

Increased Runx2 expression associated with enhanced Wnt signaling in PDLLA internal fixation for fracture treatment

ZHUOYAN LING^{1*}, LEI WU^{2,3*}, GAOLONG SHI¹, LI CHEN¹ and QIRONG DONG¹

¹Department of Orthopedics, The Second Affiliated Hospital of Soochow University, Suzhou, Jiangsu 215006;

²Centers for Disease Control and Prevention of Suzhou Industrial Park, Suzhou, Jiangsu 215021;

³School of Biology and Basic Medical Sciences, Soochow University, Suzhou, Jiangsu 215123, P.R. China

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Abstract. Poly-D-L lactide (PDLA) biodegradable implants to heal fractures are widely applied in orthopedic surgeries. However, whether the process of fracture healing is regulated differently when PDLA is used compared with traditional metal materials remains unclear. Runt-related transcription factor 2 (Runx2) and canonical Wnt signaling are essential and may interact reciprocally in the regulation of osteogenesis during bone repair. In the present study, a rat femoral open osteotomy model was used to compare the curative efficacy of a PDLA rod and Kirschner wire under intramedullary fixation for fracture treatment. The dynamic expression of Runx2 and key components of the canonical Wnt signaling in callus tissue during fracture healing was also investigated. The results of the current study indicate that at weeks 4 and 6 following fixation, the callus bone structural parameters of microCT were significantly improved by PDLA rod compared to that of Kirschner wire. In addition, at weeks 4 and 6 after fixation, the protein and mRNA expression of Runx2 and the positive regulators of canonical Wnt signaling, such as Wnts and β -catenin, were significantly increased. However, the protein and mRNA expression levels of the negative regulators of canonical Wnt signaling, such as glycogen synthase kinase-3 β , were significantly decreased in callus tissue when treated with PDLA rod compared with Kirschner wire. Collectively, these data indicate that compared to the traditional metal material, using PDLA internal fixation for fracture treatment may further improve bone formation, which is associated with the increased expression of Runx2 and the enhancement of canonical Wnt signaling.

Introduction

Since the use of biodegradable implants to heal fractures by Rokkanen *et al* in 1985, the method of using biodegradable materials has been widely applied in orthopedic surgeries (1-3). Biodegradable materials exhibit various advantages in the treatment of fractures compared with the use of traditional metal implants, including the elimination of implant removal, reduction of the ‘stress shielding’ effect, improvement of biocompatibility, reduction of radiological artifacts and utilization of magnetic resonance imaging assessment following surgery (4,5). Poly-D-L lactide (PDLA) is a material with an intermediate degradation time (PDLA begins to degrade at ~12 weeks) and may be completely replaced by bone tissue following surgery, thus, it is considered to be one of the most effective biodegradable materials for the treatment of fractures (6-8). However, with the exception of clinical outcomes, the differences in the biological processes following the use of PDLA and metal fixation to treat a fracture remain unknown.

The fracture healing process has been widely accepted to comprise a series of overlapping phases; these include inflammation, repair and remodeling events (9). This multistage repair process involves a variety of complex and well-orchestrated cellular and molecular processes, and should be considered as a specialized post-natal recapitulation of embryological skeletal development (9,10). Runt-related transcription factor 2 (Runx2) is one member of the Runx family that belongs to a small transcription factor family sharing a common runt domain (11). Previous studies indicated that Runx2 was a crucial transcriptional regulator for skeletal development and served essential roles in biological events including osteoblast differentiation, intramembranous, endochondral ossification and in the process of fracture healing, vascular invasion (12-14). However, whether the expression of Runx2 during fracture healing may be altered by the use of PDLA compared to that of a traditional metal material for fracture treatment remains unknown.

A previous study demonstrated that the canonical Wnt signaling served essential roles in regulating bone formation during the fracture repair process (15). In the canonical Wnt signaling pathway, the Wnts proteins, a family of secreted glycoproteins, bind to the membrane receptor Frizzled (Fz) leading to downstream sub-cellular events. The disheveled

Correspondence to: Professor Qirong Dong, Department of Orthopedics, The Second Affiliated Hospital of Soochow University, 1055 San Xiang Road, Suzhou, Jiangsu 215006, P.R. China
E-mail: drq0512@126.com

*Contributed equally

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protein, along with other co-receptors, is activated to inhibit the activity of glycogen synthase kinase-3 β (GSK-3 β). β -catenin is able to avoid the degradation of the protein ubiquitinylation system and translocate into the nucleus in order to associate with transcription factors, including T cell factor-1 (TCF-1), in order to regulate the transcriptional activities of relevant genes (16). A number of studies have indicated that the expression of certain Wnt proteins, including Wnt4, 5b, 10b, 11, and 13, and Fz receptors such as Fz1, 2, 4, and 5, are increased during the fracture healing process (17,18). The expression of Runx2 may also be regulated by the activity of canonical Wnt signaling through its binding with β -catenin/TCF-1 in certain promoter regions of the runx2 gene (19). However, whether and to what extent the activity of the canonical Wnt signaling in the bone repair process may be changed by PDLA, compared to that of traditional metal material for fracture treatment, has not yet been determined.

Overall, the present study established an open osteotomy model of Sprague-Dawley rats in order to determine whether and to what extent the expression of Runx2 and the activity of canonical Wnt signaling during bone repair process may be differentially regulated. The use of traditional metal material (Kirschner wire) and PDLA intramedullary rod under internal fixation for fracture treatment was assessed. The results of the present study indicate that compared to the traditional metal material, the curative efficacy of PDLA internal fixation for fracture treatment is improved, mediated by the increased Runx2 expression associated with the enhanced activity of canonical Wnt signaling.

Materials and methods

Experimental animals. A total of 36 male Sprague-Dawley rats (six months old) weighing 280-300 g were supplied by the Laboratory Animal Research Center of Soochow University (Suzhou, China). They were maintained under standard conditions for at least one week, and individually housed in plastic cages in an animal room in a temperature-controlled environment of 22-25°C and relative humidity of 40-70% with a 12-h light/dark cycle and *ad libitum* access to commercial pellets and water. Animals were randomly assigned into control and PDLA groups (n=18 per group). Following the transverse osteotomy at the mid-shaft of right femur, the traumatic fracture was stabilized intramedullary by the Kirschner wire and PDLA rod in control and PDLA groups, respectively. All procedures were approved by the Institutional Animal Care Service (Second Affiliated Hospital of Soochow University).

Surgical procedure of femoral open osteotomy model. The osteotomy was performed using a circular saw with a diameter of 1.6 cm and a thickness of 0.1 mm at mid-shaft of the right femur, under the intraperitoneal injection of 50 mg/kg sodium pentobarbital (Sinopharm Chemical Reagent, Co., Ltd., Shanghai, China). The fracture was stabilized by intramedullary insertion of a sterilized 1 mm diameter Kirschner wire (Nantong Healthcare Medical Instrument, Co., Ltd., Nantong, China) in the control group and a sterilized 1 mm diameter PDLA rod (Nantong Healthcare Medical Instrument, Co., Ltd.) in the PDLA group. At weeks 2, 4 and 6 post-surgery,

50 mg/kg sodium pentobarbital was used to anesthetize 6 rats from each group prior to sacrifice via cervical dislocation.

Alkaline phosphatase and osteoprotegerin serum levels. Blood samples (0.5 ml each) were obtained using an intravenous tube implanted in the femoral vein following 2, 4 and 6 weeks and stored at -20°C until analysis. The concentrations of alkaline phosphatase (ALP) and osteoprotegerin (OPG) were detected in the serum using commercially available ELISA kits (C506082-0096, and C506509-0048; Sangon Biotech, Co., Ltd., Shanghai, China).

MicroCT scanning and assessment of bone quality parameters. At weeks 2, 4 and 6 post-surgery, six rats randomly selected from each group were anesthetized with intraperitoneal injections of sodium pentobarbital (Sinopharm Chemical Reagent, Co., Ltd., 50 mg/kg) prior to micro-CT scanning. The femora were scanned by a desktop micro-CT system (IVIS Quantum FX; Cold Spring Biotech Corporation, Shanghai, China) with the resolution of 47 μ m and scanning time of 15 min each. Values of bone volume fraction (BV/TV), bone surface density (BS/BV), trabecular thickness (TB.TH), trabecular number (TB.N), and trabecular separation (TB.SP) were determined in each group under the certain regions of interest. The scan area was centered through the fracture line.

Western blot analysis. Protein samples were routinely extracted from callus tissue using a Total Protein Extraction kit according to the manufacturer's protocol (310003; BestBio Biotechnology Co., Ltd., Shanghai, China) 2, 4 and 6 weeks following surgery, as described previously (20). Samples of protein (80 μ g) were loaded in each lane for electrophoresis in a 4% SDS-PAGE gel (0.1% SDS and 10% polyacrylamide) and transferred to immobilon P polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). Each membrane was blocked with Tris-buffered PBS containing 5% bovine serum albumin (Chinese Academy of Sciences, Shanghai, China) plus 0.1% (v/v) Tween (TBS-T) on a gentle shaker for 1 h at room temperature, followed by incubation at 4°C overnight with rabbit polyclonal antibodies (Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA) against Runx2 (sc-10758, 1:1,000), hypoxia inducible factor 2A (HIF2A; sc-28706, 1:1,000), Wnt4 (sc-13962, 1:1,000), Wnt10b (sc-25524, 1:1,000), GSK-3 β (sc-9166, 1:1,000), β -catenin (sc-7199, 1:1,000) and TCF-1 (sc-13025, 1:1,000). Membranes were then washed three times for 10 min in TBS-T solution and incubated at room temperature with the secondary horseradish peroxidase-conjugated goat anti-rabbit antibody (1:5,000; A0208; Beyotime Institute of Biotechnology, Haimen, China) for 2 h. Proteins were then visualized using enhanced chemiluminescence detection reagents (ECL reagent RPN2232, GE Healthcare Life Sciences, Chalfont, UK) and exposed to X-ray film according to the manufacturer's protocol. The β -actin protein (primary antibody from EMD Millipore, working concentration: 1:1,000) was blotted in the same membrane as an internal control to normalize the relative density. Results were quantified and analyzed using a Kodak electrophoresis documentation and analysis system, and Kodak ID image analysis software (Kodak, Rochester, NY, USA). Three replicates were performed per tissue sample.

Table I. Comparison of microCT analysis of fracture calluses between PDLLA group and control group across time-points.

microCT parameters	Weeks after fixation	Con	PDLLA
BV/TV, %	2	2.469±0.14	2.435±0.11
	4	3.188±0.16	3.765±0.13 ^a
	6	5.132±0.18	6.123±0.13 ^a
BS/BV, 1/m	2	1.887±0.14	1.869±0.12
	4	2.025±0.11	2.183±0.12 ^a
	6	3.102±0.12	3.244±0.16 ^a
BV/TV, %	2	0.0740±0.0011	0.0738±0.0014
	4	0.0751±0.0013	0.0768±0.0011 ^a
	6	0.0831±0.0012	0.0857±0.0015 ^a
BS/BV, 1/m	2	0.334±0.031	0.333±0.043
	4	0.491±0.034	0.522±0.062 ^a
	6	0.654±0.051	0.709±0.061 ^a
BV/TV, %	2	1.106±0.0051	1.108±0.0044
	4	1.083±0.0072	1.073±0.0035 ^a
	6	1.035±0.010	1.018±0.012 ^a

Data are presented as mean ± standard deviation. ^aP<0.05. Con group, control group; PDLLA, Poly-D-L lactide treated group; BV/TV, bone volume/tissue volume; BS/BV, bone volume/bone volume.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). At weeks 2, 4 and 6 post-surgery, total tissue RNA was routinely isolated from the callus tissue using Eastep Super Total RNA Extraction kit (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol. Reverse transcription and first strand cDNA synthesis was performed using M-MLV Reverse Transcriptase according to the manufacturer's protocol (Invitrogen; Thermo Fisher Scientific Inc., Waltham, MA, USA). RT-qPCR analysis was completed for Runx2, HIF2A, Wnt4, Wnt10b, GSK-3β, β-catenin and TCF-1 gene expression in callus tissue. The GAPDH gene was used as an internal control and serial dilutions of the positive control (the synthesized tissue cDNA from rat bone; RD-107, Zyagen, San Diego, CA, USA) were performed on each plate to create a standard curve. The primer sequences (Sangon Biotech, Co., Ltd.) are as follows: For rat Runx2 are forward, 5'-CACAAAGTGC GG TGC AA ACTT-3' and reverse, 5'-AAT GACTCGGTTGGTCTCGG-3'; for rat HIF2A are forward 5'-AACCTTAAGTCGGCCACCTG-3' and reverse, 5'-TTG CTGTCCAAGGGGATGTC-3'; for rat Wnt4 are forward, 5'-TACGGATGAGGACCTGGTGT-3' and reverse, 5'-GAC GTCTTGTTGCAAGTGCG-3'; for rat Wnt10b are forward, 5'-CCGTGAGTTAGGTCGAGCAG-3' and reverse, 5'-GTG GGGAACTGTGTGGAGT-3'; for rat GSK-3β are forward, 5'-CGAACTCCACAGAGGCAAT-3' and reverse, 5'-GAG TTGGAGGCTGATGCAGA-3'; for rat β-catenin are forward, 5'-CTGCTGATCTCGGACTGGAC-3' and reverse, 5'-GGC AGCCCATCAACTGGATA-3'; for rat TCF-1 are forward, 5'-CAGAATCCACAGATACAGCA-3' and reverse, 5'-CAG CCTTGAATCTTCATC-3'; for rat GAPDH are forward, 5'-GGTGGTCTCCACGACTTTA-3' and reverse, 5'-CAA GGAGGGGCCTTTATTTC3'. RT-qPCR (iCycler iQ™ Real-time PCR detection system; Bio-Rad Laboratories, Inc., Hercules, CA, USA) was performed using 25 μl of each

sample, in 96-well plates (Takara Bio, Inc., Otsu, Japan) under the following protocol: 42°C for 30 min, 95°C for 15 min followed by 40 cycles of 95°C for 20 sec, 56°C for 1 min and 72°C for 20 sec. The amount of target gene was normalized to the GAPDH reference to obtain the relative threshold cycle ($\Delta\Delta Cq$), then $2^{-\Delta\Delta Cq}$ was calculated to determine the relative abundance for target gene expression between control and PDLLA groups (21).

Statistical analysis. All data were expressed as mean ± standard deviation. With regard to the different mRNA level of PDLLA and control groups, statistical significance was accepted (P<0.05) when the ratio of $2^{-\Delta\Delta Cq}$ exceeded 1.7. The two-way analysis of variance and independent *t*-test, where appropriate, was used to evaluate the differences between PDLLA and control groups. A statistically significant difference was accepted if P<0.05. SPSS statistical software (version 18; SPSS, Inc., Chicago, IL, USA) was used for all statistical analyses.

Results

MicroCT analysis of fracture calluses. MicroCT analysis indicated that, compared with the control, the values of trabeculae quality of the traumatic femur including BV/TV, BS/TV, TB.TH, TB.N and TB.SP were significantly improved in the PDLLA group, 4 and 6 weeks following the intramedullary fixation for femoral osteotomy (P<0.05). However, at week 2 there was no significant difference in the values of trabeculae growth between the PDLLA group compared with the control (P>0.05; Table I).

Serum values of ALP and OPG. In order to further evaluate the differences in osteogenesis between the PDLLA and

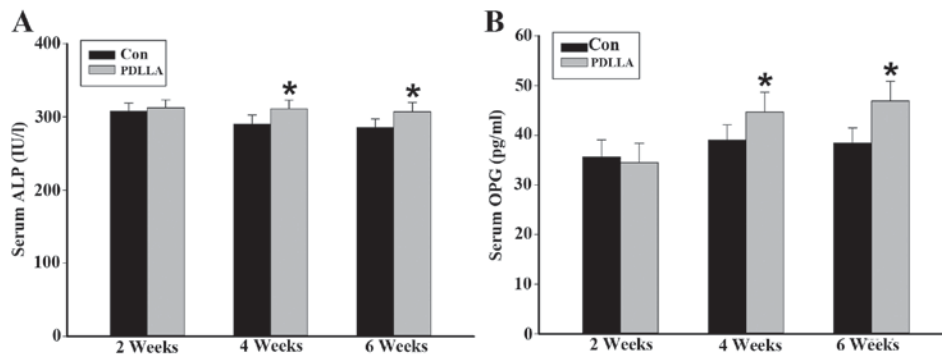


Figure 1. ALP and OPG serum levels in rats 2, 4 and 6 weeks post-surgery. (A) Serum level of ALP and (B) OPG at weeks 2, 4 and 6 post-surgery, under PDLLA intramedullary fixation (PDLLA group) compared to that of Kirschner wire (control group). Values are mean \pm standard deviation, * $P < 0.05$. Con, control group; PDLLA, Poly-D-L lactide treated group; ALP, alkaline phosphatase; OPG, osteoprotegerin.

control group, the circulatory serum levels of ALP and OPG were detected as they are widely considered to be markers of bone formation. The ELISA assay indicated that weeks 4 and 6 following the intramedullary fixation for femoral osteotomy, the serum levels of ALP and OPG were significantly increased in the PDLLA group compared with that of the control ($P < 0.05$). However, results from week 2 demonstrate that the serum levels of ALP and OPG are not significantly different in the PDLLA group compared to that of control ($P > 0.05$; Fig. 1).

Protein expression of Runx2, HIF2A, Wnts, GSK-3 β , β -catenin and TCF-1 in callus tissue. To compare the differences of cellular and molecular events involved in the process of fracture healing between the PDLLA and control groups, runx2, canonical Wnt signaling and associated regulators were dynamically examined. Western blot analysis demonstrated that 4 and 6 weeks following the intramedullary fixation for femoral osteotomy, the protein expression levels of Runx2, HIF2A, Wnts and β -catenin in callus tissue were significantly increased in the PDLLA group compared with the control ($P < 0.05$; Fig. 2A-E). Furthermore, the level of GSK-3 β protein expression in callus tissue, at week 4 and 6, was significantly decreased in the PDLLA group compared to that of control ($P < 0.05$; Fig. 2F). However, at week 2 following the intramedullary fixation, the level of protein expression for Runx2, HIF2A, Wnts, β -catenin and GSK-3 β exhibited no significant differences in the PDLLA group compared with that of control ($P > 0.05$; Fig. 2A-F). With respect to TCF-1 at weeks 2, 4 and 6 following the intramedullary fixation for femoral osteotomy, the protein expression levels exhibited no significant differences in the PDLLA group compared with that of control ($P > 0.05$; Fig. 2G).

Runx2, HIF2A, Wnts, GSK-3 β , β -catenin and TCF-1 mRNA in callus tissue. It was demonstrated following RT-qPCR that, similar to the of protein expression, 4 and 6 weeks following the intramedullary fixation for femoral osteotomy, the expression of Runx2, HIF2A, Wnts and β -catenin mRNA in callus tissue were significantly increased in the PDLLA group compared with that of the control ($P < 0.05$; Fig. 3A-E). The mRNA expression levels of GSK-3 β in callus tissue, at weeks 4 and 6 were significantly decreased in the PDLLA group compared with the control ($P < 0.05$). However, 2 weeks

after the intramedullary fixation, the level of mRNA expression observed for Runx2, HIF2A, Wnts, β -catenin and GSK-3 β exhibited no significant difference in the PDLLA group compared with the control (Fig. 3A-F; $P > 0.05$). With respect to TCF-1, at weeks 2, 4 and 6 following the intramedullary fixation for femoral osteotomy, the mRNA expression levels exhibited no significant differences in PDLLA group compared to that of control (Fig. 3G; $P > 0.05$).

Discussion

In the present study, the fracture bone repair under intramedullary fixation was improved following the use of PDLLA instead of traditional metal material. Furthermore, compared with the traditional metal material, the expression of Runx2 in callus tissue, which is closely associated with the further activation of canonical Wnt signaling, was significantly increased under PDLLA intramedullary fixation.

With the development and improvement of pixel resolution and the mathematical combination with finite element analysis, microCT has been widely applied in assessing the biomechanical features and qualities, including bone mineral density and Young's modulus, of cortical and trabecular bone (22). In the present study, microCT-scanning results indicated that following 4 and 6 weeks post-surgery, the fracture repair indices, including BV/TV, BS/BV, TB.TH, TB.N and TB.SP, were significantly improved in the PDLLA group compared with the control group. ALK and OPG were expressed in the bone tissue, specifically in osteoblastic lineages and their serum concentrations were considered as the markers for the evaluation of osteogenesis during development and bone formation throughout fracture healing (23,24). The results of the current study indicate that at weeks 4 and 6 following intramedullary fixations for osteotomy, the serum levels of ALK and OPG were significantly increased in the PDLLA group, compared with the control. Thus, the current study indicates that compared to the traditional metal material, the osteogenesis in the process of fracture healing was increasingly activated and therefore further improved the curative efficacy of PDLLA intramedullary fixation for fracture treatment.

It has been indicated that the Runx2 is extensively expressed in the callus tissue during the process of fracture

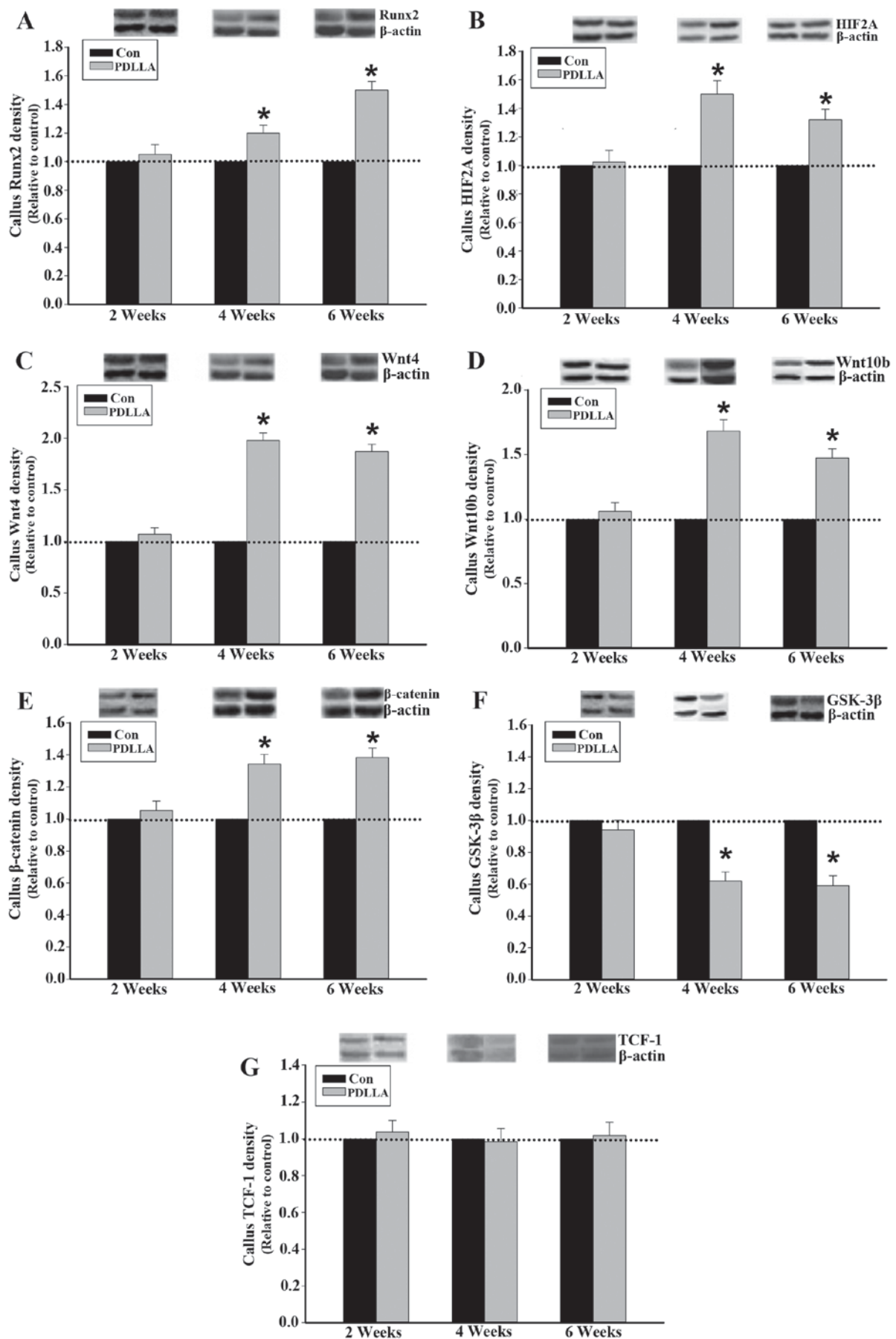


Figure 2. Protein expression in callus tissue of control and PDLLA treated rats 2, 4 and 6 weeks post-surgery. Expression level of (A) Runx2, (B) HIF2A, (C) Wnt4, (D) Wnt10b, (E) GSK-3β, (F) β-catenin and (G) TCF-1 under PDLLA intramedullary fixation (PDLLA group) compared to that of Kirschner wire (control group). B-actin is a control. Values are presented as mean ± standard deviation, *P<0.05. Con, control group; PDLLA, Poly-D-L lactide treated group; Runx2, Runt-related transcription factor 2; HIF2A, hypoxia inducible factor 2A; GSK-3β, glycogen synthase kinase-3β; TCF-1, T cell factor-1.

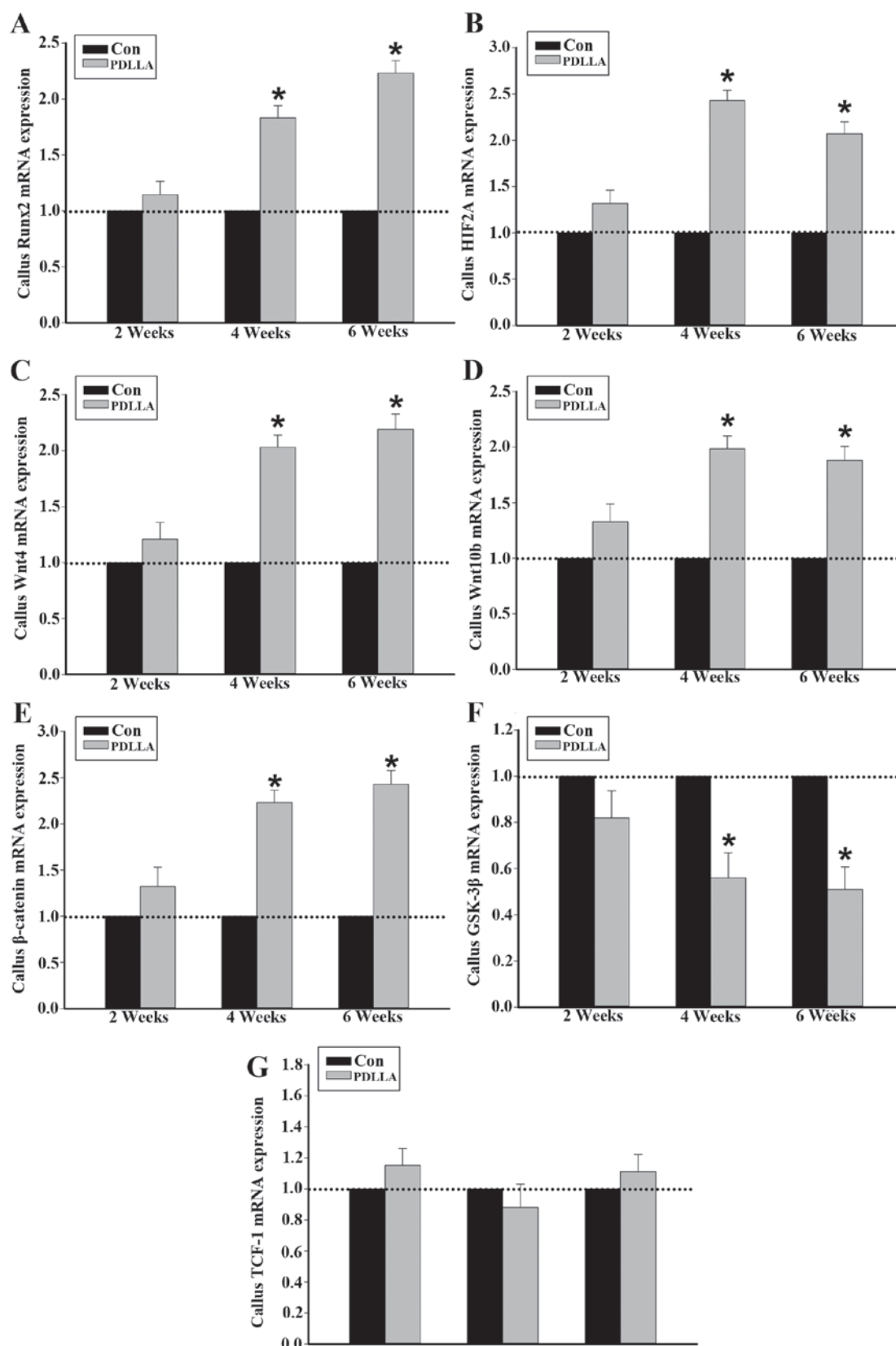


Figure 3. mRNA expression in callus tissue of control and PDLA treated rats 2, 4 and 6 weeks post-surgery. Expression level of (A) Runx2, (B) HIF2A, (C) Wnt4, (D) Wnt10b, (E) GSK-3 β , (F) β -catenin and (G) TCF-1. B-actin is a control. Values are presented as mean \pm standard deviation, *P<0.05. Con, control group; PDLA, Poly-D-L lactide treated group; Runx2, Runt-related transcription factor 2; HIF2A, hypoxia inducible factor 2A; GSK-3 β , glycogen synthase kinase-3 β ; TCF-1, T cell factor-1.

healing (25). In the present study, the level of mRNA and protein expression of Runx2 in the callus tissue of the PDLA

group were significantly increased, compared to that of control, at 4 and 6 weeks following intramedullary fixation for

osteotomy. Genetic engineering studies demonstrated that the altered expression of Runx2 may lead to abnormal and lethal bone development and ossification (26). Studies have indicated that Runx2 may serve as an essential transcriptional factor to extensively regulate the osteogenesis mediated by a number of pathways, including the differentiation and maturation of osteoblasts and osteoclasts, expression of bone matrix protein genes and angiogenesis controlled by vascular endothelial growth factor (27,28). Thus, the present study proposed that the improved curative efficacy of PDLLA in the intramedullary fixation for traumatic fracture was closely associated with the increased expression of Runx2 in callus tissue; an important regulator for osteogenesis in the process of traumatic bone repair. However, the pathways involved and the aforementioned underlying cellular and molecular mechanisms may be differentially regulated by the increased expression of Runx2, thus more studies are required.

The Runx2 gene spans ~210 kb with its two predominant transcripts, governed by P1 and P2 promoters (29,30). Runx2 RNA, driven by the P1 promoter, is extensively stimulated and highly expressed in osteoblastic lineages during skeletal development (31). Previous results have demonstrated that the specific loss of expression of the Runx2-P1 promoter in mice leads to severe developmental defects with cleidocranial dysplasia-like symptoms (32). Thus, the activity of the P1 promoter serves a crucial role in the regulation of Runx2 transcription in the process of osteogenesis. Previous experiments indicated that HIF2A may bind to the -155 and -75 bp region of the Runx2-P1 promoter and serve as upstream regulators for the basal transcription of the Runx2 gene (33). In the present study, results indicated that 4 and 6 weeks following intramedullary fixation for osteotomy, the mRNA and protein expression of HIF2A in callus tissue were significantly increased in the PDLLA group compared to the control. It was therefore hypothesized that, compared to traditional metal material, the use of PDLLA for internal fixation of fractures increases the expression of HIF2A. This further promotes the transcriptional activity of Runx2 through an interaction between HIF2A and the Runx2-P1 promoter during the process of traumatic bone repair (34). Notably, various studies indicated that, from the second week following PDLLA implantation, the material began to be degraded (35,36). The biodegraded monomer would then enter into the citric acid cycle to yield carbon dioxide and water (35). As a consequence, when compared to the traditional metal material, the oxygen level in the local repair tissue decreases at weeks 4 and 6 following intramedullary fixations by PDLLA. Thus, under PDLLA internal fixation for fracture treatment, the expression of HIF2A may have increased due to the relative hypoxia condition in the callus tissue. However, the concerned data supporting this hypothesis in this study is lacking and the detailed mechanism requires further study to be elaborated.

It is acknowledged that the canonical Wnt signaling may govern the lineage commitment and differentiation of progenitor cells, specifically the mesenchymal stem cells, into the chondrocytes and osteoblasts, so as to serve the critical roles in the process of osteogenesis (37). Gain- and loss-of-function mutations in canonical Wnt signaling components including Wnt ligands have elaborated the critical functions of canonical Wnt signaling during endochondral bone formation (15). The

canonical Wnt signaling is initiated by the binding of Wnt proteins and its Fz receptors. Following the activation of the Fz receptor complex, the protein phosphorylation cascade, triggered by GSK-3 β , may be inhibited to stabilize the intracellular β -catenin levels (38). Subsequently, β -catenin is translocated to the nucleus to form a heterodimeric DNA-binding complex with TCF-1 to regulate the transcription activity of downstream genes such as connexin 43 and Runx2 (39). The current study demonstrated that at weeks 4 and 6 following intramedullary fixation for osteotomy, the mRNA and protein expression levels of Wnt4, Wnt10b and β -catenin were significantly increased, while mRNA and protein expression levels of GSK-3 β were significantly decreased in the PDLLA group compared with the control. Consequently, it is suggested that, compared to the traditional metal material, the canonical Wnt signaling pathway was further activated under PDLLA internal fixation for fracture treatment. Notably, previous findings indicated that, the heterodimeric DNA-binding complex of β -catenin and TCF-1 may target the -97 and -93 bp region of the Runx2-P1 promoter, enhancing the transcription activity of Runx2 gene (19). Although the expression of TCF-1 was not changed, the increased expression of β -catenin may, at least in part, contribute to further activation and transcriptional activity of Runx2 gene under PDLLA intramedullary fixation compared to that of metal material. The binding sites for β -catenin, TCF-1 and HIF2A in the P1 promoter region of the Runx2 gene overlapped, the current study hypothesized that the function of β -catenin, TCF-1 and HIF2A may synergistically involve the transcriptional regulation of Runx2 gene.

It should be noted that at week 2 following the intramedullary fixation for osteotomy, compared with traditional metal materials, all parameters detected in the current study presented little difference following the use of PDLLA biodegradable material. A number of potential reasons for this are as follows: In one aspect, previous results have indicated that PDLLA degrades relatively slowly in the first two weeks *in vivo* (40). This indicated that the mechanical strength PDLLA is greater than the callus tissue and the released monomer and changes to microenvironment in local tissue are limited. As such, compared to the traditional metal material, the differences concerning stress shielding and local inflammatory effects induced by PDLLA would be negligible. Secondly, during the first two weeks following the fracture in rats, the bone repair process is still in the inflammatory and initial repair phases (9,10). This indicates that the biological processes of fracture healing were in the most active period and may not be clearly altered by the relatively subtle differences between the traditional metal and PDLLA materials. Over time, the bone repair process moves into the late repair and remodeling phases and new blood vessels also become mature. Furthermore, the degradation process of PDLLA material *in vivo* would become more active and the differences between traditional metal and PDLLA materials, including stress shielding and local inflammatory effects, would become progressively apparent. However, further investigation is required to assess which of the aforementioned factors serve as the trigger and are therefore important for changing the pathways examined in this study.

In conclusion, the findings of the present study provide a novel insight into the differences of cellular and molecular

pathways in the process of bone repair under the internal fixation for traumatic fracture treatment using PDLA, a biodegradable material compared to that of traditional metal materials. Although these results were obtained from the rat model, the present study may facilitate further studies of the relevant biological mechanisms underlying PDLA in clinical use.

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