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RESEARCH ARTICLE

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The temporal and spatial expression of sclerostin and Wnt signaling factors during the maturation of posterolateral lumbar spine fusions

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Abstract

The bone healing environment in the posterolateral spine following arthrodesis surgery is one of the most challenging in all of orthopedics and our understanding of the molecular signaling pathways mediating osteogenesis during spinal fusion is limited. In this study, the spatial and temporal expression pattern of Wnt signaling factors and inhibitors during spinal fusion was assessed for the first time. Bilateral posterolateral spine arthrodesis with autologous iliac crest bone graft was performed on 21 New Zealand White rabbits. At 1-, 2-, 3-, 4-, and 6-weeks, the expression of sclerostin and a variety of canonical and noncanonical Wnts signaling factors was measured by qRT-PCR from tissue separately collected from the transverse processes, the Outer and Inner Zones of the fusion mass, and the adjancent paraspinal muscle. Immunohistochemistry for sclerostin protein was also performed. Sclerostin and many Wnt factors, especially Wnt3a and Wnt5a, were found to have distinct spatial and temporal expression patterns. For example, harvesting ICBG caused a significant increase in sclerostin expression. Furthermore, the paraspinal muscle immediately adjacent to the transplanted ICBG also had significant increases in sclerostin expression at 3 weeks, suggesting new potential mechanisms for pseudarthroses following spinal arthrodesis. The presented work is the first description of the spatial and temporal expression of sclerostin and Wnt signaling factors in the developing spine fusion, filling an important knowledge gap in the basic biology of spinal fusion and potentially aiding in the development of novel biologics to increase spinal fusion rates.

KEYWORDS

lumbar spine, osteogenesis, sclerostin, spinal fusion, Wnt signaling

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1 | INTRODUCTION

The development of de novo bone following a posterolateral spine arthrodesis surgery is a complex temporal process that involves many tissue types and molecular pathways. Despite state-of-the-art surgical techniques, failure to fuse the spine still occurs in 20% of primary cases.[1] In addition to significantly worse clinical outcomes, the high costs required to continue to treat patients with a spinal pseudarthrosis represents a major economic burden on our global healthcare system.[2]

This difficulty in achieving a solid fusion is largely because the healing environment within the posterolateral spine is inherently challenging. Unlike fractured long bones or critical size bone defects, bone does not naturally exist in the intended fusion bed and it is a large, uncontained space. Also, current bone graft options continue to have serious limitations. Autologous iliac cres bone graft (ICBG) remains the gold standard for spinal fusion, but its harvest is associated with long-term donor site pain in up to 25% of patients.[3] A variety of alternative bone graft options are available (allograft, demineralized bone matrix, and cellular bone matrices), but none have proven to be suitable and effective substitutes for ICBG in the posterolateral spine.[4,5] Alternatively, biologic bone grafts substitutes such as bone morphogenetic protein (BMP) can be used either as an adjunct to autograft/allograft or it can be used as a standalone osteoinductive agent. However, its widespread use has been limited due to the unexpectedly high doses required in humans,[6] which is not only cost-prohibitive, but is also associated with a variety of local side-effects.[7]

New biological approaches that can modulate the local biology within the developing fusion bed are therefore greatly needed. One potential strategy to address this challenge is to leverage the Wnt/β-catenin signaling pathway by using locally delivered biologics at the time of spinal fusion surgery. This is an attractive strategy because the cumulative literature suggests that promotion of canonical Wnt signaling via blockade of its inhibitor sclerostin achieves an anabolic bone forming effect while simultaneously decreasing bone resorption.[8-10] As such, sclerostin inhibition is one of the only known anabolic strategies for uncoupling bone formation and resorption,[11] which is particularly attractive in the setting of a spinal fusion where de novo bone formation is required.

Wnt proteins are classified into canonical (ie, Wnt1, 2, and 3) and noncanonical (ie, Wnt4 and 5a) based upon downstream signaling effects (β-catenin dependent or independent, respectively). Sclerostin, a primary inhibitor of Wnt/β-catenin signaling, is a secreted glycoprotein that inhibits bone formation by binding to the extracellular portion of the Wnt receptor, LRP5/6 (low-density-lipoprotein-related protein5/6). While much work has been performed in studying the role of sclerostin in the setting of fracture healing[12,13] and osteoporosis,[14,15] there is relatively very little known about the role that sclerostin and Wnt signaling play in the setting of spinal fusion. To our knowledge, the only study that has investigated this was performed by Shaffer et al,[16] in which a rat posterolateral spine fusion model was used to demonstrate that systemically administered anti-sclerostin monoclonal antibodies dramatically increased spinal fusion bone mass and volume when compared to controls. While only a single small-scale study, these findings warrant further investigation to better understand the role of these potent regulators of osteogenesis in spinal fusion.

The purpose of this study was to fill a fundamental knowledge gap in our understanding of the basic biology of spinal fusion by examining for the first time the temporal and spatial pattern of the canonical and noncanonical Wnt signaling factors known to be most important in bone development and homeostasis.[17] Using a well-established posterolateral intertransverse process (TP) arthrodesis model in rabbits,[6,18-21] we herein report distinct temporal changes in the gene expression profiles of sclerostin and a variety of canonical and noncanonical Wnt signaling factors from various spatial locations within the fusion bed. These data are important in helping understand the timing and location within the maturing spine fusion mass that sclerostin and Wnt signaling may be most biologically active. As such, these data will potentially aid in the design of local delivery strategies for anti-sclerostin biologics to achieve more consistent spinal fusions.

2 | MATERIALS AND METHODS

2.1 | Rabbit spine fusion surgeries

Upon approval by the Institutional Animal Care and Use Committee at the Atlanta Veteran Affairs Medical Center (#V005-14), 21 adult 8-month-old female New Zealand White rabbits (*Oryctolagus cuniculus*) (Covance, Princeton), all weiging >4 kg, underwent bilateral inter-TP arthrodesis at L5-L6, as previously described.[19] In short, each rabbit had ~2.5 $cm^3$of bone harvested from each posterior iliac crest through separate fascial incisions. The TPs were then decorticated with a 3 mm high-speed bur and ICBG was placed directly overlying the TPs on each side of the spine. After closure, rabbits were allowed to eat and perform activities ad lib, and were treated for pain in compliance with institutional guidelines. Intravenous pentobarbital was used to euthanize n = 4 rabbits at 1-, 2-, 3-, 4-weeks, and n = 5 rabbits at 6-weeks after arthrodesis. It is important to note that, in the model used, successful fusion using ICBG occurs reliably in 4 to 5 weeks and that if not fused by 6 weeks, it can safely be considered a pseudarthrosis. We therefore chose a 6-week endpoint to confidently determine fusion vs nonunion.[19,21] Following euthanasia, tissue biopsies were immediately taken from the right side of the fusion bed for gene expression analysis. These samples were collected from four distinct regions within the developing fussion mass: (a) muscle attached to the transplanted ICBG (designated as *Muscle*), (b) the decorticated TPs themselves, (c) ICBG between the TPs (*Inner Zone*), and (d) ICBG directly overlying/adjacent to the TPs (*Outer Zone*). All tissue was immediately snap-frozen in liquid nitrogen and stored at −80° C until further processing. Paraspinal muscle was included because it has been shown to be actively involved in regulating BMP signaling within the fusion bed.[22]

Also, the left side of each spine was harvested en bloc at each timepoint and fixed in 10% formalin for subsequent histology/IHC. In

addition, two rabbits did not undergo spinal fusion surgery and served as controls. Following euthanasia of these controls, ICBG, TPs, and paraspinal muscle were harvested for gene expression analysis in an identical manner as described above.

2.2 | Radiographic assessment of fusion

Plain radiographs and μCT (Scanco Medical, 55kVp, 145 μA, 200 ms integration time, 30 μm voxel size) of the explanted spines were taken immediately after harvest. μCT images were reconstructed in the sagittal and coronal plane. Successful fusion, defined as continuous bridging bone between the TPs, was assessed by three spine surgeons. All reviewers had to agree in order for a fusion to be considered successful.

2.3 | Quantitative real-time PCR analysis

RNA was isolated from tissue at −196°C during a single step (Spex 6875 Freezer/Mill, Stanmore, UK), ensuring the isolation of high-quality RNA.[23] Total RNA was extracted using a RNeasy mini kit (Qiagen, Germantown, Maryland). cDNA was reverse-transcribed (Invitrogen, Waltham, Massachusetts) and real-time PCR was performed in duplicates following the manufacturer's recommendations. Expression of sclerostin, canonical Wnts (Wnt1, Wnt2, and Wnt 3a), noncanonical Wnts (Wnt4 and Wnt5a), β-catenin, GSK3b, LRP6, and GAPDH was measured (primers shown in Table 1). Data was analyzed using ΔΔCt method.[24] mRNA levels of the target genes were normalized to the 18S ribosomal RNA level of the same tissue. In the control group, the normalized gene level was expressed as a ratio to that of the muscle (designated as 1). In all rabbits undergoing surgery, the normalized gene level was compared to that of the corresponding tissue in the control group.

TABLE 1 Custom and ready-made primers

Primer	Sequence	
Forward SOST	TGG TCC TGA CTC TGC CAC TTG	
Reverse SOST	GCC TCT GTT TCT GTC TCC CTC TC	
Forward LRP6	GCT TGG CAC TTG TAT GTA AA	
Reverse LRP6	TGG GCT AAG ATC ATC AGA CT	
Forward CTNNB1	CAT CTG TGC TCT CCG TCA TCT GA	
Reverse CTNNB1	CAA CTG AAC TAG GCG TGG AAT GG	
Forward Wnt4	GGA TGC TCT GAC AAC ATC GCC TA	
Reverse Wnt4	CAC TTG ACG AAG CAG CAC CA	
Wnt1	Bio Rad Assay ID	qOcuCID00113361
Wnt2	Bio Rad Assay ID	qOcuCED0009090
Wnt3A	Bio Rad Assay ID	qOcuCED0014153
Wnt5A	Bio Rad Assay ID	qOcuCED00110200
DKK1	Bio Rad Assay ID	qOcuCED00112635
GSK3B	Qiagen Cat #.	PPN03338A
18S rRNA	Thermo Fish. Sci Cat #.	4319413E

2.4 | Histologic and immunohistochemical analysis

After fixation, the fusion masses were decalcified and embedded in paraffin. Serial sagittal sections were cut (4-μm thickness) and then stained with either hematoxylin and eosin or Gomori's trichrome. For IHC, paraffin-embedded sections were dewaxed, serially incubated in xylene, and then rehydrated with serial alcohol washes. After antigen retrieval with citrate buffer, slides were incubated in blocking solution (10% normal goat serum) and then incubated with primary antibody (1:50 dilution, anti-sclerostin antibody, Abcam). An isotype control antibody (Rabbit DA1E mAb IgG XP, Cell Signaling Technology, Danvers, Massachusetts) was used at the same working concentration as the primary antibody. A 1.5% serum-treated section was used as a negative control. After incubation with Signal boost secondary antibody, sections were stained with SignalStain (Cell Signaling Technology) and counterstained with hematoxylin.

2.5 | Quantification of antibody staining

Three to five images per slide, of the TPs only, were acquired at the same exposure and magnification using cellSens Entry software on a BX53 Upright Microscope (Olympus, Center Valley, Pennsylvania). For quantification, 5 to 10 representative regions of equal dimensions were acquired from each sample using the ImageJ.[25] Images were deconvoluted with Fiji.[26] Mean intensity values were converted to optical density (OD) as follows: OD = log (max intensity/mean intensity). For manual quantification, the number of sclerostin-positive cells were manually counted in the same images used for staining intensity analysis. Staining was represented as a percentage of sclerostin-positive cells to total cells. Of note, the investigator involved in assessment of the IHC was blind to the timepoint, condition, and fusion status of each specimen.

TPs were specifically chosen for IHC quantification to confirm the qPCR data, because: (a) the TPs had the most dynamic changes in sclerostin expression over time compared to the Inner Zone or Outer Zone (excluding muscle) and (b) the TPs provide easy landmarks of consistent size (regions of interest) across all histology sections. In contrast, due to the heterogeneity inherent in each fusion mass within the Inner and Outer Zones, the amount of bone present on a single thin histological slice makes quantification technically difficult across multiple samples.

2.6 | Statistical analysis

All values throughout are presented as Mean ± SD. PCR data were compared using a paired sample *t*-test. For IHC, one-way ANOVA followed by a Tukey's multiple comparisons test was performed using

v8.1.1 Prism (GraphPad, San Diego, California). Only two-tailed *P*-values were used throughout. For all analyses, significant differences were identified by $P < .05$.

3 | RESULTS

All rabbits tolerated surgery well without complication, maintaining a normal diet and healthy weight throughout the duration of the study. No infections were observed.

3.1 | Radiography

At 1-, 2-, and 3-weeks after surgery, there was no evidence of fusion on plain radiographs or μCT (Figure 1). At week-1 and -2, the ICBG still had clear margins and TPs showed no apparent changes. At week-3, however, early evidence of graft consolidation was present. At week-4, implanted ICBG became more radio-opaque, indicating fusion maturation. μCT showed increased consolidation of ICBG at week-4, as well as bridging between the TPs and new bone formation in the Outer Zone. Two of the week-4 rabbits (50%) revealed solid fusion on μCT. At week-6, radiographs revealed a continued increase in graft consolidation and more continuous trabecular bone spanning the TPs, but only in those spines that fused. In nonunions, however, well-corticated but disparate pieces of bone in the Inner Zone were seen. In the five rabbits at the 6-week timepoint, μCT showed a 60% fusion rate at 6-weeks (3/5), which is consistent with previously published studies.[19,22]

3.2 | Histology

Histology grossly correlated with radiographic findings. At weeks 1 to 2, the Inner Zone was filled with graft fragments, necrotic myofiber, and tissue debris (Figure 2A,B). ICBG in the Inner and Outer Zones was outlined with osteoblasts depositing new osteoid (images not shown). At week-3, cartilage and newly formed woven bone were evident in the Inner Zone. Newly formed bone was obvious at the muscle-ICBG interface in the Outer Zone as well (Figure 2C,D). By week-4, the bone marrow cavity of the TPs was continuous with that of new bone in the Outer Zone, with new membranous bone extending toward the Inner Zone (Figure 2E,F). During this phase, new bone formation in the Inner Zone was characterized by endochondral ossification. At week-6 in the spines that went on to fusion, the medullary canals of both TPs were continuous with the fusion mass, showing increased trabecular bone and marrow volume throughout. In the Inner Zone, the majority of cartilage matrix present up to this point was replaced by new bone via further endochondral ossification. ICBG was remodeled and integrated into the newly formed bone (Figure 2G,H). In those spines with a nonunion, however, the ICBG became well-corticated, but the medullary canals were discontinuous with surrounding autograft and there was no bridging bone from TP to TP (Figure 2I,J).

3.3 | Gene expression

The temporal expression profiles of the assesed factors are shown for the TPs (Figure 3A,B), the Outer (Figure 3C,D) and Inner Zones

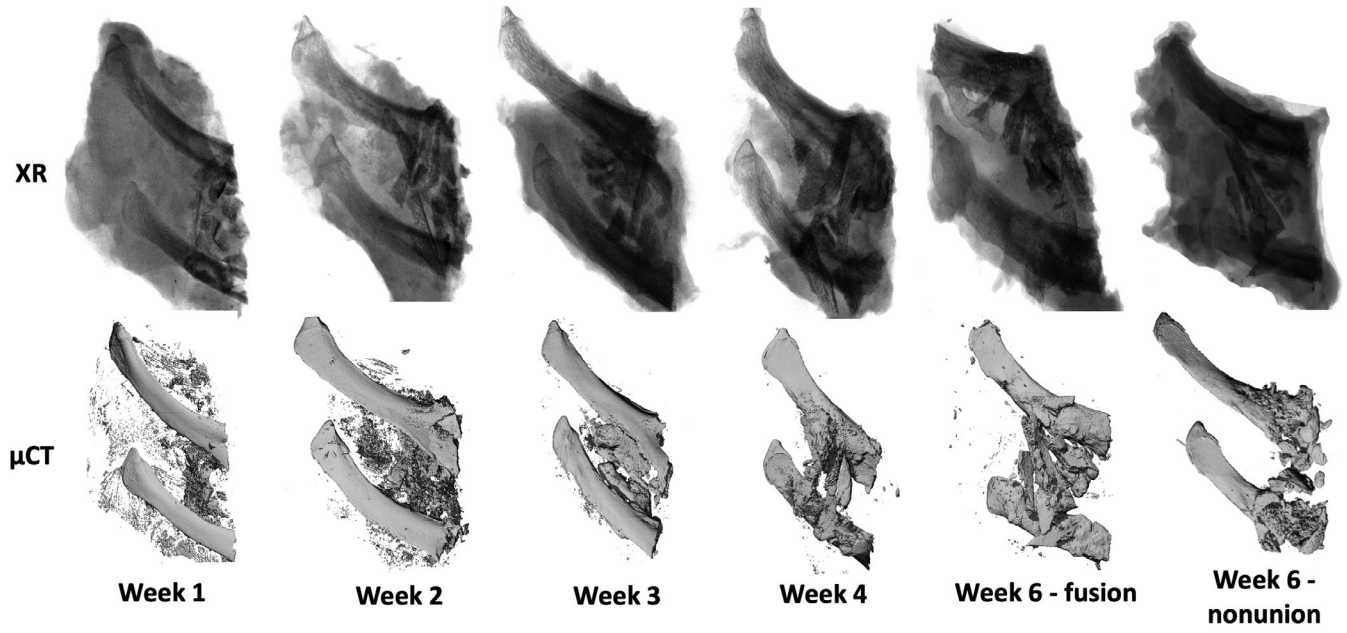

FIGURE 1 Plain radiographs and μCT of representative rabbit lumbar spines after arthrodesis. At early timepoints, the grafted bone had clear margins and no evidence of consolidation. By 4 weeks, however, the ICBG became more radiopaque and there was evidence of bridging between the TPs and ICBG of the Outer Zone. There was continuous trabecular bone connecting the TPs at 6 weeks in those that fused, but well-corticated and discontinuous bone in spines with a nonunion

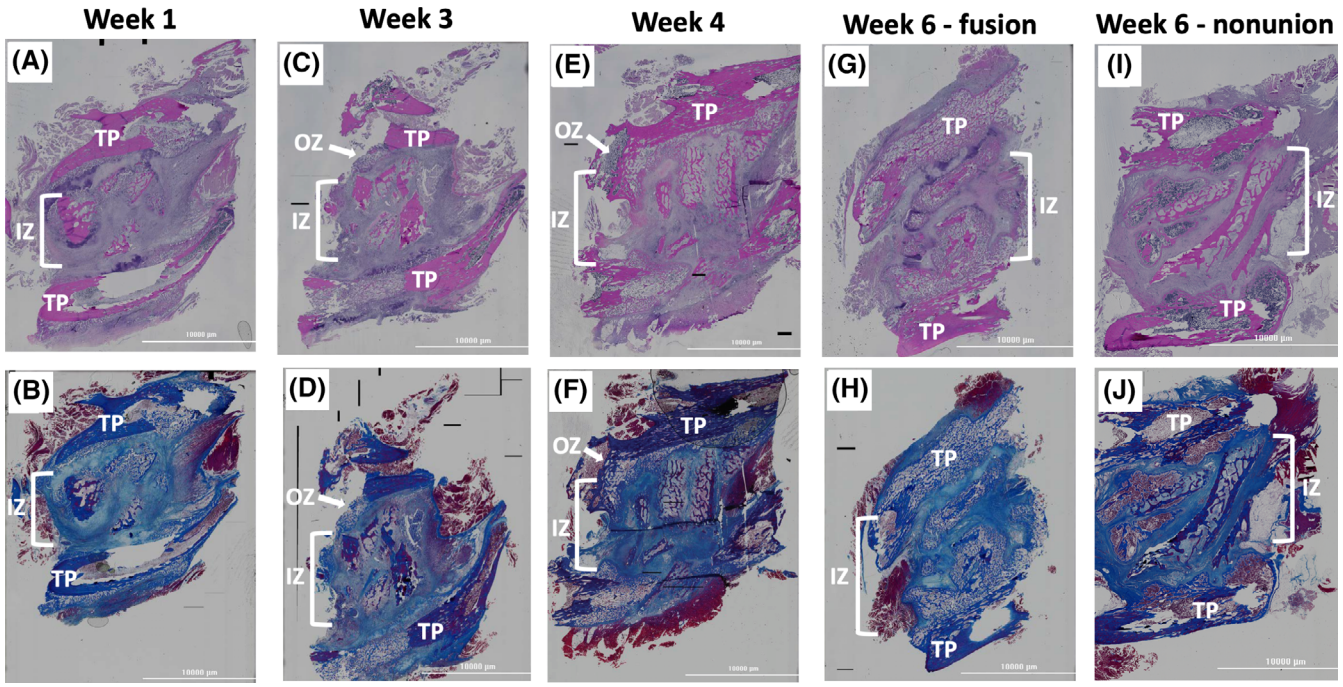

FIGURE 2 Hematoxylin and Eosin staining, A,C,E,G,I and Gomori's trichrome, B,D,F,H,J of histologic specimens are shown. At early timepoints (weeks 1-2), there was no evidence of graft consolidation, A,B. At the 3-week timepoint, newly formed bone was obvious at the muscle-ICBG interface in the Outer Zone, C,D. By 4 weeks, the bone marrow cavity of the TPs was continuous with that of new bone in the Outer Zone, with new membranous bone formation extending toward the Inner zone, E,F. At the 6-week timepoint in fusions, the medullary canals of the two TPs were continuous with the fusion mass, which showed increased trabecular bone and marrow volume throughout, G,H. At 6 weeks in nonunions, however, there was evidence of well corticated bone in the IZ that did not have continuity of its medullary canal with adjacent bone, I,J. *Of note, the black bars indicate software artifact from image stitching. Scale bars: 10000 μm. IZ, inner zone; OZ, outer zone; TP, transverse process

(Figure 3E,F) of ICBG, and the muscle in direct contact with the transplanted ICBG (Figure 3G,H). These values are shown for all rabbits in the surgery group, regardless of whether their spines fused or not. In the controls, TPs, and ICBG demonstrated no significant differences in baseline expression of sclerostin or any other Wnt signaling factor when compared to muscle (data not shown).

In the TPs, the expression of sclerostin trended down from baseline for the first 4 weeks, but then significantly increased at week-6 (4.7-fold increase compared to baseline, $P < .05$; Figure 3A). GSK3b expression also increased significantly in the TPs at week-3, -4, and -6. Likewise, Wnt3a expression steadily increased over time, with a 500-fold increase compared to baseline at week-6 ($P < .05$; Figure 3A). The noncanonical Wnt, Wnt5a, also increased over the first 4-weeks, reaching a >60-fold increase in expression at week-4 ($P < .05$), before decreasing again at week-6 (Figure 3B).

The Inner and Outer Zones, compared to control iliac crest, also demonstrated distinct differences in the spatial and temporal regulation of gene expression. For example, at earlier timepoints (week-1 and -2), the expression of canonical Wnts was significantly higher than that of noncanonical Wnts in the Inner Zone, while the Outer Zone showed significantly higher expression of the noncanonical Wnt, Wnt5a, at the same timepoints. This relationship between canonical and noncanonical Wnt expression was then reversed in the Outer Zone at later timepoints. In general, when sclerostin expression was high, the expression of noncanonical Wnts were at their greatest and vice versa. This is consistent with previous knowledge about how noncanonical Wnt signaling can act in a compensatory manner when canonical Wnt signaling is repressed.[27-29]

Gene expression was also dynamically regulated in Muscle. The expression of Wnt3a and GSK3b significantly increased in Muscle at week-1 (>19 500- and >2100-fold increase, respectively; $P < .05$) and then returned to baseline levels by week-3. Sclerostin expression, conversely, slowly increased in Muscle over the first 2 weeks, but then increased sharply at week-3 (13.4-fold increase, $P < .05$) before returning close to baseline at weeks-4 and -6.

When comparing the rabbits whose spines were fused at 6 weeks (n = 3) to those with a pseudarthrosis (n = 2), several differences were found in gene expression. In the Outer Zone, the expression of Wnt2, Wnt3a, and GSK3b was significantly higher ($P < .05$) in spines with a nonunion compared to those that successfully fused (Figure 4A). This is perhaps an attempt to continue to provide an osteogenic response in these animals via the canonical Wnt signaling pathway. There were no differences in expression of noncanonical Wnts, or any other of the assessed factors, in the Outer Zone between those animals that fused and those that did not. The only other difference found between fusions and nonunions at 6 weeks was in the expression of LRP6 and GSK3b in the Inner Zone, which is the region known to be most responsible for the development of nonunions (Figure 4B).[18] LRP6 and GSK3b expression were significantly increased in the animals with a pseudarthrosis at

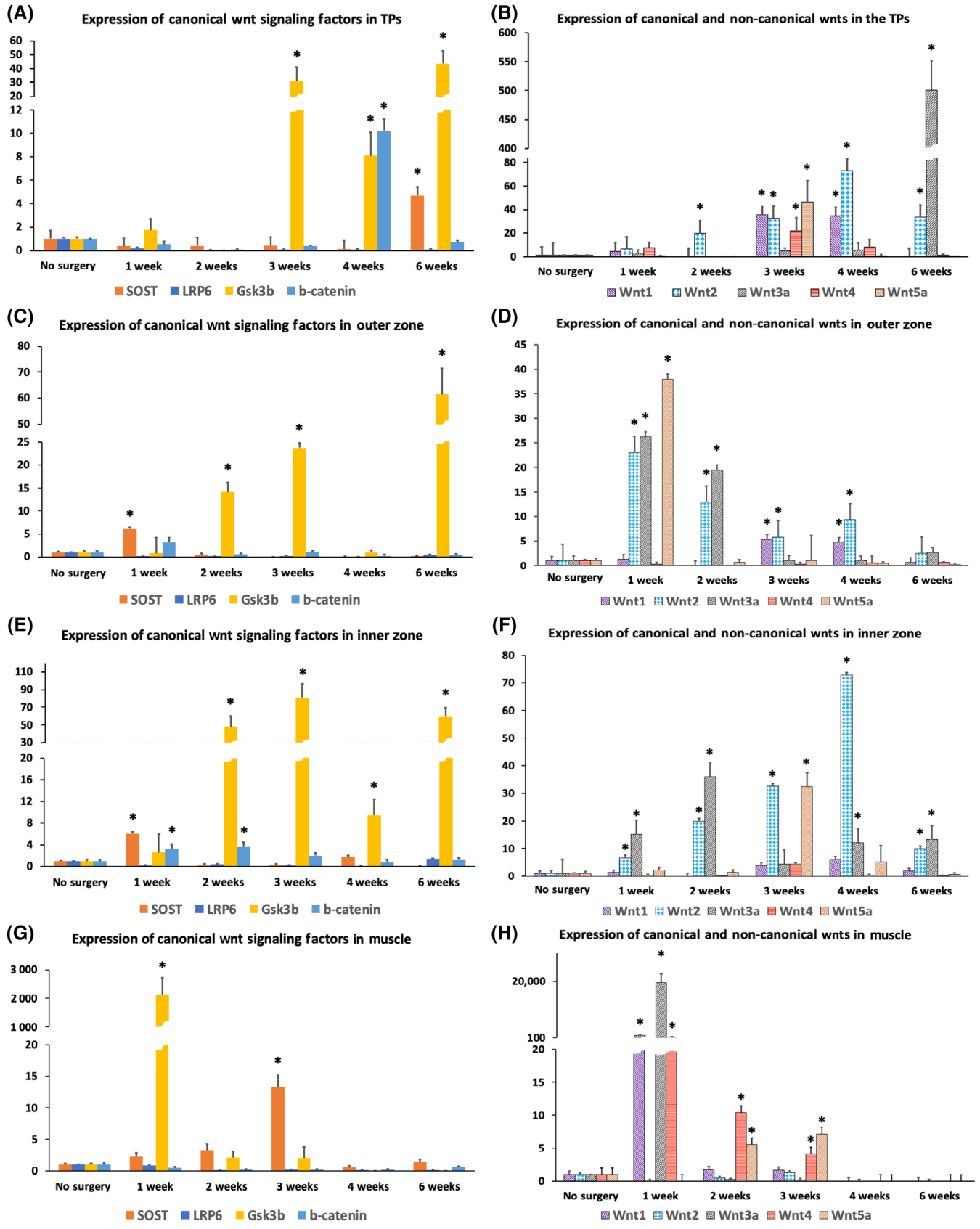

FIGURE 3 Gene expression of sclerostin and various canonical and noncanonical Wnt signaling factors in different parts of the fusion mass over time. Differential expression patterns for each of the assessed signaling factors were found to be unique for each of the regions in the spinal fusion bed. mRNA levels of the target genes were normalized to the 18S ribosomal RNA level of the same tissue. In the control group, the normalized gene level was expressed as a ratio to that of the muscle (designated as 1). In all rabbits undergoing surgery, the normalized gene level was compared to that of the corresponding tissue in the control group. SOST = sclerostin. "*" indicates statistical significance, $P < .05$. Error bars indicate SD

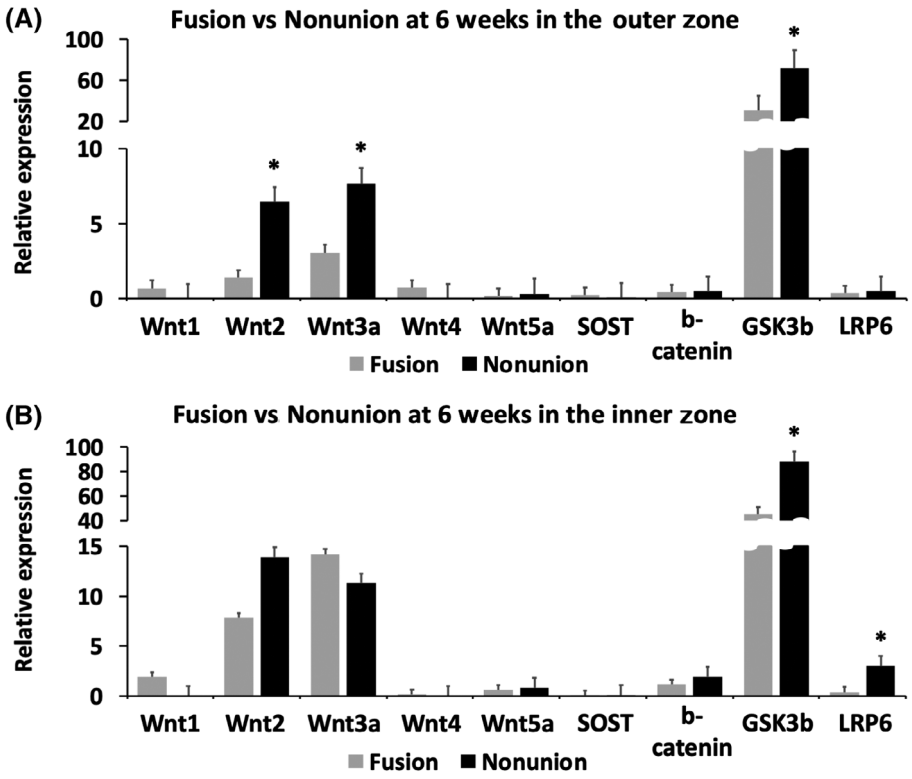

FIGURE 4 Differences in gene expression between those rabbits whose spines successfully fused and those who had a pseudarthrosis at 6 weeks. Significant differences in Wnt2, Wnt3, and GSK3b expression levels were found between these two groups in the Outer zone, A, as well as in the expression of LRP6 in the Inner zone, B. No other differences were found in any of the regions assessed (data not shown). "*" indicates statistical significance, $P < .05$. Error bars indicate SD

6-weeks compared to those that were successfully fused (7.55-fold and 2.00-fold increase, respectively, $P < .05$). Again, no other differences for any of the assessed factors were found in the Inner Zone, and there were similarly no differences that reached significance between fusions and nonunions in the TPs or the Muscle (data not shown).

3.4 | Immunohistochemistry

Anti-sclerostin IHC demonstrated temporal changes in the amount of sclerostin protein present in the decorticated TPs. Figure 5A shows positive antibody staining in embedded osteocytes of the TPs, with a steady increase in sclerostin staining over time. This increase was evident from the greater staining intensity per field as the spinal fusion matured (Figure 5B), and was corroborated by manual cell counts that revealed higher numbers of osteocytes staining positive for sclerostin at week-6 compared to week-1 (Figure 5C). This indicates that sclerostin increased significantly in the fusion bed over time via a greater number of embedded osteocytes expressing sclerostin. Among all the 6-week rabbits, there were no significant difference in the amount of sclerostin staining between those that fused and those with a pseudarthrosis (data not shown). Of note, no significant staining was shown with either isotype control or secondary antibody (Figure S1).

4 | DISCUSSION

An important barricade to the successful translation of potential biologic strategies into the clinic is the lack of understanding of the molecular mechanisms and cues that direct bone formation in a developing spinal fusion. This data fills an important knowledge gap in our understanding of how the gene expression of various Wnt signaling factors most implicated in bone development and homeostasis[17] are differentially regulated over time in the setting of a posterolateral spine fusion.

The expression of sclerostin, one of the primary inhibitors of Wnt/β-catenin signaling, was found to be reproducibley differentially regulated in varying regions of the developing fusion mass over time. Most notably, the act of harvesting and transplanting ICBG into the posterolateral spine resulted in significant increases in sclerostin expression at week 1 (Figure 3C,E). This significant increase in sclerostin expression occurred in transplanted ICBG in both the Inner and Outer Zones of the fusion bed, with both areas showing a >6-fold increase in sclerostin expression 1 week following arthrodesis, suggesting that the increases seen are likely due to the process of ICBG harvest and transplantation and not dependend on the location of the fusion bed in which it was placed. This sharp increase in sclerostin early in the healing process, and the expected subsequent blockdade of osteogenic Wnt signaling, could potentially be problematic for the development of successful spine fusions. Also, it was noted that the muscle in direct contact with ICBG had a threefold increase in sclerostin expression at week-2 and a >13-fold increase at week-3. Interestingly, this data further supports other previous studies that found that surrounding paraspinal muscle may play an active role in osteogenic signaling cues.[18,22] Therefore, it may be important for the surgeon to pay particularly close attention to the amount of muscle left in the fusion bed during arthrodesis, as it may potentially have a negative impact on osteogenesis. Lastly, it was noted that

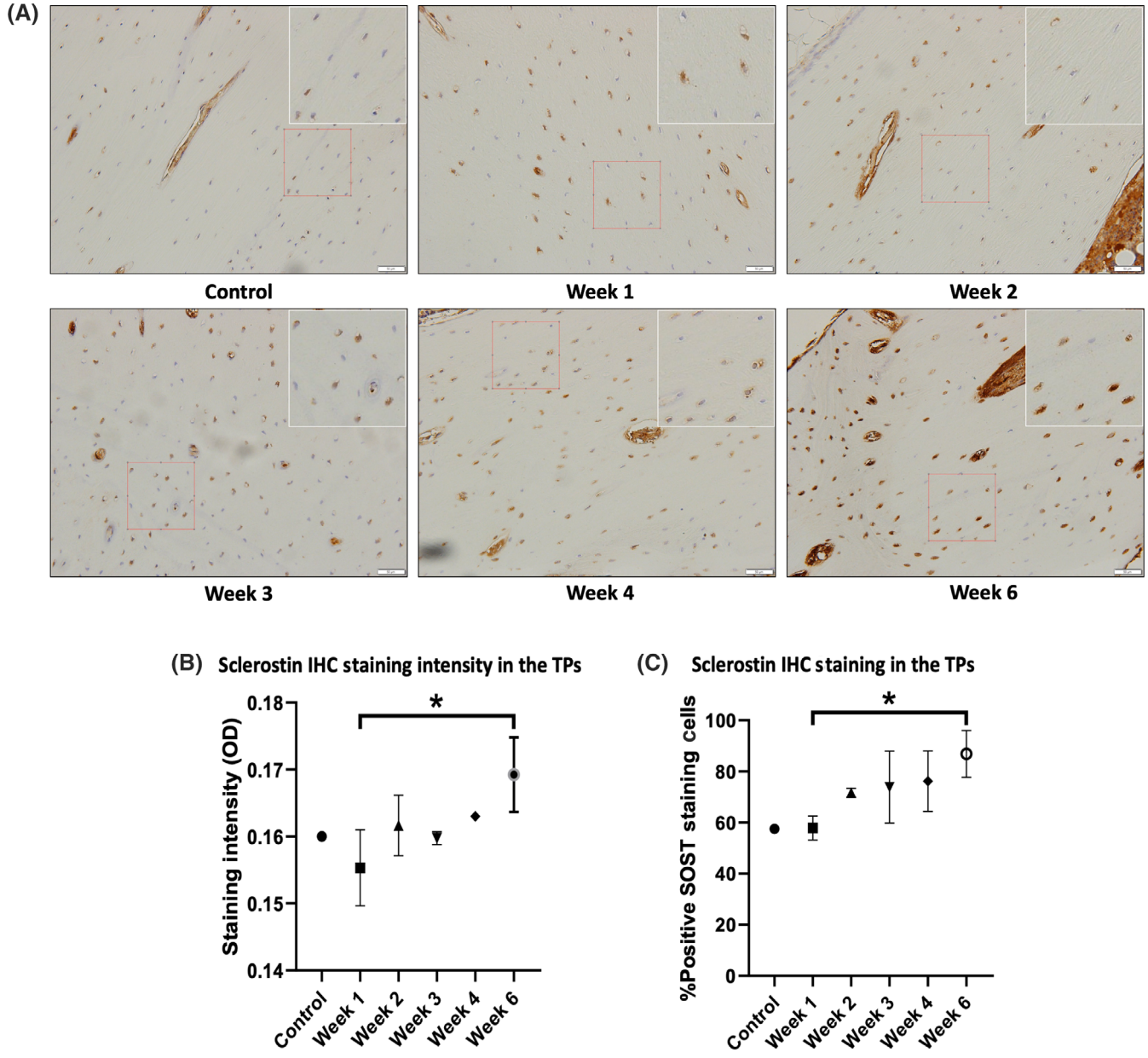

FIGURE 5 Immunohistochemical staining of sclerostin on tissue sections of the developing spinal fusion mass from the TPs, A. Quantitative measurements of sclerostin staining intensity, B, and the percentage of cells stained positive for sclerostin, C, are shown as well. It was found that, overall, sclerostin levels increased over time in the fusion mass. SOST, sclerostin. Scale bars: 50 μm. "*" indicates statistical significance, $P < .05$. Error bars indicate SD

sclerostin expression was significantly increased in the TPs at week-6 (~5-fold increase). This could indicate that sclerostin acts in a negative feedback loop, with increased expression upon bridging of the fusion mass from TP to TP.

Looking at individual factors across all tissue types, Wnt3a was the most dynamically expressed factor assessed, with a 500-fold increase in expression in the TPs at week-6, a 26- and 20-fold increase in the Outer Zone at week-1 and week-2, respectively, as well as a 36-fold increase at week-2 in the Inner Zone. Wnt5a, on the other hand, was the most dynamically expressed noncanonical signaling factor, with a 38-fold increase in expression at week-1 in the Outer Zone and a 33-fold increase at week-3 in the Inner Zone.

The rabbits with a pseudarthrosis at 6 weeks had significantly increased expression of Wnt2, Wnt3a, and GSK3b in the Outer Zone compared to those who fused (Figure 4A). Differences in non-canonical Wnts, however, were not seen between fusions and non-unions, suggesting that Wnt/β-catenin signaling may play a more important role in posterolateral spine fusions, at least in later stages. The persistence of high levels of expression of these canonical Wnt factors in the rabbits with a nonunion suggests the existence of a feedback loop in which a successful fusion results in suppression of canonical Wnt expression. Therefore, it may be possible to "rescue" a failing spinal fusion, or even delayed union, and obtain bridging bone by administering a biologic that blocks antagonists of canonical Wnt

signaling like sclerostin. This is especially true given our finding that LRP6 expression in the Inner Zone was more than threefold higher in rabbits with a nonunion (Figure 4B). Given the high amounts of sclerostin present in the fusion bed at this late timepoint (Figure 3A and Figure 5), a biologic strategy of sclerostin inhibition to achieve spinal fusions may therefore be possible by taking advantage of the higher levels of canonical Wnts, GSK3b, and LRP6 in the nonunions to provide additional osteogenic cues to the fusion mass.

There are some important limitations to this study worth mentioning. First, sclerostin was the only factor whose expression data was verified at the protein level (and only in the TPs). Because most antibodies are produced by immunizing rabbits with purified antigen,[30] IHC on rabbit tissue is technically challenging due to high levels of background binding. This limited our ability to perform IHC for other factors. A second limitation is that only one of the primary inhibitors of Wnt signaling was evaluated (sclerostin). DKK1 (Dickkopf-Related Protein 1), the other primary inhibitor of Wnt/β-catenin signaling,[31] was not considered in this study and may also play an undefined role in spinal fusion. It was decided to focus on sclerostin in this study because it is more widely studied and it is actively being investigated as a druggable target for other clinical applications.[12,14] A third limitation is the small sample size available to compare the 6-week rabbits that fused and those that did not. While this initial study was not intended or powered to detect differences between these subset of rabbits, the differences in Wnt2, Wnt3, GSK3b, and LRP6 were large enough to reach statistical significance, suggesting that a further follow-up study may be warranted to investigate this in more depth. Moreover, with the present data, those follow-up studies will be able to be better powered to detect meaningful differences. Last, while the rabbit spine fusion model is considered the gold standard, in that it most closely reproduces the human condition and at reasonable cost, there may still be important differences between rabbit and human biology that cannot be accounted for in this study.

In conclusion, the present study is the first to explore the role of Wnt signaling and its inhibitors in the setting of spinal fusion. We demonstrate that there is a reproducible spatial and temporal expression pattern of sclerostin and various Wnt signaling factors as a posterolateral spine fusion develops, suggesting their distinct roles in regulating osteogenesis in this setting. Furthermore, the large increases in sclerostin expression from transplanted ICBG at week-1, as well as the paraspinal muscle immediately adjacent to the transplanted ICBG at week-3, both suggest new potential mechanisms for pseudarthroses following spinal arthrodesis. These potential molecular mechanisms, as well as our proposed novel biologic strategy to overcome these anti-osteogenic signals by achieving sclerostin inhibition in the fusion bed using locally delivered anti-sclerostin inhibitors, warrant further investigation.

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CONFLICT OF INTEREST

The authors have no relevant financial conflicts of interest to disclose.

AUTHOR CONTRIBUTIONS

Study design: Scott D. Boden, Hicham Drissi, and Steven M. Presciutti. Study conduct: John Rodriguez-Feo, Lorenzo Fernandes, Anuj Patel, Thanh Doan, and Steven M. Presciutti. Data collection: John Rodriguez-Feo, Anuj Patel, and Steven M. Presciutti. Data analysis: Lorenzo Fernandes, Thanh Doan, and Steven M. Presciutti. Data interpretation: Scott D. Boden, Hicham Drissi, and Steven M. Presciutti. Drafting manuscript: Hicham Drissi and Steven M. Presciutti. Approving final version of manuscript: John Rodriguez-Feo, Lorenzo Fernandes, Anuj Patel, Thanh Doan, Scott D. Boden, Hicham Drissi, and Steven M. Presciutti. Steven M. Presciutti takes responsibility for the integrity of the data analysis.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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