-/-}$ arrests osteoclast formation by disrupting the cytoskeletal matrix and inhibiting the transmigration through the osteoblast layer.[34] Previous studies have also demonstrated that normal fracture healing requires activation and differentiation of macrophages by DAP12.[17] Stimulation of DAP12 receptor is involved in giant cell formation by affecting endogenous transcripts of IL-6.[17] Signaling via DAP12 influences the Janus kinase/Stat signaling, which are known cascades in pro-inflammatory (M1) cell differentiation into macrophages.[35] The mechanisms of signaling cascades that results in differentiation are still not completely understood.

It is important to discuss the limitations of the present study. The DAP12$^{-/-}$ and B6 mice were not littermates. Studies with littermates limit other confounding variables that may arise from different genetic linages. These genotypic variances may cause altered inflammatory and fracture healing responses. Additionally, the non-parametric Wilcoxon test was chosen to determine significant differences

between DAP12$^{-/-}$ and B6 mice. Perhaps with a larger sample size, the data may display a more normal distribution, which allows the use of a parametric test.

5. CONCLUSION

Our study demonstrates DAP12 as a critical component in the normal fracture healing process. DAP12 plays a role in the initial inflammatory response along with the cartilage to trabecular bone remodeling. Enhancing the understanding behind the mechanism of fracture repair can lead to improve healing strategies and potential therapeutics. Medical professionals are always seeking ways to promote fracture healing, improve the body's natural mechanisms, and eliminate patients' pain and suffering. Further investigation on DAP12 is recommended, and this study attests to its value and potential benefits.

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