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LDB2 regulates the expression of DLL4 through the formation of oligomeric complexes in endothelial cells

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Delta-like ligand 4 (DLL4) expression in endothelial cells is intimately associated with angiogenic sprouting and vascular remodeling, but the precise mechanism of transcriptional regulation of DLL4 remains incompletely understood. Here, we showed that LIM-domain binding protein 2 (LDB2) plays an important role in regulating basal DLL4 and VEGF-induced DLL4 expression. Knockdown of LDB2 using siRNA enhanced endothelial sprouting and tubular network formation *in vitro*. Injection of *ldb2*-morpholino resulted in defective development of intersegmental vessels in zebrafish. Reduction or over-expression of LDB2 in endothelial cells decreased or increased DLL4 expression. LDB2 regulated DLL4 promoter activity by binding to its promoter region and the same promoter region was occupied and regulated by the LMO2/TAL1/GATA2 complex. Interestingly, LDB2 also mediated VEGF-induced DLL4 expression in endothelial cells. The regulation of DLL4 by the LDB2 complex provides a novel mechanism of DLL4 transcriptional control that may be exploited to develop therapeutics for aberrant vascular remodeling. [BMB Reports 2018; 51(1): 21-26]

INTRODUCTION

Well-coordinated sprouting angiogenesis is essential for formation of functional vascular networks during development and adulthood. DLL4 plays a critical role in endothelial sprouting and vascular remodeling relevant in physiologic and pathologic angiogenesis (1, 2). DLL4 expression is mainly detected in the restricted region of nascent vessels, particularly in tip cells, which is associated with VEGF signaling. VEGF induces the tip cell phenotype and DLL4 expression (3, 4) and triggers Notch signaling in adjacent cells, and this, in turn, attenuates VEGF signaling and inhibits tip cell behavior (5, 6), thereby balancing vessel sprouting. Gene inactivation or pharmacological inhibition of DLL4 or Notch results in excessive sprouting with an increased number of tip cells at the vascular front and hyperdense vascular networks (3, 4, 6). Despite the central role of DLL4 in vascular remodeling and angiogenesis, the precise transcriptional regulatory mechanism of DLL4 is less well defined.

LIM-domain binding proteins (LDBs) were initially identified as co-factors for LIM-homeodomain (LHX) transcription factors and LIM-only (LMO) proteins (7), which play critical roles in cell fate determination, differentiation, and tissue development (8, 9). LDBs were also shown to interact with other transcription factors, including GATA, bHLH, and Otx family members (10, 11). The LDB family is composed of two members, LDB1 and LDB2, which are highly homologous in two well conserved domains: an amino-terminal homodimerization domain (DD) and a carboxyl-terminal LIM interaction domain (LID) (9). These domains play important roles in mediating transcription through higher-order transcription complexes containing TAL1/E47/GATA1/LMO2/LDB. Of particular interest is that LDB1 mediates long-range promoter-enhancer interactions through these multimeric complexes, which regulate erythroid gene transcription (11, 12). Although the roles of LDB1 in diverse developmental processes, including erythroid differentiation, are relatively well established, the function of LDB2 has not yet been clearly determined. In this study, we determined that LDB2 plays a key role in angiogenic sprouting by regulating DLL4 through higher-order transcription complexes in endothelial cells.

RESULTS

Depletion of LDB2 in endothelial cells leads to aberrant tubular network formation and endothelial sprouting

To identify transcription factors that are critical for the angiogenic activity of endothelial cells, we screened transcription factors whose expressions were significantly altered during endothelial progenitor cell (EPC) differentiation from a

microarray analysis (GEO accession number GSE12891). The mRNA of LDB2 was dramatically induced during EPC differentiation, whereas mRNA expression of LDB1, a homologue, was not significantly changed (Supplementary Fig. 1). To address the role of LDB2 in endothelial cells, we performed tubular network formation assay on Matrigel with endothelial cells transfected with siRNA targeting LDB2 (Supplementary Fig. 2A-C). Number of meshes formed on Matrigel increased in LDB2-deficient endothelial cells when compared to control cells (Supplementary Fig. 2B and C) A 3D-fibrin-gel bead assay with LDB2-deficient endothelial cells displayed more sprouting, and the length of sprouts were increased compared to control cells (Fig. 1A-C). To investigate the role of *ldb2* during angiogenesis *in vivo*, we knock-downed *ldb2* gene expression using morpholino and observed intersegmental trunk vessels (ISV) in transgenic zebrafish embryos with fluorescent blood vessels (refered to as Tg(*flk-1:egfp*) embryos). At 72 hpf, control MO-injected embryos showed no apparent vascular abnormality (Fig. 1D and F, Control MO). By contrast, *ldb2*
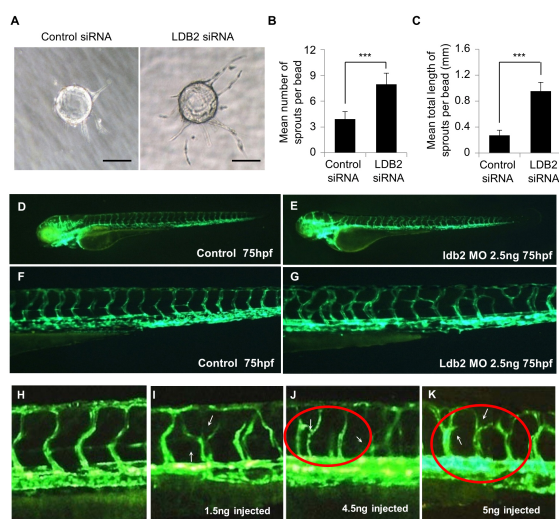
MO-injected Tg(*flk-1:egfp*) embryos displayed vessel-branching defects (Fig. 1E and G, *ldb2* MO). High-magnification images confirmed that ISVs of *ldb2* MO-injected embryo was disorganized and hyper-branched at this stage in a dose-dependent manner (Fig. 1H-K).

LDB2 regulates transcription of DLL4 in endothelial cells

The increased sprouting and hyper-dense network formation by LDB2-knockdowned endothelial cells is reminiscent of the behavior of DLL4-deficient endothelial cells (13). To elucidate the relationship between DLL4 and LDB2 in endothelial cells, DLL4 protein levels were measured in LDB2-deficient endothelial cells. The level of DLL4 protein expression was significantly reduced (Fig. 2A). Because LDB2 is a transcription co-factor, we examined transcription of DLL4 after LDB2 knockdown in HUVECs and found that the transcript level and promoter activity of DLL4 decreased (Fig. 2B and C). Conversely, DLL4 expression was increased when LDB2 was overexpressed in HUVECs (Fig. 2D). DLL4 promoter activity was also increased with LDB2 expression in a dose-dependent manner, although the induction level was relatively low (Fig. 2E). This moderate change may be attributed to the fact that LDB2 is a transcription co-factor, which requires other transcription factors for full induction of transcription of a target promoter.
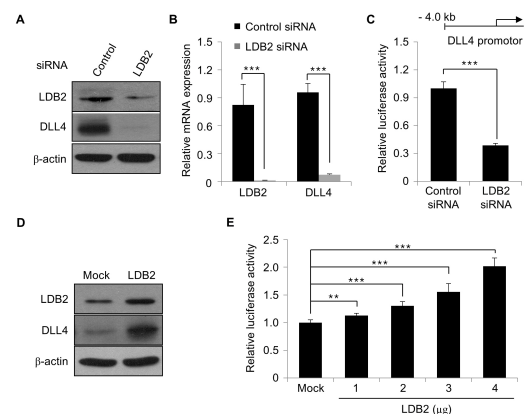

Fig. 1. Depletion of LDB2 in endothelial cells results in abnormal endothelial sprouting. (A-C) HUVECs were transfected with control or LDB2 siRNA (80 nM) and 3-D fibrin gel assay was performed. (A) Representative images of sprouting from HUVECs transfected with indicating siRNA (80 nM). Sacale bars: 100 µm (B and C) Quantification of sprouts in a 3-D-fibrin gel assay. Beads coated with HUVECs were seeded and cultured in fibrin gel for 5 days. (n = 4; 15 beads were assayed in each experiment). (D-G) Control morpholino (D and F) or *ldb2* morpholino (E and G) was micro-injected at one cell stage of zebrafish and pictures were taken at 75 hpf to examine the vessel development. High magnification of control embryo's trunk region (F) showing no abnormality and *ldb2* morpholino-injected embryo's trunk region (G) showing aberrant development of intersegmental vesseles. (H-K) Control or *ldb2* morpholino of 1.5 ng, 4.5 ng and 5 ng was injected. *ldb2* morpho-lino-injected embryos show disorganized and hyper-branched intersegmental vessels (I-K, red rounds and arrows). Error bars represent mean ± SD. ***P < 0.001. Student's *t* test.

Fig. 2. LDB2 regulates transcription of DLL4 in endothelial cells. (A, B) HUVECs were transfected with control or LDB2 siRNA (80 nM) and DLL4 protein and transcript level was determined by Western blot analysis (A) and RT-PCR (B). (C) Luciferase activity in cells co-transfected with LDB2 siRNA and luciferase reporter containing human DLL4 promoter (−4.0 kb). (D) HUVECs were transfected pCMV2-FLAG mock (1 µg) or pCMV2-FLAG-LDB2 vector (1 µg) and DLL4 proteins level was determined by Western blot analysis. (E) HUVECs were transfected with the DLL4 promoter-luciferase reporter vector along with increased amount of LDB2- expression vector. Luciferase activity was measured. Each experiment was repeated at least 3 times. Error bars represent mean ± standard deviation (SD). **P < 0.01; ***P < 0.001. Student's *t* test.

LDB2 regulates DLL4 transcriptionally through the multimeric complex consisting of LMO2, TAL1, and GATA2 onto DLL4 promoter

LDB2 regulates genes by interacting with transcriptional factors that directly bind to DNA, and several transcription factors have been identified as binding partners of LDB proteins. LDB proteins are known to bind TAL1/GATA transcription factors, and this interaction is mediated by LMO2. Interestingly, TAL1/GATA binding sites were found −1.9 kb from the DLL4 promoter region, and this organization is highly conserved in mice and humans (Fig. 3A and Supplementary Fig. 3). Activation of the mouse DLL4 promoter
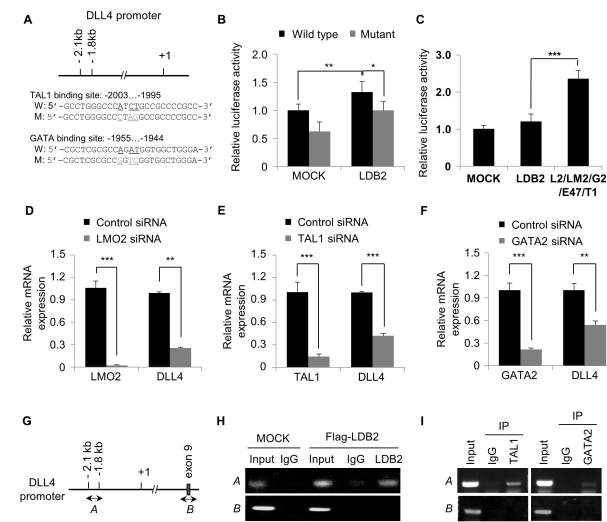
by LDB2 protein was suppressed when the promoter region containing TAL1/GATA binding sites was deleted (Supplementary Fig. 3). Site-directed mutagenesis of these sites also suppressed LDB2-induced and basal DLL4 promoter activity (Fig. 3B).

To determine whether this transcription factor complex regulates transcription from the DLL4 promoter, the components of the complex were overexpressed in HUVECs along with the DLL4 promoter fused to luciferases. The promoter activity of DLL4 was significantly enhanced by cotransfection with TAL1, E47, GATA2, LMO2, and LDB2 (Fig. 3C). In accordance with this, DLL4 transcription was reduced when TAL1, GATA2, or LMO2 was knocked-down by siRNA in HUVECs (Fig. 3D-F). These results suggest that the TAL1/E47/GATA2/LMO2 complex bound by LDB2 may play a significant role in transcriptional regulation of DLL4 in endothelial cells. To further elucidate the transcriptional regulation of DLL4 by LDB2, we performed chromatin immunoprecipitation in LDB2-overexpressing HUVECs. LDB2-FLAG was immunoprecipitated at the TAL1 and GATA2 binding sites of the DLL4 promoter (Fig. 3G and H). Endogenous TAL1 and GATA2 were also detected at the same promoter region (Fig. 3I).

LDB2 mediates VEGF-induced DLL4 expression

It is well known that VEGF induces DLL4 transcriptionally in endothelial cells, and thereby restricts tip cell formation in adjacent cells. To determine whether DLL4 induction by VEGF is regulated by LDB2 in endothelial cells, HUVECs transfected with a dominant negative form of LDB2 (LDB2 DN; the
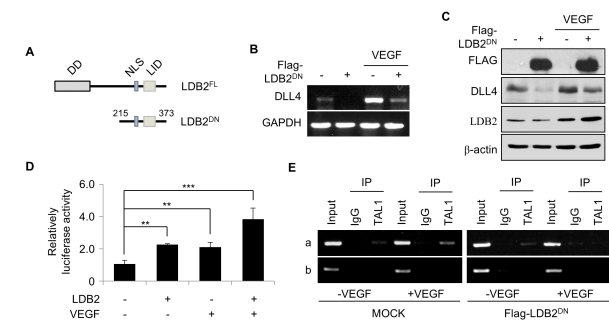

Fig. 3. LDB2 regulates DLL4 promoter activity via TAL1 and GATA2 binding on DLL4 promoter. (A) The human DLL4 promoter region containing the E-box-GATA2 element (binding sites for TAL1 and GATA2) is depicted. Wild-type (W) and mutant (M) sequences are shown. The point mutations are underlined. (B) HUVECs were transfected with wild-type or mutant DLL4 promoters fused to a luciferase reporter along with a pCMV2-FLAG or pCMV2-FLAG-LDB2 expression vector. Luciferase activity was measured 24 h after transfection. (C) HUVECs were transfected with a mock vector, an LDB2 vector, or an LDB2 (L2), LMO2 (LM2), GATA2 (G2), E47, and TAL1 (T1) vector along with a DLL4 promoter-luciferase reporter. The total amount of transfected DNA was adjusted to 4 μg. (D-F) DLL4 transcripts were measured in HUVECs transfected with indicated siRNA by qRT-PCR. Error bars represent mean ± SD. **$P < 0.01$, ***$P < 0.001$ by Student's *t* test. (G) Primers corresponding to the region that encompasses the E-box-GATA2 element of the DLL4 promoter are denoted as A and primers corresponding to the DLL4 coding region are denoted as B for CHIP analysis. (H) HUVECs were transfected with the pCMV2-FLAG mock vector or the pCMV2-FLAG-LDB2 vector, and FLAG-LDB2 was immunoprecipitated with an anti-FLAG antibody. Co-immunoprecipitated and input DNAs were amplified with the primers indicated in G. (I) Endogenous TAL1 and GATA2 were immunoprecipitated in HUVECs with anti-TAL1 and anti-GATA2 antibodies, respectively. Co-immunoprecipitated and input DNAs were amplified with primers indicated in G. Each experiment was repeated at least 3 times.

Fig. 4. LDB2 mediates VEGF-induced DLL4 expression. (A) The dominant negative form of LDB2 (LDB2 DN, a.a. 215-373) was depicted. LDB2 FL, full length form of LDB2; NLS, nuclear localization sequence; LID, LIM-interacting domain. (B and C) HUVECs were transfected with pCMV2-FLAG or pCMV2-FLAG-LDB2 DN and treated with VEGF (50 ng/ml). (B) mRNA of DLL4 was analyzed by RT-PCR. (C) Expression of DLL4 and LDB2 protein was detected by western blotting. (D) HUVECs transfected with mock vector or with LDB2 vector and with the DLL4 promoter-luciferase reporter were treated with VEGF. Luciferase activity was determined. (E) CHIP assays were performed with lysates from HUVECs transfected with mock vector or with the LDB2 DN expression vector. Endogenous TAL1 was immunoprecipitated 1 h after VEGF treatment. Each experiment was repeated at least 3 times. Error bars represent mean ± SD. **$P < 0.01$, ***$P < 0.001$ by Student's *t* test.

dimerization-domain deletion mutant) (Fig. 4A). Transcription of DLL4 was induced by VEGF in control cells, and this induction of DLL4 mRNA by VEGF was suppressed in LDB2 DN-expressing cells (Fig. 4B). Expression of DLL4 protein was also induced by VEGF. Similar to DLL4 expression, endogenous LDB2 protein was increased by VEGF treatment. However, the induction of expression of DLL4 protein by VEGF was inhibited by LDB2 DN (Fig. 4C). The reporter activity of the DLL4 promoter was increased by VEGF treatment, and this was further enhanced by LDB2 expression (Fig. 4D). Moreover, chromatin immunoprecipitation results revealed that binding of TAL1 to the DLL4 promoter region was also increased by VEGF treatment and this increase was suppressed by expression of LDB2 DN (Fig. 4E). These results suggest that LDB2 is involved in VEGF induction of DLL4 through its oligomerization.

DISCUSSION

LDB transcription co-factor proteins have been suggested to be important for the regulation of cell selection and cell fate determination via their interactions with LIM domain containing transcription factors (7, 14). In this study, we showed that LDB2 is expressed in endothelial cells and that it plays a unique role in vascular remodeling by regulating expression of DLL4. Despite the incomparable role of DLL4 expression on vascular morphogenesis, transcriptional regulation of DLL4 in endothelial cells has remained largely unclear. A few transcription factors, including Wnt signaling effector, β-catenin, and FOXC transcription factors that regulate DLL4 expression in endothelial cells have been identified (15-17), but these studies did not provide direct evidence of their roles in VEGF-induced DLL4 expression. The current study provides a new insight into the transcriptional regulation of DLL4 expression and shows that an LDB2-containing higher-order transcriptional complex mediates basal and VEGF-induced DLL4 expression. ETS transcription factors have also been identified as regulators of DLL4 expression and they primarily function during arterial specification (18). SOXF transcription factors and the RBPJ/NICD complex also regulate DLL4 transcription during arterial specification (19). Because these studies focused on regulation of arterial-specific DLL4 expression during development, they did not evaluate regulation of DLL4 expression during endothelial sprouting. Further, the regulatory elements bound by these factors are in intron 3 of *DLL4* rather than in the promoter sites identified in this study. Thus, it is likely that the mechanisms regulating DLL4 expression during arterial specification and during angiogenic sprouting are distinct.

TAL1, a transcription factor initially identified in human acute T-cell leukemia, has been shown to be involved in both developmental and postnatal angiogenesis (20, 21). TAL1 deletion mutant mice showed defective yolk sac angiogenesis due to the defect in *Tal1*-/- endothelial cells. TAL knockdown in HUVECs resulted in defective cell migration, which led to a lack of tubular network formation on Matrigel (22). VE-cadherin has been identified as a target of the TAL1 complex in endothelial cells (23). Deletion of LMO2, which interacts with LDB2 and TAL1/GATA2, also caused a severe angiogenesis defect in mouse embryos (24). The vascular defects caused by these genetic deficiencies were not due to impaired formation of the primitive vascular plexus, but rather were due to defects in subsequent angiogenic remodeling processes. The results from these studies and our study suggest that misregulated expression of DLL4 by the lack of these transcription factors may be the cause of the abnormal vascular remodeling observed in these mutant mice. Furthermore, LDB2 was identified as an upregulated gene during the morphogenic stages of angiogenesis in a chick chorioallantoic membrane assay (25), and LDB2 expression in blood vessels in the lungs of mice was confirmed (25). There was no significant vascular phenotype in LDB2 mutant mouse embryos presumably because the homologue LDB1 may compensate for LDB2. However, more detailed investigations on blood vessel development in mutant mice and further elucidation of the role of LDB2 in pathological angiogenesis is required.

In conclusion, this study suggests that LDB2 plays a role in endothelial sprouting by regulating DLL4 expression via an oligomeric complex and that manipulation of the LDB2 complex could be exploited for the development of therapeutic strategies to inhibit aberrant angiogenesis.

MATERIALS AND METHODS

Cell cultures
Primary human umbilical vein endothelial cells (HUVEC) were purchased from Lonza Inc. and used between passage 4 and 8. Endothelial cells were cultured in gelatin-coated plates in Medium 199 (Hyclone) containing 20% FBS (Lonza), 3 ng/ml basic-FGF (R&D System), 5 units/ml heparin (Sigma), and 1% penicillin-streptomycin (Gibco).

Tube formation assay and *in vitro* morphogenesis on 2-D Matrigel
Tube formation was assayed as previously described (26). Briefly, 300 μl of Matrigel (BD Biosciences) was added to the wells of a 24-well plate and allowed to polymerize for 20 min at 37°C. HUVECs were harvested, resuspended in Medium 199, and seeded onto Matrigel (0.8×10^5 cells/well).

Transfection of siRNA and DNA
HUVECs of 60-80% confluence were transfected with control siRNA (Santa Cruz Biotech.; sc-37007) or LDB2 specific siRNA (Santa Cruz Biotech.; sc-38023) using Lipofectamine (Invitrogen). HUVECs were transfected with plasmid DNAs using Lipofectamine LTX and Plus reagent following manufacturer's instruction (Invitrogen). Total amount of DNA transfected was adjusted with the control vector plasmid.

Morpholino injection

Micro-injections of morpholino oligonucleotides were carried out as previously described (26). Briefly, embryos were injected at the single-cell stage with 2-5 ng of control morpholino (Gene Tools). The sequences for the morpholino oligonucleotides used in this study are: *ldb2* splicing morpholino: 5′-CGTACACACCTGAGAAAGCAGACAT-3′; *ldb2* ATG morpholino: 5′-CATGTTGATTCCGTGTGCCGTTTGC-3′; mismatch control for *ldb2* ATG morpholino; 5′-CATCTTCA TTGCGTCTGCCCTTTGC-3′.

3-D Fibrin gel assay for endothelial sprouting

Endothelial sprouting assays were performed as previously described (27) with some modifications. HUVECs were trypsinized and mixed with Cytodex microcarrier beads (Amersham Pharmacia) at a ratio of 10^6 cells per 2,500 beads for 4 h at 37°C. After incubation for 24 h at 37°C, the coated beads were embedded into the fibrinogen with thrombin. The clots were overlaid with 2×10^4 human lung carcinoma fibroblasts (WI-38) and incubated with 2 ml of EGM-2 medium per well. Media was replaced every 2 days and vessel formation was analyzed after 3-5 days.

qRT-PCR

Total RNA was extracted and purified using the RNeasy kit (Qiagen). qRT-PCR was performed with $1\times$ SYBR Green Mix (Invitrogen) with a Bio-Rad real-time PCR detection system. For each reaction, 100 ng of total RNA was transcribed at 50°C for followed by a denaturing step at 95°C for 5 min, 40 cycles of 95°C for 30 s and 60°C for 30 s. Fluorescence data were collected and analyzed using an I-cycler (Bio-Rad). For normalization, human *GAPDH* was used as the endogenous control.

Western blotting

Cell pellets were lysed on ice with RIPA buffer supplemented with phosphatase inhibitor cocktail tablets (Roche) and then were mixed with $6\times$ sample buffer. Protein samples were electrophoresed by SDS-PAGE and transferred to PVDF membranes. The membranes were probed with anti-LDB2 (1:500, Abcam), anti-DLL4 (1:500, Cell Signaling), and anti-β-actin (1:1,000, Santa Cruz) antibodies and detection was performed with an ECL Plus detection kit (Amersham Bioscience).

Luciferase assay

The luciferase reporter assay was performed as described previously (28, 29). HUVECs that were plated onto a gelatin-coated dish were transfected with different expression vectors along with reporter plasmids as described in the figures. The total amount of transfected plasmid DNA was adjusted with empty vector DNA. Luciferase activity was assayed 24 h after transfection. Cells were lysed with Passive lysis buffer (Promega), and luciferase activities in cell extracts were determined 24 h after transfection using a Dual luciferase assay system (Promega).

CHIP assay

The CHIP assay was performed using a CHIP kit (Millipore) as previously described, with some modification (30). Genomic DNA and protein were cross-linked by adding formaldehyde (1%, final concentration) directly to the culture medium and incubating for 10 min at 37°C. Then, the cells were harvested, lysed, and sonicated to generate 0.3-1.0 kb DNA fragments. After centrifugation, the cleared supernatant was incubated with anti-TAL1 (Santa Cruz), anti-GATA2 (Santa Cruz), anti-FLAG (Sigma), and IgG for immunoprecipitation. Co-immunoprecipitated and input DNAs were amplified by PCR, and amplified DNA was separated on a 1.5% agarose gel and visualized with ethidium bromide.

Statistical analysis

Statistical analysis was carried out using GraphPad Prism 4.0. Data are presented as mean ± standard deviation (SD). Differences between groups were performed through an unpaired Student's *t*-test. A value of $P < 0.05$ was considered significant.

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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