Published in final edited form as:

J Cell Biochem. 2019 March ; 120(3): 3362–3366. doi:10.1002/jcb.27606.

TIEG1 is upregulated in Lrp5/6-mediated valve osteogenesis

Nalini M Rajamannan^{1,2}

¹Department of Cardiology, Most Sacred Heart of Jesus Cardiology and Valvular Institute, Sheboygan, Wisconsin

²Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, Minnesota

Abstract

We have previously demonstrated that Lrp5/6/ β -catenin plays an important role in valve calcification with a specific osteogenic phenotype defined by increased bone mineral content and overall valve thickening. Recent studies indicate that TIEG1 may be involved in mediating the Wnt signaling pathway in bone, which is known to play critical roles in osteoblast differentiation and bone mineralization. Therefore, we sought to test the role of TIEG1 in mediating Wnt signaling, in an established model of hypercholesterolemic valve disease. Our previous model treated null mice with cholesterol diets: Lrp5^{-/-}/ApoE^{-/-} mice versus wild-type control (n = 180). Group I (n = 60) normal diet, Group II (n = 60) 0.25% chol diet (w/w), and Group III (n = 60) 0.25% (w/w) chol diet + atorv was tested for gene expression for TIEG1, Lrp6, and Runx2. Real-time polymerase chain reaction confirmed that there is upregulation of the gene expression for TIEG1 and Runx2 in the hypercholesterolemic double knockout and single knockout valves as compared with controls with a mild increase in Lrp6. To confirm the mechanism, coexpression of β -catenin, TIEG1, and LEF1 in valve cells in vitro, led to the coactivation of the TOPFLASH reporter, which was further confirmed by the observation that TIEG1 and β -catenin colocalize with one another in the nucleus of valvular interstitial cells (VICs) following stimulation with transforming growth factor- β treatment, an established activator of TIEG1. Taken together, these data implicate an important role for TIEG1 in mediating valve osteogenesis.

Keywords

lipids; Lrp5; osteoblastogenesis; TIEG1

1 | INTRODUCTION

Previously, we have demonstrated that experimental cholesterol diet can upregulate Lrp5/6 receptors in aortic valve osteogenesis. This model demonstrates that Lrp6 can be upregulated and cause aortic valve calcification, but only in the presence of hypercholesterolemia.¹ Recently, TIEG1 has been shown to upregulated in Wnt osteogenic bone formation.² TIEG1 is a novel Kruppel-like transcription factor known to regulate bone osteogenesis in osteoblast cells³ via Wnt signaling.²

In this brief communication, we tested the valves from our previous model of valve calcification for TIEG1 expression, and the regulation of TOPFLASH reporter in valve interstitial cells, to further demonstrate that TIEG1 is a critical regulator in Wnt signaling in valve osteogenesis¹ in addition to the recently established role in osteoblastogenesis.²

2 | METHODS

2.1 | ApoE^{-/-}/Lrp5^{-/-} experimental hypercholesterolemia mouse model

ApoE^{-/-} mice were purchased from Jackson Laboratories (Bar Harbor, ME), and Lrp5^{-/-} were purchased from Taconic laboratories (Germantown, NY). ApoE^{-/-}:Lrp5^{-/-} were produced by cross breeding. Mice aged 6 to 8 weeks (male and female mice) were assigned to a control (N = 60), a 0.2% cholesterol (w/w) diet (Harlan Teklad 88137), (N = 60) and a 0.2% cholesterol (w/w) diet (Harlan Teklad 88137). All animals were fed ad libitum for 23 weeks. Control mice were fed a standard diet. Following this 23-week period, the mice were euthanized with inhalation CO₂. All experiments were performed in an animal facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, Inc (ACUC-A3283-01, 1-08-382). Immediately after dissection from the heart one leaflet from each aortic valve was fixed in 10% buffered formalin for 24 hours and then embedded in paraffin. Valves were also snap frozen in liquid nitrogen and stored in -80° freezer for gene expression experiments. Real-time polymerase chain reaction (RT-PCR)^{2,4} was performed to measure Lrp6, TIEG1, and Runx2 in the cardiac valves.

2.2 | Cell lines and culture conditions

Valve Interstitial cells were isolated as described previously.⁵ Briefly, the valves were dissected out, cleaned of all tissue, rinsed in PBS, and minced. Valves were subjected to three collagenase digestions and interstitial cells were isolated from the third digestion were plated in α -Minimal Essential Media (α -MEM) (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS) (Gemini Bio-Products, West Sacramento, CA) and 1% antibiotic/antimycotic (Thermo Fisher Scientific, Waltham, MA), and propagated in a humidified incubator with 5% CO₂. Analysis of variance-statistical analysis will be performed to test the differences in the different treatment groups (a *P* value of less than 0.05 is significant.)

2.3 | Transient transfection and luciferase assays

Valve interstitial cells were plated at a density of 50% in 12-well plates in replicates of six. As indicated, cells were transfected with 250 ng of the TOPFLASH reporter and/or various expression vectors (empty pcDNA4.0, Flag-tagged TIEG1, Flag-tagged Lef1, constitutively active β -catenin, or Xpress-tagged TIEG1 domain expression constructs² using Fugene-6 (Roche, Indianapolis, IN) as specified by the manufacturer. Empty vector was added to transfections as necessary to normalize the total amount of DNA transfected across each condition. Twenty-four hours following transfection, cells were lysed in passive lysis buffer (Promega, Madison, WI), lysates were quantitated for protein content, and equal amounts of protein were used to measure luciferase activity using Luciferase Assay Reagent (Promega) and a glomax-dual luminometer (Promega).

2.4 | Confocal microscopy

Valvular interstitial cells were plated on coverslips at low confluence and allowed to adhere overnight. Cells were transfected as indicated or treated with transforming growth factor- β (TGF- β) (2 ng/mL) for 24 hours. Cells were fixed in 1% paraformaldehyde for 30 minutes and washed twice with 1X PBS followed by permeabilization with 0.2% Triton-X in PBS for 30 minutes and blocked for an additional 30 minutes in heat-inactivated 5% FBS. Subsequently, cells were incubated with a polyclonal TIEG1 antibody produced by our laboratory (#992) and a monoclonal β -catenin antibody (clone 14/ β -catenin [RUO]) for 60 minutes. Cells were washed twice with PBS and stained with Texas Red- and fluorescein isothiocyanate-conjugated secondary IgG antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) for an additional 60 minutes. DAPI (4',6-diamidino-2-phenylindole) was used as a counterstain. Immunofluorescence images were captured with a Zeiss LSM 510 confocal microscope (Carl Zeiss, Jena, Germany).

3 | RESULTS

To understand if $Lrp5^{-/-}/ApoE^{-/-}$, hypercholesterolemic aortic valves osteogenesis is regulated via upregulation of TIEG1 expression, we performed RT-PCR on the valves from our established model of valve osteogenesis.¹ Figure 1A is the quantification of the gene expression for the hypercholesterolemic wild-type (WT) valves versus the hypercholesterolemic $Lrp5^{-/-}$ valves, $ApoE^{-/-}:Lrp5^{-/-}$ valves. The RT-PCR confirmed an upregulation of TIEG1 and Runx2 in the $Lrp5^{-/-}$ valves and double knockout $ApoE^{-/-}:Lrp5^{-/-}$, and again a significant increase in $Lrp6$ in the $Lrp5^{-/-}$ valves with a mild increase in the $Lrp6$ in the $ApoE^{-/-}:Lrp5^{-/-}$ valves hypercholesterolemic aortic valve. This current study demonstrates an increase in the TIEG1/Runx2/ $Lrp6$ gene expression in the hypercholesterolemic aortic valves from the single and double knockout mice as compared with the WT control mice, confirming the role of elevated lipids in the osteogenic gene cascade, with the novel finding of TIEG1 upregulation in these valves.

Coexpression of both β -catenin and LEF1 led to the coactivation of the TOPFLASH reporter when transfected into valvular interstitial cells (VICs). When TIEG1 was coexpressed with LEF or β -catenin, a significant increase in the reporter activity was observed. These data suggested that TIEG1 regulates LEF and β -catenin to form a transcriptionally active protein complex, leading to enhanced Wnt signaling in VICs as shown in Figure 1B. Figure 1C, outlines the signaling pathway in valve osteogenesis via $Lrp5/6$ /TIEG1 signaling. This possibility was further confirmed by the observation that TIEG1 and β -catenin colocalize with one another in the nucleus of VICs following stimulation with TGF- β treatment, a known regulator of TIEG1 expression,³ as shown in Figure 1D.

Figure 2A demonstrates the aortic valve histology and MicroCT for the model of aortic valve calcification, demonstrating the need for $ApoE$ null mice to induce calcification in the aortic valve via $Lrp6$.¹ Figure 2B demonstrates the $ApoB$ levels increasing ($P < 0.05$) in the $ApoE$ null mice further implicating the role of $ApoE$ and $ApoB$ in the signaling of osteogenesis in aortic valve calcification.

4 | DISCUSSION

This brief communication further confirms the role of TIEG1 in osteogenic bone signaling. Previously, we have shown that the valve interstitial cells differentiate in the presence of hypercholesterolemia,⁶ abnormal oxidative stress, and Lrp5/6 receptor-mediated calcification.¹ Recently, our group confirmed the role of TIEG1 in Wnt Signaling in osteogenic bone formation.² We tested the valves from our previous study of hypercholesterolemic valve calcification and discovered that TIEG1 is also upregulated and active in the calcifying aortic valve tissue from our mouse model. This finding will help in the future to further understand the role of TIEG1 in the transcriptional biology of Wnt Signaling in osteogenic bone formation. In addition, consideration of lipid lowering including LDL-C and ApoB levels via PCSK9 inhibitors may play a future role in the treatment of this disease. Further studies are necessary to test the role of TGF- β , WNT secretion from aortic valve endothelial cells⁶ in the future understanding the transcriptional signaling of osteogenesis in the aortic valve.

ACKNOWLEDGMENTS

This study was completed with the support of 5R01HL085591 and 3R01HL085591S1 to NM Rajamannan. NM Rajamannan is the inventor of a patent for methods to slow progression of valvular heart disease and of a patent for the TIEG1 null mouse. These patents are owned by the Mayo Clinic, and the author does not receive any royalties from this patent. The author would like to thank Dr Thomas C Spelsberg, Dr Malayannan Subramaniam, and Dr John Hawse for their excellent career mentorship, support, and helpful suggestions over the past 17 years of collaboration. Finally, Dr. NM Rajamannan thanks post-humously Dr Muzaffer Cicek and collaborator and colleague Dr Thomas Spelsberg for their collaboration on the ARRA award to develop this hypothesis.

REFERENCES

1. Rajamannan NM. The role of Lrp5/6 in cardiac valve disease: experimental hypercholesterolemia in the ApoE^{-/-}/Lrp5^{-/-} mice. *J Cell Biochem.* 2011;112:2987–2991. [PubMed: 21678468]
2. Subramaniam M, Cicek M, Pitel KS, et al. TIEG1 modulates beta-catenin sub-cellular localization and enhances Wnt signaling in bone. *Nucleic Acids Res.* 2017;45:5170–5182. [PubMed: 28201653]
3. Subramaniam M, Oursler MJ, Rasmussen K, Riggs BL, Spelsberg TC. TGF-beta regulation of nuclear proto-oncogenes and TGF-beta gene expression in normal human osteoblast-like cells. *J Cell Biochem.* 1995;57:52–61. [PubMed: 7721958]
4. Hawse JR, Iwaniec UT, Bensamoun SF, et al. TIEG-null mice display an osteopenic gender-specific phenotype. *Bone.* 2008;42:1025–1031. [PubMed: 18396127]
5. Johnson C, Hanson M, Helgeson S. Porcine cardiac valvular subendothelial cells in culture: cell isolation and growth characteristics. *J Mol Cell Cardiol.* 1987;19:1185–1193. [PubMed: 3327949]
6. Rajamannan NM. Oxidative-mechanical stress signals stem cell niche mediated Lrp5 osteogenesis in eNOS^{-/-} null mice. *J Cell Biochem.* 2012;113:1623–1634. [PubMed: 22359381]

FIGURE 1.
A, The RT-PCR results for the ApoE^{-/-}:Lrp5^{-/-} valves versus WT control mice on hypercholesterolemic diets. B, The regulation of TOPFLASH reporter after transfection with TIEG1, LEF, and β-catenin. C, The role of TIEG1 in aortic valve osteogenesis via Wnt signaling. D, The confocal microscopy of TIEG1 translocation to the nucleus in the presence of TGF-β. RT-PCR, real-time polymerase chain reaction; TGF, transforming growth factor; TGF-β, transforming growth factor-β; WT, wild type

FIGURE 2.
A, The aortic valve histology, MicroCT in the ApoE^{-/-}:Lrp5^{-/-} valves versus WT control mice on hypercholesterolemic diets (used with permission).¹ B, The apoB lipid levels in the ApoE^{-/-} on control versus hypercholesterolemic diets. WT, wild type