The Effect of Systemic Administration of Sclerostin Antibodies in a Mouse Model of Distraction Osteogenesis

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Table of Contents

Abstract

Distraction osteogenesis (DO) is a surgical technique widely used to treat complex orthopaedic conditions. One limitation of this technique is the long period the external fixator needs to be left in place until the newly form bone is completely consolidated. This might lead to significant morbidities in terms of persistent pain, risk of pin tracts infection and negative psychological impact on patients and their families. Although the use of sclerostin antibodies (Scl-Ab) has shown promising results to enhance bone repair in various animal models, its effect in DO remains to be determined. We hypothesized that the systemic administration of Scl-Ab can accelerate bone regeneration in a mouse model of DO. A total of 110 mice were randomized to saline versus Scl-Ab injection groups. After DO surgery in the right tibiae, mice were injected intraveounsly once weekly with Scl-Ab (100mg/kg) versus saline (0.1 ml). Mice were sacrificed at four time points, day 11 (mid-distraction phase), day 17 (end of distraction), day 34 (mid-conslidation) and day 51 (end of conslidation). Radiographic (Faxitron), microstructural (μCT), and qualitative histological analysis were performed for the lengthened tibiae at all time points. In addition, biomechanical testing was performed at day 34 and 51. Micro-CT results showed an increase of bone volume in the Scl-Ab treated group at day 11 ($P=0.009$) when compared to the saline group. A trend toward increase bone volume was observed in the Scl-Ab groups at day 17, 34 and 51 ($P>0.05$). Histological results showed predominately presence of chondrocytes and fibrocartilages in Scl-Ab group at day 11 when compared to the saline group. Radiographic bone scores were higher in the Scl-Ab treated groups at all time points with $P=0.04$ at day 11. Biomechanical analysis revealed a trend toward higher values of ultimate force and work

to ultimate point in Scl-Ab treated groups at day 34 and 51 ($P>0.05$) when compared to the saline groups. In conclusion, our data demonstrate the benefits of Scl-Ab on acceleration of bone regeneration and suggest its potential utility in clinical situations to reduce the treatment period with an external fixator during DO procedures.

RÉSUMÉ

La distraction osseuse (DO) est une technique chirurgicale largement utilisé pour traiter des conditions orthopédiques complexes. Une des limites de cette technique est le temps que le fixateur externe doit être laissé en place jusqu'à l'os nouvel soit complètement consolidé. Cela pourrait conduire à des comorbidités significatives en termes de douleur persistante, augmenter le risque d'infection des broches et l'impact psychologique négatif sur les patients et leurs familles. Sclerostin, est une glycoprotéine sécrétée qui interagit avec la protéine liée à la lipoprotéine receptor-5 (LRP5) et inhibe la voie de signalisation Wnt intracellulaire, ce qui conduit à une diminution de l'activité de la formation osseuse par les ostéoblastes. Lorsque Sclerostin est inactivée, la formation osseuse est donc stimulée. Nous émettons l'hypothèse que l'administration systémique d'anticorps sclérostine (Scl-Ab) peut accélérer la régénération osseuse dans une modèle de souris de la DO. Un total de 110 souris ont été randomisés à injections salées contre les groupes d'injections Scl-Ab. Après la chirurgie DO dans la tibia droit, les souris ont reçu une injection une fois par semaine avec Scl-Ab intraveineuse (100 mg / kg) par rapport à une injection de solution saline (0,1 ml). Les souris ont été sacrifiées à quatre points differents, dans le jour 11 (phase mi-distraction), 17 jours (fin de la distraction), 34 jours (mi-conslidation) et à 51 jours (fin de conslidation). L'analyse radiographique (Faxitron), de la microstructure (μCT), et histologique qualitative ont été effectuées aux tibias allongé à tous les points de temps. Aussi, les tests biomécaniques ont été réalisée au jour 34 et 51. Les résultats de Micro-CT ont montré une augmentation du volume osseux dans le groupe traité Scl-Ab à 11 jours ($p = 0,009$) par rapport au groupe de solution saline. Une tendance vers le volume augmentation osseuse a été observée dans les groupes Scl-

Ab au jour 17, 34 et 51 (P> 0,05). Les résultats histologiques ont montré principalement la présence de chondrocytes et fibrocartilages dans le groupe Scl-Ab à 11 jours par rapport au groupe de solution saline. Les scores radiographiques de remplissage osseous étaient plus élevés aux groups avec Scl-Ab à tous les points de temps avec P = 0,04 au jour 11. L'analyse biomécanique révélé une tendance vers des valeurs plus élevées de force ultime et le travail à point ultime aux groupes avec Scl-Ab à 34 jours et 51 (P> 0,05) par rapport aux groupes avec salins. En conclusion, nos données démontrent les avantages de Scl-Ab sur l'accélération de la régénération osseuse et suggère son utilité potentielle dans des situations cliniques afin de réduire la période de traitement avec un fixateur externe au cours des procédures DO.

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CONTRIBUTIONS OF AUTHORS

Asim Makhdom: Methodology planning, performed surgical procedures, harvested specimens, micro CT scans, X-rays, biomechanical testing, statistical analysis, manuscript preparation and editing.

Dominique Lauzier: Contributed to histological data analysis, manuscript editing.

Frank Rauch: Methodology planning, overall data analysis, manuscript preparation and editing

Reggie Hamdy: Methodology planning, overall data analysis, manuscript preparation and editing

ABBREVIATIONS

BMPs: bone morphogenetic proteins

BV :Bone volume

BV/TV: Bone volume/Tissue volume

DO: Distraction Osteogenesis

Dkk: Dickkopf

FGF: fibroblast growth factor

Fz: Frizzled

GFs: Growth factors

IGF: insulin growth factor

LRPR 5/6: low-density lipoprotein receptor-related protein 5 or 6

PDGF: platelet-derived growth factor

sFRPs: secreted frizzled-related proteins

SOST: sclerostin

Scl-Ab: Sclerostin Antibody

TGF-β: transforming growth factor-β

VEGF: vascular endothelial growth factor.

SECTION 1: INTRODUCTION:

{A} Rational and Objectives:

Distraction osteogenesis (DO) is a surgical technique widely used to treat complex orthopaedic conditions. Although very successful, one limitation of this technique is the long time the external fixator needs to be left in place until the new bone is completely consolidated. This can lead to significant morbidities in terms of persistent pain, increase risk of pin tracts infection and negative psychological impact on patients and their relatives (1-3). The question remains, how can we accelerate bone regeneration in patients undergoing DO, so that the external fixation device can be removed in a shorter period and therefore minimize the potential complications. Several biological, mechanical and biophysical techniques have been described in the literature to accelerate bone formation in DO(4, 5). However, these techniques currently have limitations and have not achieved clinical consistent satisfactory outcomes(6-10). The inhibition effect of sclerostin on WNT signaling pathway and hence on bone regeneration is currently well studied(11, 12). Targeting this protein with the sclerostin antibody {Scl-Ab} has been a promising and successful strategy in many studies to accelerate bone formation(13-15). However, to the best of our knowledge, there is no study that has examined the effect of Scl-Ab in the context of DO. The objective of this thesis was to determine the in vivo effect of the systemic administration of Scl-Ab on acceleration of bone regeneration in a mouse model of DO. Through an experimental study design, we examined the role of Scl-Ab on bone regeneration at 4 different time points; day 11 (mid-distraction), day 17 (end of distraction),day34 (mid-consolidation) and day 51 (end of consolidation) . This was performed by means of x-rays, microcomputed tomography {Micro-CT},

biomechanical testing and qualitative histology.

{B} Review of The Literature:

Distraction Osteogenesis and Its Clinical Importance:

There is an intrinsic capacity for bone to heal spontaneously following injury. However, this capacity cannot extend beyond a certain critical size defect and therefore an external intervention becomes necessary(16). Several techniques are currently available to treat these large defects including the gold standard bone grafts(17). These procedures have limitations {huge financial cost} in cases of severe bone loss or when large portions of bone need to be lengthened(18). Distraction osteogensis {DO} is considered an excellent option in such circumstances. Since its introduction by Ilizarov in early 1950s, DO technique has been used worldwide to treat many complex orthopedic and craniofacial conditions with satisfactory results. These conditions include nonunions, congenital and acquired longitudinal bone deficiencies, and severe bone loss secondary to infections and bone tumors(19). DO technique is a controlled surgical procedure that has the ability to achieve spontaneous bone regeneration by using the mechanical forces to stimulate the endogenous biological response. This technique is performed as follows: the proximal and distal ends of the bone are stabilized and fixed by using an external fixator device followed by a low energy osteotomy to divide the bone in two segments (proximal and distal). Then, a latency phase of 5-10 days is required to allow for the hematoma formation. Subsequently, the distraction phase is initiated in which the two-bone segments are gradually distracted until the desired lengthening is obtained. The consolidation phase follows in which the distraction is stopped and the two-bone

segments are held in place by the fixator until the new bone in the distraction gap is completely fused. Each one centimeter of lengthening typically requires one month period of consolidation {Figure-1}(20). The external fixator can be only removed once complete bone healing is achieved.

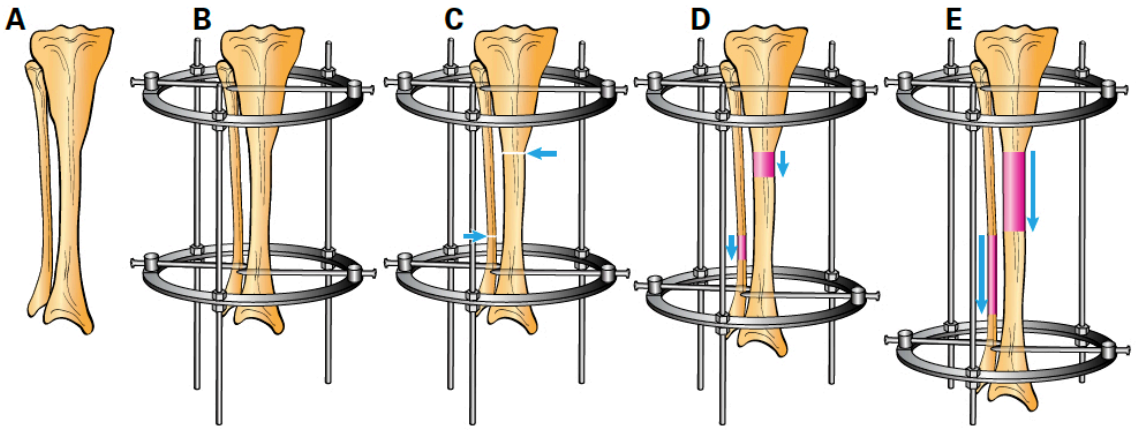

Figure 1. Description of distraction osteogenesis technique. (A) Showing the tibial bone that need to be lengthened. (B) Application of external fixator at the proximal and distal end. (C) Tibial and fibular osteomty. (D) Distraction phase. Note the new bone formation in the distraction gap (E) consolidation phase. Adapted from Makhdom et al 2014 with permission {15}.

Molecular Response and Mechanobiology in Distraction Osteogenesis:

During the latency phase, an intense local inflammatory reaction eliciting secretion of cytokines and growth factors occurs immediately after the low energy osteotomy. This includes secretion of interleukin-1, interleukin-6, TGF-β, BMPs, PDGF, FGF, IGF and VEGF and activation of Wnt signaling pathway(4, 21, 22). This enables local distribution, differentiation and proliferation of mesenchymal cells, fibroblasts, and osteoprogenitors as well as fibrin/collagen matrix edification and capillary invasion. The osteogenic potential of these pathways is achieved by inducing the expression of bone-specific genes (e.g. Runx2, Osterix) (23). Finally, differentiation of osteoblasts is associated with an increased expression of type 1 collagen and alkaline phosphatase(24). Once these are achieved, a soft bone formation {callus} that surrounds the osteotomy bone ends and between the endosteal and periosteal surfaces is formed. In the distraction phase, this callus formation is exposed to tensile stresses meant to facilitate bone regeneration in the distraction gap(25). The mesenchymal stem cells that migrated and proliferated into the callus differentiate primarily into fibroblast like cells(21). They adopt a well-defined direction that is parallel to the vector of distraction. During this phase, there is increased blood flow, neovascular proliferation and ongoing up-regulation of growth factors, Wnt signaling pathway and matrix proteins(26).

The physical forces and strains are transformed into biochemical signals which are then incorporated into molecular and cellular responses {mechanotransduction}(27, 28). This is responsible for maintaining the dynamic balance between bone regeneration and bone resorption. The mechanical forces applied to the bone are related to the flow past the osteocytic processes in their canaliculi. The osteocytes can sense the flow of fluid and

then produce signaling molecules that regulate osteoblast-mediated bone formation and osteoclast-mediated bone resorption. Formation of a new bone that is strong enough to sustain physiological loadings requires accurate surgical technique and osteotomy, stable fixator and specific distraction rate and rhythm(29).

Limitations of Distraction Osteogenesis:

DO is considered the best in vivo tissue engineering techniques as it has the ability to accomplish spontaneous regeneration of de novo native bone without the need for bone grafts. However, this technique has complications and drawbacks due to the need for keeping the external fixation device for long periods until the bone is completely consolidated(2). These complications include pin sites infection, osteopenia, negative psychological impact on patients and their families(3). Furthermore, long treatment periods is associated with huge financial burden to the family and health care institution. One solution to decrease the complications rates is by accelerating the bone formation in the distraction gap and therefore removing the external fixation device at a shorter period.

Methods to accelerate bone regeneration in Distraction osteogenesis:

Several biological, mechanical, and biophysical techniques have been described in the literature to accelerate bone formation in DO.

One of the promising biological methods is the exogenous application of growth factors {GFs} to promote cellular migration, differentiation, and bone growth(30). Of these, bone morphogenic protein (BMP) signaling pathway is well recognized for its significant contribution in the bone regenerative process(31). Interestingly. BMPs are the only osteoinductive GFs that play a role in early differentiation process of undifferentiated mesenchymal cells(32). In our laboratory and others, the exogenous

application of BMPs has shown promising results from the animal models of DO(33-36). However, the use of BMPs is still limited in the clinic. This is related to the rapid clearance of these proteins from the circulation, short resident time in tissues and short half-life(37). Therefore, large doses are required in order to achieve the desired outcome. This is associated with adverse outcomes such as toxicity and huge cost.

Several studies have reported on the biophysical methods to accelerate bone formation in DO. The low-intensity pulsed ultrasound (LIPUS) is one option to accelerate fracture healing(38, 39). This device is applied locally at the skin and corresponding to the point of distraction gap. The ultrasound may increase the blood flow at the site of osteotomy. However, the critical role of LIPUS in DO is still unknown due to lack of data and prospective clinical trials. Extracorporeal Shock waves therapy has been also investigated as another biophysical method to accelerate bone healing with little available data(40).

Mechanically, changing the rate and rhythm or adding compression during the distraction phase has been investigated in an experimental animal model. Hente et al. noted that the amount of periosteal callus formation was up to 25 times greater on the compression side of the distraction gap when compared to the distraction side in an experimental model of tibial fractures, using an external fixator(41). In the context of DO, this concept still in its experimental level and future studies are required.

All methods of acceleration in bone regeneration are summarized in Figure-2. For detailed information on this topic the reader can refer to a book chapter by Hamdy et al(5).

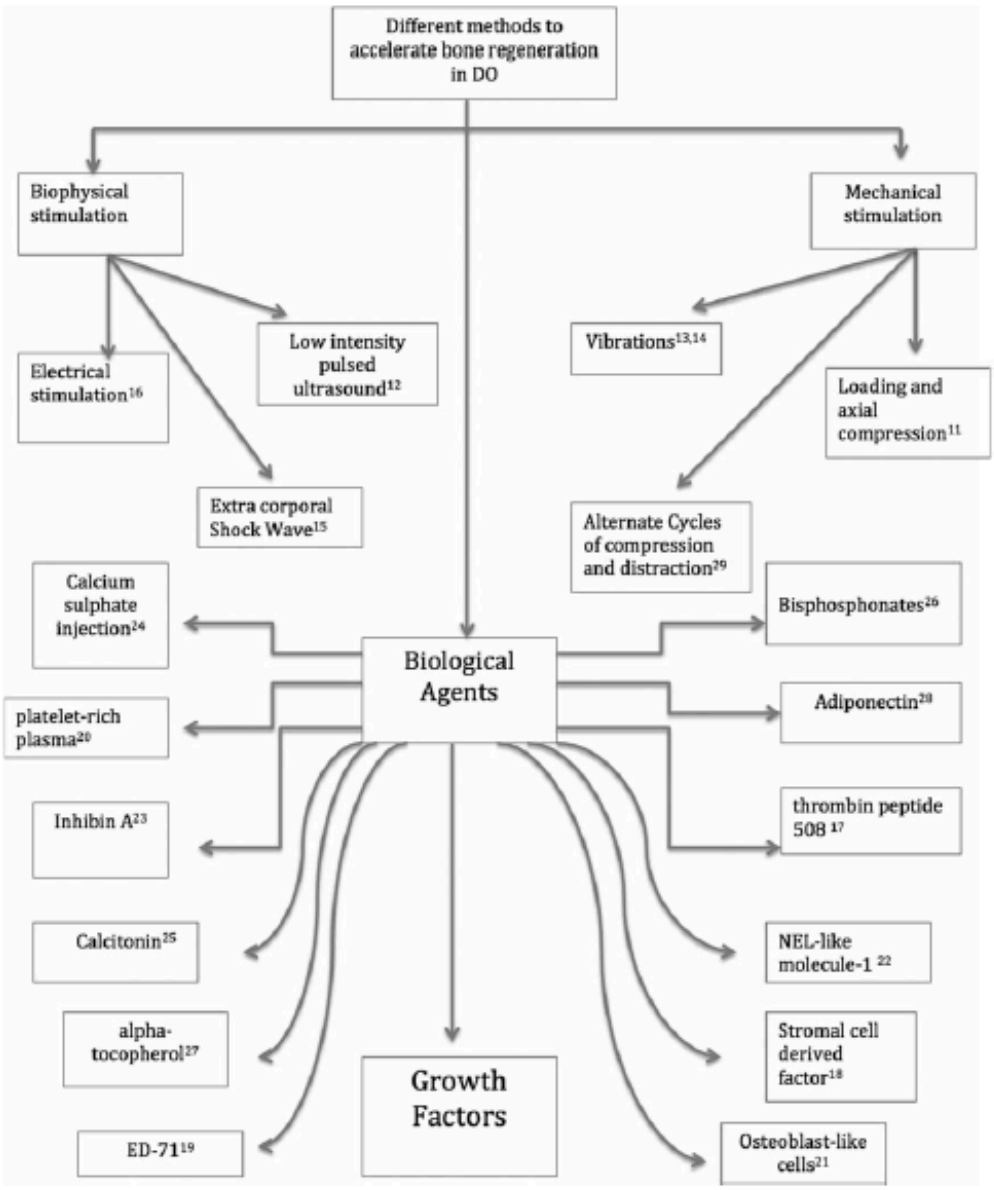

Figure-2: Different methods to accelerate bone regeneration in distraction Osteogenesis. Adapted with permission from Makhdom and Hamdy 2013 {4}.

Wnt Signaling pathway and The Role of Sclerostin in Bone formation:

Canonical Wnt signaling pathway has been recently recognized as a critical regulator in the bone regenerative process(11). WNT molecules belong to a family of 19 secreted proteins that are involved in various biological processes, particularly osteogenesis(42, 43). Wnt proteins form a complex with the receptors of low-density lipoprotein receptor-related protein 5 or 6 (LRP5/6) and Frizzled (Fz) to initiate Wnt signaling cascade. Subsequently, this will result in an accumulation and nuclear translocation of B-catenin, which will interact with T-cell factor/lymphoid enhancer factor to activate the transcription of Wnt downstream target genes for osteoblasts differentiation. The biological activity of Wnt signaling is tempered by negative feedback mechanisms that antagonize Wnt ligand–receptor interactions such as sclerostin {SOST}, secreted frizzled-related proteins (sFRPs) and Dickkopf {Dkk}(44) **{Figure-3}**.

Interestingly, sclerosteosis and van Buchem disease led to the discovery of sclerostin(13). These are rare bone disorders characterized by high bone mass secondary to a deficiency of the expression of sclerostin (encoded by SOST gene). Sclerostin is a glycoprotein that is exclusively secreted by osteocytes to interact with the LRP5/6 receptor and inhibits the intracellular Wnt signaling pathway, leading to decreased bone formation activity(11). In addition, sclerostin was found to inhibit bone morphogenic protein (BMP) pathway predominantly by decreasing the secretion of BMP7 in osteocytes(45). Researchers have found that targeting the Sclerostion and Wnt signaling pathway is a promising strategy to increase bone formation with numerous applications. These will be discussed in the following section.

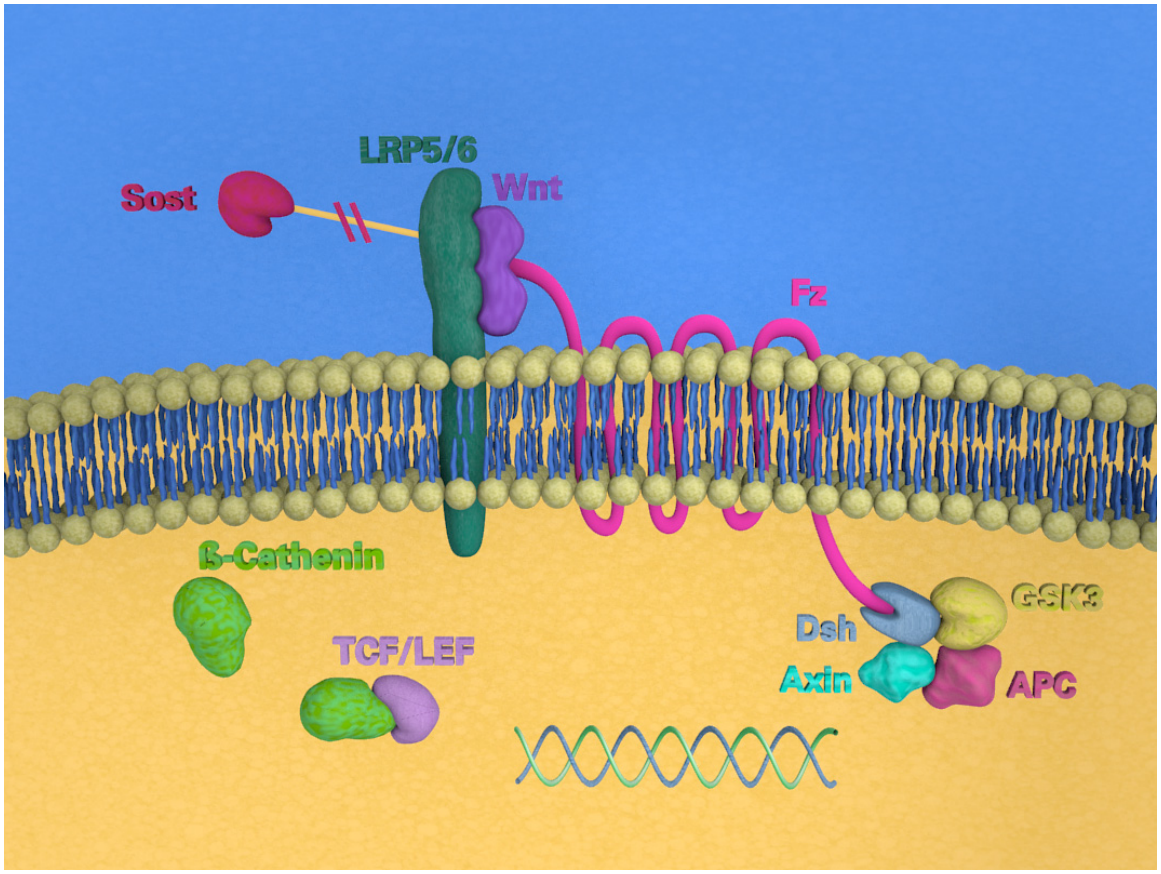

FIGURE .3

The illustration shows canonical Wnt signaling pathway. Wnt ligands (Wnt) form a complex with the receptors low-density lipoprotein receptor-related protein 5 or 6 (LRP5/6) and Frizzled (Fz). Disheveled (Dsh) is then able to bind to Fz. Dsh forms a complex with glycogen synthase kinase 3ß (GSK3ß), Axin, and adenomatous polyposis coli (APC). This complex protects ß-catenin from proteasomal degradation. Subsequently, ß-Catenin can accumulate in the cytosol and translocate to the nucleus. In the nucleus it interacts with the T-cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors, leading to gene transcription. Sclerostion (SOST) binds to (LRP5/6) and prevents Wnt binding.

Sclerostin Inhibition: What Does The Literature Say?

Previous experimental and clinical trials have shown that antagonizing the sclerostin function would improve bone mass in osteoporotic bones. Ominsky et al have examined the effect of Scl-Ab in ovariectomized female rats(14). These animals were left for 1 year to induce significant estrogen deficiency–induced bone loss. Subsequently, rats were randomized to Scl-Ab treated group versus sham control group for a duration of 5 weeks. The treated groups with Scl-Ab have shown significant increases in bone formation in trabecular, periosteal, endocortical, and intracortical surfaces. Interestingly, the effect of Scl-Ab did not only result in reversal outcome at several skeletal sites but also has increased the bone mass to levels greater than those found in non-ovariectomized control rats. Furthermore, Padhi et al have conducted a randomized, double-blind, placebo-controlled study in 72 healthy healthy men and postmenopausal women who were treated with single dose of Scl-Ab(46). Dose-depended increases in the bone regeneration markers procollagen type 1 N-propeptide (P1NP), bone-specific alkaline phosphatase (BAP), and osteocalcin were observed. Additionally, statistically significant results were found in terms of increase in bone mineral density up to 5.3% at the lumbar spine and 2.8% at the hip when compared with placebo at day 85. All study participants have generally tolerated the Scl-Ab with no serious side effects. This human study is very promising and considered a major advancement in the field of osteoporosis research. However, long term effects of the Scl-Ab remain to be determined.

Several experimental studies have also proven the potential benefits of Scl-Ab in bone implant fixation and fracture healing. Virk et al have investigated the role of inhibiting the sclerostin in femoral critical size defect in a rat model(47). The authors

have randomized the rats into sclerostin antibody {Scl-Ab} treatment group versus saline injection group. These samples were examined by using Micro-CT. At 12 weeks, the treatment group demonstrated a significant increase in new bone formation ($p < 0.05$) when compared with the control group. Similarly, Virdi et al examined the role of Scl-Ab on the fixation strength of titanium cylinders which were placed in the femoral medullary canal of 90 rats(48). These rats were randomized to a Scl-Ab treatment group versus saline injection group. At 4 and 8 weeks, the authors found significant higher fixation strength in rats treated with Scl-Ab. These results were affirmed by Aghlome et al who have studied the effect of Scl-Ab on the pull out strength of screw fixation(49). The treated rats had significant increase in the pull-out force by 50% when compared with controls after 2 and 4 weeks. Additionally, Micro-CT results revealed that the Scl-Ab led to a 30% increase in bone volume fraction in a region surrounding the screw.

Taken together, the current body of the literature has confirmed the utility of Scl-Ab to enhance bone regeneration in various bone applications.

WNT Signaling Pathway in Distraction Osteogenesis:

Although the canonical WNT signaling pathway has been recently identified as a critical regulator in modulating bone formation and bone mass, very little is known about its role in DO. In our laboratory, we have examined the spatial and temporal expression of WNT signaling proteins in a mouse model of distraction osteogenesis(22). Forty healthy adult female wild-type mice were used for the experiment. After DO surgery, these mice were sacrificed at 4 time points; day 5 (end of latency phase), days 11 and 17 (middle and end of distraction phase), days 34 and 51 (middle and end of consolidation phase). Immunohistochemistry was then carried out and the results have revealed an increased

expression of WNT ligands (WNT4 and WNT10A), receptors (FZD1 and 2, LRP5 and 6), β -catenin, and pathway antagonizers (Sclerostin, DKK1; sFRP1, 2, and 4) during the distraction phase. These molecules were then down-regulated during the consolidation phase. Furthermore, predominance of chondrocytes was found during the early stages of DO (day 11 and 17), which coincided with an upregulation of WNT signaling. This study confirmed the important role of WNT pathway in DO and opened the door for therapeutic strategies and regenerative medicine for accelerating bone regeneration in DO.

SECTION 2: MANUSCRIPT

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The Effect of Systemic Administration of Sclerostin Antibodies in a Mouse Model of Distraction Osteogenesis

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Abstract

Distraction osteogenesis (DO) is a successful technique for bone lengthening, but one problem is the need to keep an external fixator in place until bone completely regenerates. We hypothesized that the systemic administration of sclerostin antibodies (Scl-Ab) can accelerate bone regeneration in a mouse model of DO. A total of 110 mice were randomized to receive one intravenous injection per week of either Scl-Ab (100 mg per kg body weight) or saline after DO surgery. Mice were sacrificed on day 11, 17, 34 or 51 post-surgery. Microcomputed tomography showed that bone volume per tissue volume of the Scl-Ab treated group was significantly higher on day 11 ($P=0.009$). Histological examinations indicated that chondrocytes and fibrocartilage predominated in the Scl-Ab group at day 11. The radiographic score of bone healing was also higher in Scl-Ab treated animals at day 11. There was a trend towards higher ultimate force and work to failure in Scl-Ab treated groups on day 34 and 51 ($P>0.05$). These data suggest the potential utility of Scl-Ab to reduce the time during DO when an external fixator is required.

Key words: Bone regeneration; Distraction Osteogenesis; Sclerostin antibody, Wnt signaling

Introduction

Distraction osteogenesis (DO) is a widely used surgical technique to treat many complex craniofacial and orthopedic conditions, including limb length discrepancy, nonunion, acquired and congenital bone defects, and bone loss secondary to infections and bone tumors [1]. The technique involves osteotomy followed by gradual distraction of the two bone segments with an external fixator. This stimulates the endogenous biological response to create new bone (2). Although very successful, one major limitation of this technique is the long time that the external fixator needs to be left in place until the newly formed bone is completely consolidated. This can be associated with adverse events such as increase risk of infection, osteopenia, persistent pain, and negative psychological impact on patients and their families [3-6]. Accelerating bone regeneration during DO would allow removing the external fixator in a shorter time and might limit these adverse events.

Several biophysical, mechanical and biological methods have been investigated to accelerate bone regeneration during DO (2). One of these is the exogenous application of growth factors, such as bone morphogenetic proteins, to promote cellular migration, differentiation, and growth of bone tissue. Others and we have found promising results in animal studies evaluating bone morphogenetic proteins [7-10], but the rapid clearance of these proteins from the circulation, short resident time in tissues and short half-life mean that large doses are required, increasing the risk for adverse effects [11-13].

The canonical Wnt signaling pathway is a critical regulator in the bone regenerative process, which makes this pathway an interesting target for the acceleration of bone regeneration [14, 15]. Wnt signaling can be modulated by systemic application of

antibodies against sclerostin [16]. Sclerostin is a glycoprotein that is exclusively secreted by osteocytes, interacts with the LRP5/6 receptor and thereby inhibits the intracellular Wnt signaling pathway, leading to decreased bone formation activity [17]. Sclerostin may also decrease the secretion of bone morphogenetic protein 7 in osteocytes [17]. Studies suggest that antagonizing sclerostin can enhance bone formation during fracture healing and implant fixation, and may be useful to treat low bone mass in the context of estrogen-deficient osteoporosis and osteogenesis imperfecta [18-21]. However, to the best of our knowledge, the effect of sclerostin inhibition during DO has not been assessed. The aim of this study was therefore to determine if systemic delivery of sclerostin antibodies (Scl-Ab) can accelerate bone regeneration in a mouse model of DO.

Materials and Methods

Study Design

Osteotomy and DO of the tibia were performed on male mice (day 0). After surgery, animals were randomly assigned to receive injections with Scl-Ab or normal saline and outcomes were analyzed on four different time points. The study was approved by the McGill University Animal Care Committee.

A total of 110 white male wild-type FBV mice (Charles River Inc, Montreal) were utilized. Mice were 8 to 12 weeks of age, and had an average weight of 23 g. The dose of Scl-Ab (supplied by Novartis Inc.) was 100 mg per kg body weight, the dose of saline injection was 0.1 ml. The dose of Scl-Ab was chosen based on previous results by Novartis. Both Scl-Ab and saline injections were delivered by the intravenous tail route

immediately after the surgery and then once weekly until the time of sacrifice.

Mice were sacrificed at four different time points: Day 11 (mid-distraction phase; n=20), day 17 (end of distraction; n=20), day 34 (mid-consolidation; n=34) and day 51 (end of consolidation; n=36). Of the 110 mice, 15 mice (6 mice from the Scl-Ab group and 9 mice from the saline group) were euthanized post operatively due to infection (n=4), foot necrosis (n=5) and wound dehiscence (n=2), while 4 mice were euthanized post-operatively secondary to a fracture below (n=3) or above the external fixator (n=1), leaving 95 mice that were included in the present study**.** DO procedures and final sample allocation across time points and tests are summarized in Figure 1.

Immediately after sacrifice, all samples underwent μCT and radiographic analyses. Samples for histological analysis were immersed in buffered formaldehyde. The soft tissue for theses samples was kept over the distracted bone specimens. Samples for biomechanical testing were immersed in phosphate buffered saline.

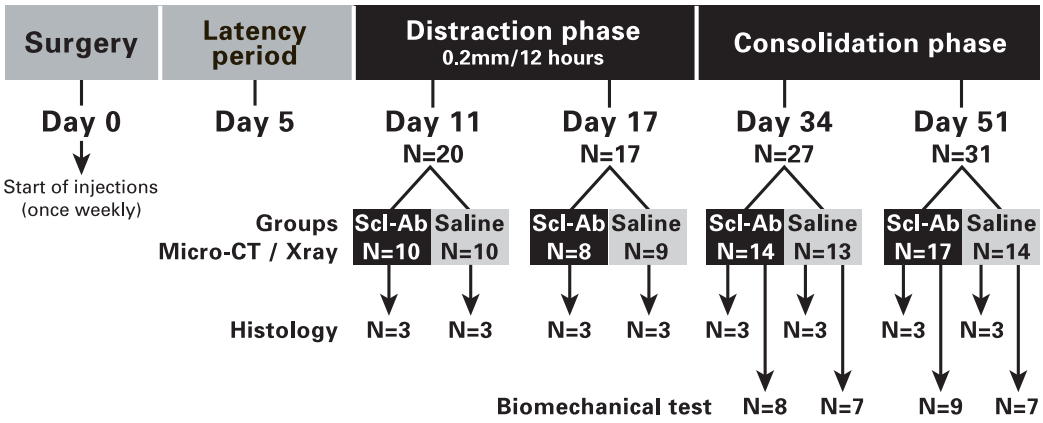

Figure 1. Schematic representation of the study design and the sample distribution.

Surgical Procedures

Murine tibial DO was performed using a miniature Ilizarov fixator (Paolo Alto, CA), as previously described by our group [22-24]. A set of two 0.25 mm pins was drilled perpendicular to each other into the proximal and distal metaphysis of the right tibia. These pins were locked into position using two parallel rings and eight hexagonal nuts. Three threaded rods were used to connect the two parallel rings. Subsequently, a transverse low-energy osteotomy was performed along the middle diaphysis of the right tibia, between the proximal and distal pins, using a no. 11 surgical scalpel (Fisher Scientific, Osaka, Japan). The fibula was then broken using the back end of the scalpel.

A latency period of 5 days was allowed and followed by distraction at rate of 0.2 mm every 12 hours for 12 days. All surgeries were performed under general anesthesia using inhaled isoflurane and subcutaneously injected with two doses (before and after surgery with 6 hours interval) of buprenorphine (0.1 mg/kg) and 4 doses (intraoperative and then within 24 hours interval) of carprofen (5mg/kg) for postoperative pain management. All animals were monitored immediately after surgery and then daily throughout the study period until the time of sacrifice. The mice were euthanized by CO_2 asphyxia under general anesthesia at the time of sacrifice. The entire callus located between the distracted bone fragments of the operated tibiae was dissected for subsequent analysis. Cuts were made proximal and distal to the distracted region to avoid disturbing the bony callus.

Microcomputed Tomography and Radiography

A SkyScan 1072 device (Aartselaar, Belgium) was used to perform the μCT analysis. Distracted tibiae were scanned at 45 KeV/255 μA with 25 X magnification (11.5 μm pixel size). Image reconstruction was performed using NRecon (version 1.4.4, SkyScan). The CT Analyzer (1.8.0.2, SkyScan) was used to measure static histomorphometric parameters of the region of interest, which was defined as distracted area between the proximal and distal bone ends. These parameters included tissue volume (mm^3), bone volume (mm^3), bone volume per tissue volume (BV/TV, %), trabecular number (1/mm), trabecular separation (mm), and trabecular thickness (mm).

A Faxitron MX-20 device (Faxitron X-Ray Corporation,Wheeling, IL) was used to produce radiographs of the distracted specimens. The results of radiographs were unlabeled and then graded by three blinded observers using 4-point bone fill score as previously described [22, 23, 25]. This score is as follow; 0 = no bone, 1 = 0% to less than 50% bone fill, 2 = 50% to less than 100% bone fill, and 3= complete bone fill.

Qualitative Bone Histology

After completion of μCT and radiological analyses, three specimens from each group at each time point (3 specimens x 2 groups x 4 time points = 24 specimens in total) were processed for histology. Samples were fixed in buffered formalin, then decalcified in formic acid for 9 days, embedded in paraffin, and sectioned using a Leica RM 2255 microtome (Leica Microsystems, Richmond Hill, ON, Canada).

Following deparaffinization and hydration, sections were stained using Trichrome Goldner. Pictures were taken under various magnifications using a Leica microscope

(Leica Microsystems, Richmond Hill, ON) attached to a Q-Imaging camera (Olympus DP70, Japan) to detect non-mineralized (red stained) and mineralized (green-stained) regions.

Biomechanical Testing

Eight specimens from each group at day 34 (total N=16) and 9 specimens from each group at day 51 (total N=18) were sent for biomechanical testing. Of these specimens, one specimen (saline group) at day 34 and two specimen (saline group) at day 51 had a persistent defect in the distracted zone. Therefore these 3 specimens were excluded from the biomechanical analysis.

A three-point bending test was performed at McGill Centre for Bone and Periodontal Research of McGill University (Montreal, Canada) using the Mach-1^{TM} Micromechanical Systems device (Bio Syntech Canada, Inc., Laval, QC). The three-point bending test was chosen over other methods of biomechanical testing based on previously reported studies in mice [23, 26]. A bending load was applied downwards on the mid-shaft of the posterior surface of the lengthened tibia at a rate of 50 mm/s until failure. Failure loads were analyzed using the Mach-1^{TM} Motion and Analysis Software (version 3.0.2, Bio Syntech Canada). A load-displacement curve was generated using this software to measure four biomechanical parameters including stiffness (N/mm), ultimate force (N), ultimate displacement (mm), and work to failure (N*mm).

Statistical Analyses

Means and standard deviations were used for descriptive statistics. Mann-Whitney test was used to compare between the Scl-Ab and saline groups at separate time points in terms of bone fill scores, μCT and biomechanical testing results. A P value <0.05 was considered statistically significant. Calculations were performed using SPSS version 20.0 (SPSS Inc., Chicago, IL, USA).

Results

Tissue volume and bone volume were higher in the Scl-Ab group at day 11 when compared to the saline group (Table 1, Figure 2). No statistically significant group differences in these parameters were found at day 17, 34 and 51, but at each time point the average bone volume was numerically higher in the Scl-Ab group. No significant group differences were found for relative bone volume (BV/TV), trabecular thickness, number and separation at any time point (Table 1). The average bone fill score was numerically higher in the Scl-Ab group at each time point, but the group difference reached statistical significance only on day 11 (Table 2, Figure 2).

Qualitative histological evaluation showed phenotypic differences at day 11, where the specimens from Scl-Ab treated mice showed more chondrocytes and fibrocartilage when compared to the control group (Figure 3).

Biomechanical testing showed that ultimate displacement was higher in the Scl-Ab than in the saline treated group at day 34 but not at day 51 (Figure 4). Stiffness, ultimate force and work to ultimate point were all numerically higher in the Scl-Ab group, but none of the differences reached statistical significance.

Variable	11 days			17 days			34 days			51 days		
	Scl-AB (N=10)	Saline (N=10)	P	Scl-AB (N=8)	Saline (N=9)	P	Scl-AB (N=14)	Saline (N=13)	P	Scl-AB (N=17)	Saline (N=14)	P
Tissue volume (mm^3)	10.2±7.5	6.4±9.6	0.01	12±12	9.6±4.8	0.88	32±20	30±24	0.46	27±20	22±12	0.72
Bone volume (mm^3)	0.47±0.7	0.3±0.86	0.009	0.7±1.4	0.4±0.8	0.63	1.2±1.1	0.8±0.9	0.13	1.4±0.7	1.1±1.0	0.19s
BV/TV (%)	4.1±6.0	2.2±2.5	0.4	3.4±3.5	3.2±5.6	0.44	4.0±2.5	2.9±1.8	0.15	7.2±4.6	5.5±4.7	0.23
Trabecular thickness (mm)	0.09±0.09	0.04±0.02	0.05	0.1±0.3	0.08±0.03	0.70	1.1±2.0	0.4±0.9	0.26	1.8±1.8	0.93±1.1	0.08
Trabecular number (1/mm)	0.4±0.3	0.34±3.6	0.38	0.5±0.6	0.5±0.4	0.96	0.5±0.3	0.46±25	0.33	0.7±0.5	1.08±2.0	0.08
Trabecular separation (mm)	0.4 ±0.14	0.4±2.6	0.24	1.01+0.14	1.2±0.4	0.48	2.2±2.0	1.4±0.9	0.17	2.8±1.8	1.67±1.0	0.40

Table-1: Micro-CT results across all time points.

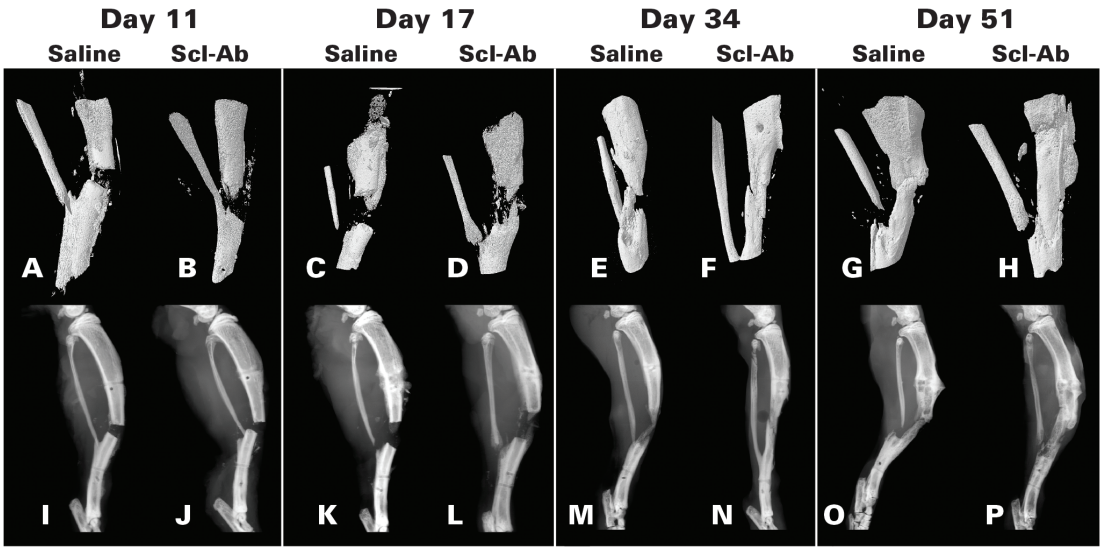

Figure 2. Micro-CT (Top panel) and radiological images (bottom panel) of lengthened tibias, analyzed using micro-CT (A–H) and Faxitron x-ray (I–P).

Time point	Average bone fill score		
	Scl-Ab group	Saline group	P value
Day 11	0.7	0.5	0.049
Day 17	1.08	1.03	0.70
Day 34	1.6	1.3	0.12
Day 51	2.1	1.9	0.18

Table-2: Bone-fill scores across all time points.

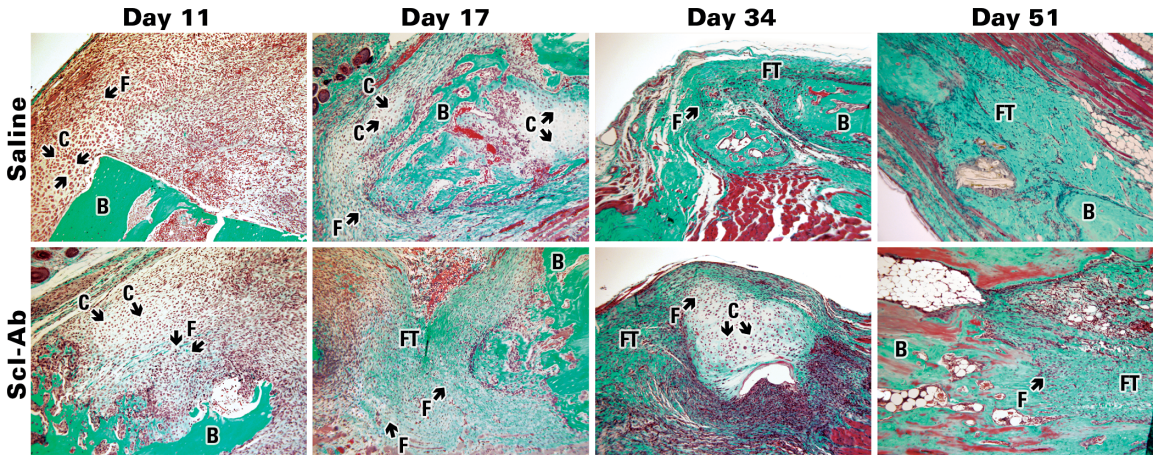

Figure 3. Histological images of distracted tibias stained using the Goldner-Trichrome technique. At 11 days, Scl-Ab specimens show a predominance of chondrocyte and fibrocartilage when compared to the control group as indicated by the arrows. At the other time points, both groups contained varying levels of mineralized (green) and nonmineralized (red) tissue. Chondrocytes and fibrous tissue were also present in the distracted samples, as indicated by the arrows in the diagram (magnification 100X).

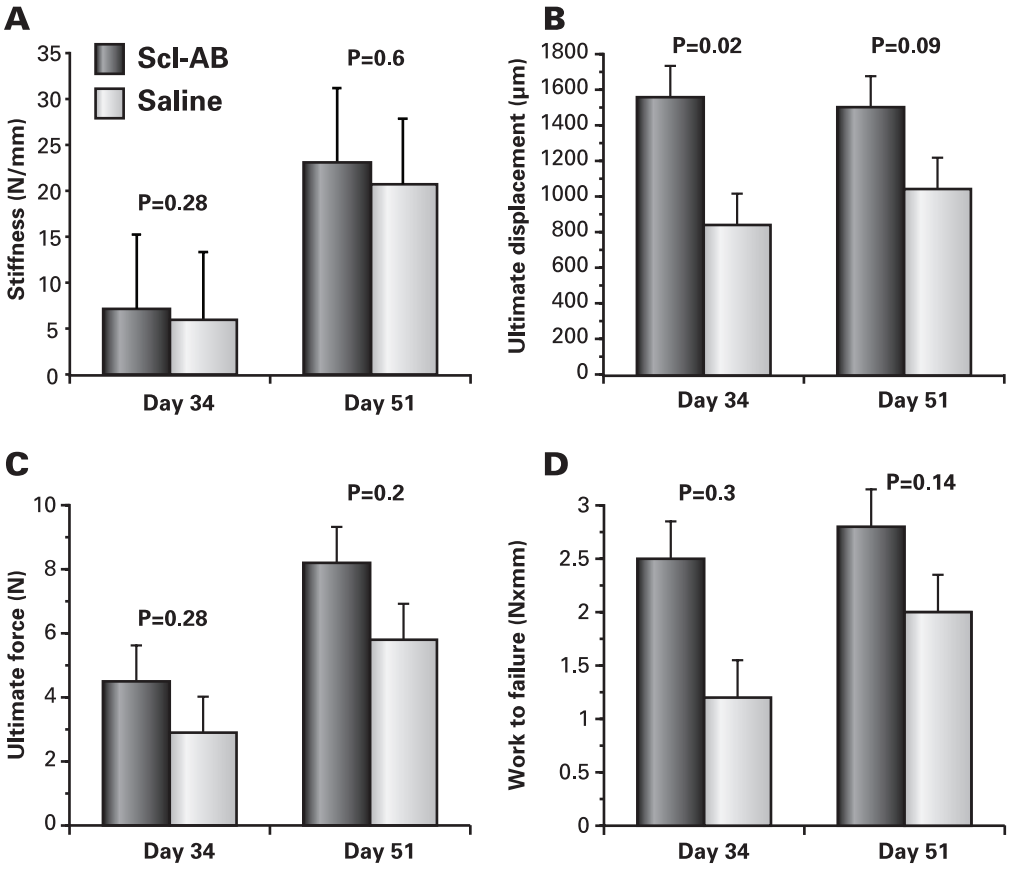

Figure 4. Biomechanical testing results. Biomechanical testing parameters to compare sclerostin-antibody (Scl-Ab) injected and saline-injected (control) groups at 34 days and 51 days post-surgery.

Discussion

In the present study we found that the systemic administration of Scl-Ab led to some acceleration of bone regeneration during DO. Higher values of μCT parameters, bone fill scores and biomechanical parameters were observed in Scl-Ab group when compared to the control group.

Bone volume was significantly higher in the Scl-Ab group at day11 and qualitative histological analysis also showed some differences at the same time point. These findings suggest that Scl-Ab was effective mostly during the distraction phase of DO. This corroborates the evidence of our previous report in which we found that positive regulators of Wnt signaling were most highly expressed during the distraction phase, while their expression decreased during the consolidation phase [15].

Biomechanical results also showed some promising results. Even though group differences were significant only for ultimate displacement on day 34, it should be noted that work to failure was already numerically higher in Scl-Ab group at day 34 than in the control group at day 51. This is in line with the view that Scl-Ab injections accelerated the bone regeneration process. Our results are thus in accordance with the results of other investigators, who found that Scl-Ab injections accelerated the healing process after osteotomy (50), improved the mechanical fixation of medullary implants [28], and improved healing of bone defects [29, 30].

In bone repair and implant fixation models, there is some discussion whether Scl-Ab has its maximal effect during the repair phase (early after injury) or in the remodeling phase (late after injury) [28, 31, 32]. Our data suggest that the maximum benefit of the systemic administration of Scl-Ab occurs during the distraction phase. It would therefore be

interesting to investigate whether a short-term intervention limited to the distraction phase is as effective as the injection of Scl-Ab throughout the follow-up period. Although the mechanism of the beneficial effect of Scl-Ab in the context of bone regeneration is not clear, it is interesting to note that sclerostin levels in fracture hematoma are significantly higher than in serum [33]. It could thus be that systemically administered Scl-Ab has a particularly strong effect on fracture or osteotomy regions, given the availability of large quantities of the target antigen.

Although systemic injections of Scl-Ab are thought to be safe and are generally well tolerated in adults [19], the effect of this approach in children remains to be investigated. In the context of DO where a local effect is desired, the development of local delivery methods may be advantageous.

A limitation of the present study is that results had quite a large within-group variability, which made it difficult to detect statistically significant results. Variability in the stability of the external fixator certainly contributed to the variability in results. In addition, variations in the age of the mice (between 8 and 12 weeks) also may have contributed to increase variability. Another limitation is the use of a single Scl-Ab injection protocol, which precluded the determination of the optimal dose and dosing regimen. The dose of the Scl-Ab was based on previous results by Novartis Inc, who provided the antibody. It should be noted that the present antibody is different than the one that was used in the previously mentioned experimental bone healing studies [27-30], and therefore direct comparisons of dose and dosing interval are not feasible.

In conclusion, the systemic delivery of Scl-Ab led to acceleration of bone regeneration during the distraction phase of DO. Future studies on treatment dose and treatment interval are needed to better define the potential role of Scl-Ab in the context of DO.

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Summary and Conclusion

DO technique is used widely to treat many complex orthopaedic conditions. However, one major limitation is the long time the fixator is left in place until the bone is completely consolidated. Currently, the use of Scl-Ab has shown promising results in various orthopaedic applications including osteoporosis, implant fixation and crtical size defects. Our study has also shown the potential utility of Scl-Ab in DO. Future experimental research should be focused in optimizing the Scl-Ab dose during DO and possibly finding a local injectable form of Scl-Ab to be delivered at distraction gap.

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