

The effect of systemic administration of sclerostin antibody 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.

Keywords: 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 tumors¹. 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 bone². 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 associ-

ated with adverse events such as increase risk of infection, osteopenia, persistent pain, and negative psychological impact on patients and their families³⁻⁶. 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 DO². 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. We and others have found promising results in animal studies evaluating bone morphogenetic proteins⁷⁻¹⁰, 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 effects¹¹⁻¹³.

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 sclerostin¹⁶. 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

The authors have no conflict of interest. The sclerostin antibody that was used in this study was provided by Novartis Inc.

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Edited by: J. Rittweger
Accepted 28 January 2014

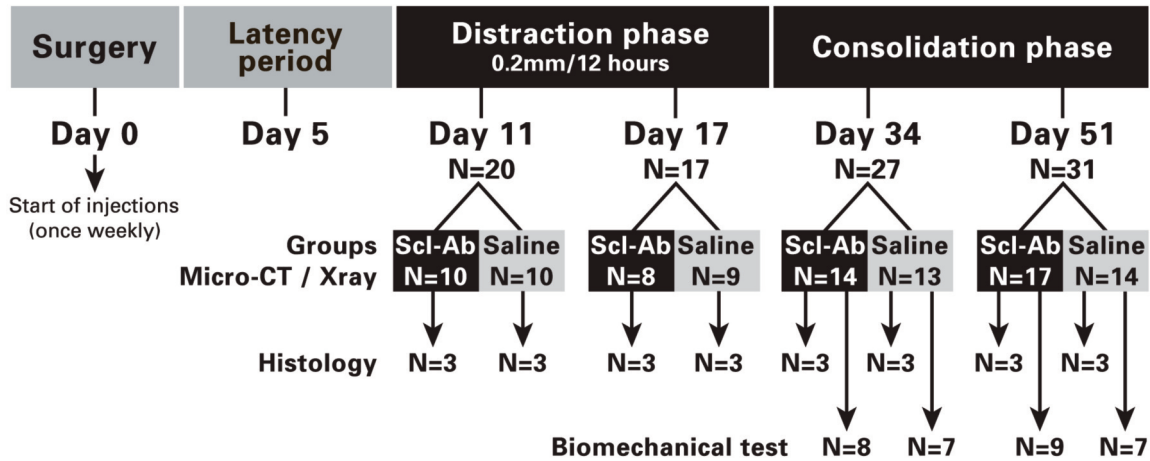


Figure 1. Schematic representation of the study design and the sample distribution. Day 11 (mid-distraction phase), day 17 (end of distraction), day 34 (mid-consolidation) and day 51 (end of consolidation).

formation activity¹⁷. Sclerostin may also decrease the secretion of bone morphogenetic protein 7 in osteocytes¹⁷. 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 imperfecta¹⁸⁻²¹. 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 μ CT and radiographic analyses. Samples for histological analysis were immersed in buffered formalin. The soft tissue for these samples was kept over the distracted bone specimens. Samples for biomechanical testing were immersed in phosphate buffered saline.

Surgical procedures

Murine tibial DO was performed using a miniature Ilizarov fixator (Paolo Alto, CA), as previously described by our group²²⁻²⁴. 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 (5 mg/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 CO₂ asphyxia under general anesthesia at the time of sacrifice. The entire callus located between the distracted bone fragments of the operated



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).

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.

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 μ CT analysis. Distracted tibiae were scanned at 45 KeV/255 μ A with 25 X magnification (11.5 μ m 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 (mm³), bone volume (mm³), 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 μ CT 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™ 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™ 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, μ CT 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

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.

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.

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 μ CT 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 day 11 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 phase¹⁵.

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 osteotomy²⁷, improved the mechanical fixation of medullary implants²⁸, 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 serum³³. It could

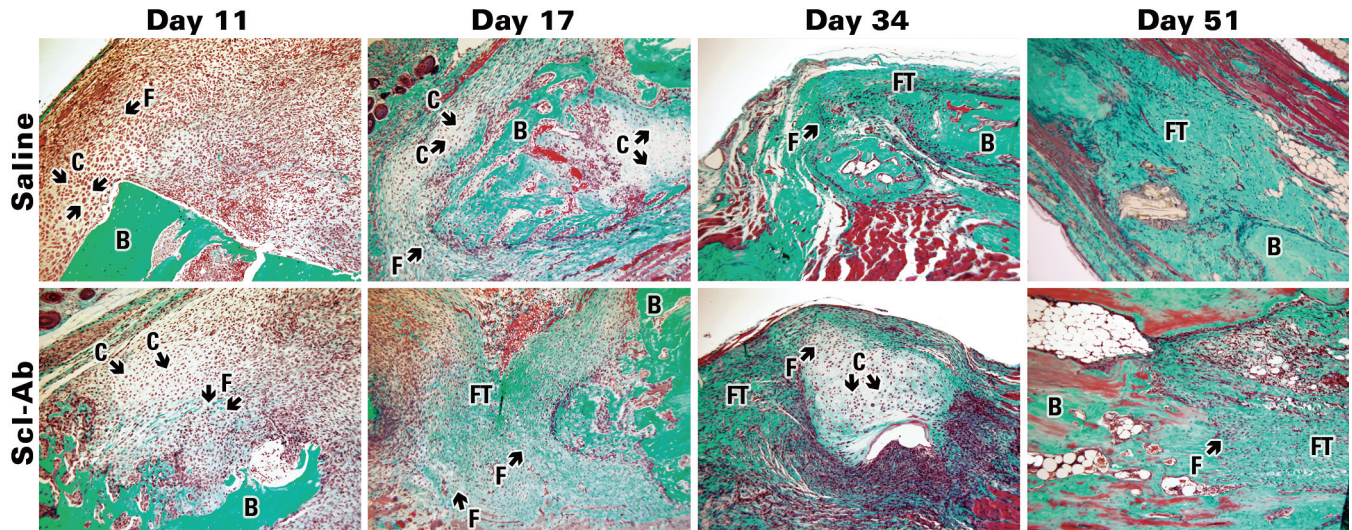


Figure 3. Histological images of distracted tibias stained using the Goldner-Trichrome technique. At 11 days, Scl-Ab specimens show a predominance of chondrocyte and fibrocartilage when compared to the control group as indicated by the arrows. At the other time points, both groups contained varying levels 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). C: Chondrocyte, F: Fibrocartilage, FT: Fibrous tissue, B: Bone.

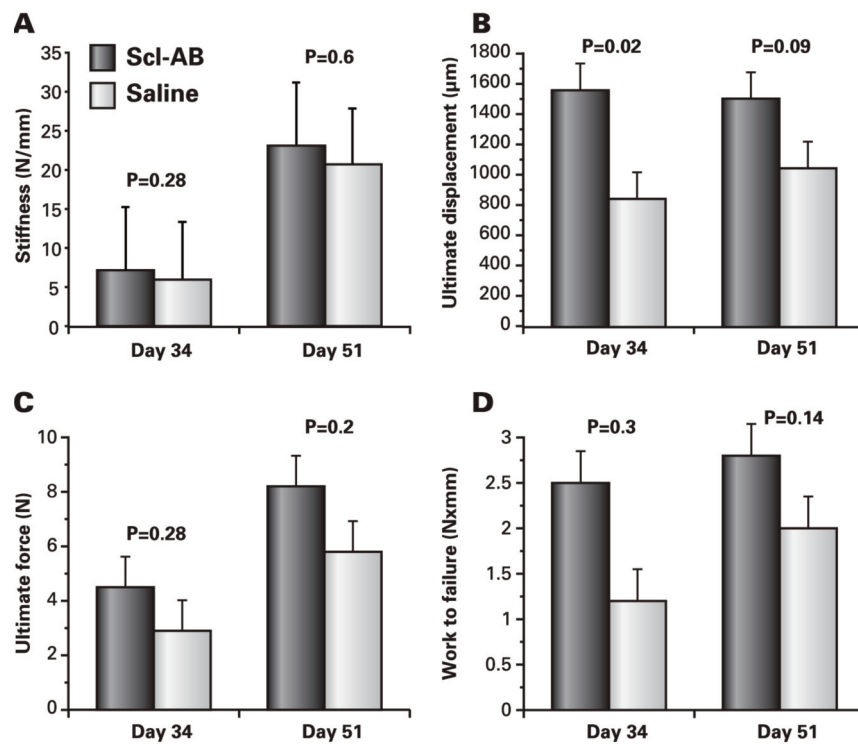


Figure 4. Biomechanical testing results. Biomechanical testing parameters to compare sclerostin-antibody (Scl-Ab) injected and saline-injected (control) groups at 34 days and 51 days post-surgery.

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 adults¹⁹, 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 studies²⁷⁻³⁰, 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.

Acknowledgements

We thank M. Kneissel, Novartis Institutes of Biomedical Research (NI-BR) for the provision of the antibody, which was generated in collaboration with MorphoSys AG. We are indebted to Guylaine Bedard for preparation of the figures. This study was supported by the Shriners of North America, The Canadian Institute for Health Research (Canada), and King Abdulaziz University (Jeddah, Saudi Arabia).

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