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A DNA Binding Mutation in Estrogen Receptor-α Leads to Suppression of Wnt Signaling Via β-Catenin Destabilization in Osteoblasts

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ABSTRACT

Estrogen receptors (ERs) play vital roles in the function and remodeling of bone. Their cellular mechanisms can broadly be categorized into those involving direct DNA binding (classical) or indirect DNA binding (non-classical). The generation of non-classical ER knock-in ($ER\alpha^{-/NERKI}$) mice provides a unique opportunity to define these pathways in bone. We previously demonstrated that $ER\alpha^{-/NERKI}$ mice exhibit an osteoporotic phenotype; however, the mechanism(s) for this remain unresolved. Gene expression analyses of cortical bone from $ER\alpha^{-/NERKI}$ mice revealed suppression of *lymphoid enhancer factor-1* (*Lef1*), a classic Wnt-responsive transcription factor that associates with β-catenin. Since Wnt signaling is generally considered bone anabolic, this observation leads to the hypothesis that NERKI-induced suppression of Wnt signaling may contribute to the low bone mass phenotype. We generated $ER\alpha^{-/NERKI}$ mice crossed with the Wnt-responsive TOPGAL transgenic mouse model and observed significantly less β-galactosidase activity in $ER\alpha^{-/NERKI}$ mice, confirming suppression of Wnt activity in vivo. Adenoviral expression of the NERKI receptor using an in vitro cell system resulted in the induction of several secreted antagonists of Wnt signaling. Furthermore, expression of NERKI abrogated Wnt10b-dependent Wnt activation using a lentiviral-mediated reporter assay. Finally, expression of NERKI destabilized β-catenin cellular protein levels and disrupted ER/β-catenin interactions. Collectively, these data suggest the osteoporotic phenotype of $ER\alpha^{-/NERKI}$ mice may involve the suppression of *Lef1*-mediated Wnt signaling through both the stimulation of secreted Wnt inhibitors and/or disruption of normal β-catenin function. J. Cell. Biochem. 113: 2248–2255, 2012. © 2012 Wiley Periodicals, Inc.

KEY WORDS: ESTROGEN RECEPTOR; NERKI; Wnt; β-CATENIN; BONE

Bone homeostasis relies on the coordinated interactions among the bone forming osteoblasts, bone resorbing osteoclasts, and the mechanosensing osteocytes [Monroe et al., 2006]. Considerable research has focused on the role of estrogen receptors (ERα and ERβ) in these cell types and their roles in both normal and pathological bone states [Windahl et al., 1999; Windahl et al., 2001; Sims et al., 2002; Bonnelye and Aubin, 2005; Imai et al., 2009]. Although the molecular mechanisms of ER action are varied and complex, their main recognized roles are as modulators of gene expression either through direct DNA binding to estrogen response elements (classical pathway) or indirect DNA binding via protein interactions with other transcriptional regulators (non-classical pathway) [Jakacka et al., 2001; Syed et al., 2005]. The generation of non-classical ER knock-in ($ER\alpha^{-/NERKI}$) mice, which harbor a mutation in the first zinc finger that eliminates classical but preserves non-classical signaling [Jakacka et al., 2002], provides a unique model to define these pathways in bone. It was previously demonstrated that $ER\alpha^{-/NERKI}$ mice exhibit an osteoporotic phenotype [Syed et al., 2005, 2007], however the molecular mechanism(s) have remained elusive.

The Wnt/β-catenin signaling pathway has garnered much attention due to its widely recognized role in bone metabolism (reviewed in [Baron and Rawadi, 2007; Monroe et al., 2012]). The classical Wnt signaling pathway, which is recognized as the major Wnt pathway in bone, is initiated through the binding of Wnt glycoproteins to frizzled (Fzd) receptors and the low-density lipoprotein receptor-related protein (Lrp)-5/6 coreceptors. This induces a complex signal transduction cascade that ultimately leads

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to the nuclear translocation of hypophosphorylated β-catenin where it modulates T-cell factor (Tcf) and lymphoid enhancer binding factor (Lef)-dependent gene expression. Interactions between the Wnt and ER signaling pathways have just begun to be identified and explored. Armstrong et al. [2007] demonstrated that bone responses following mechanical stress require both β-catenin and ERα signaling, and that β-catenin nuclear transport is facilitated by ERα. Consequentially, regulation of fewer Wnt-responsive genes in response to mechanical loading was observed in cells from mice lacking ERα. Kouzmenko et al. [2004] demonstrated a physical association between ERα and β-catenin on specific estrogen response element and Tcf/Lef target promoters. Thus, it is clear that interactions between the ER and Wnt signaling pathways are extremely important for osteoblast function.

In this study, we utilize a custom QPCR array approach to identify differentially expressed osteogenic genes from cortical bone between $ERα^{+/+}$ and $ERα^{-/NERKI}$ mice. Using this approach, we provide evidence that the Wnt signaling pathway is suppressed in $ERα^{-/NERKI}$ mice. In vitro cellular analyses further demonstrate that the presence of the NERKI receptor stimulates expression of specific Wnt inhibitors, suppresses global Wnt activity and destabilizes β-catenin protein. Understanding the molecular mechanisms by which a mutant ERα (e.g., NERKI) causes bone loss may aid in the identification of therapeutic targets for clinical interventions in the treatment of bone diseases such as osteoporosis.

MATERIALS AND METHODS

ANTIBODIES AND KITS

The rabbit anti-β-catenin antibody (06-734) was purchased from Millipore (Billerica, MA). The Flag-M2 antibody and β-Galactosidase Reporter Gene Staining Kit were purchased from Sigma–Aldrich (St. Louis, MO). The α-tubulin antibody (H-300) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The fluorescein horse anti-mouse IgG antibody (FI-2000) and Texas red goat anti-rabbit IgG antibody (TI-1000) were purchased from Vector Laboratories, Inc. (Burlingame, CA). The Cignal Lenti TCF/LEF Reporter (luc) Kit and Mouse Osteogenesis RT^2 Profiler™ PCR Array were purchased from (SABiosciences, Frederick, MD). The BCA Protein Assay Kit was purchased from Thermo Scientific (Rockford, IL). The Luciferase Assay Reagent Kit was purchased from (Promega, Madison, WI).

ANIMALS

Three-month-old female wild-type ($ERα^{+/+}$) or $ERα^{-/NERKI}$ mice, both in a C57/BL6 genetic background, which harbor a mutation in the ERα DNA-binding domain that abolishes direct DNA binding [Jakacka et al., 2001], were used for isolation of cortical bone RNA. In an independent experiment, $ERα^{+/+}$ or $ERα^{-/NERKI}$ mice were crossed with a Tcf/Lef1-β-gal reporter mouse strain [Jackson Laboratories, 004623 Tg(Fos-lacZ)34Efu/J]), to create $ERα^{+/+}$//TOPGAL and $ERα^{-/NERKI}$//TOPGAL hybrids and analyzed at 6 weeks of age. All animal studies were conducted in accordance with the principles and procedures outlined in the National Institute of Health Care and Use of Animals under Protocol Number A38108.

PLASMID CONSTRUCTIONS

Mouse estrogen receptor-alpha (ERα) was PCR amplified from mERα-pcDNA3.1 containing an N-terminal Flag-epitope tag (DYKDDDDK) and subcloned as a *Hin*dIII/*Bam*HI fragment into the expression vector Dual-CCM (Vector Biolabs, Philadelphia, PA) resulting in ERα-Dual. The NERKI-Dual construct was created by introducing a double-point mutation (E207A/G208A) in ERα-Dual to correspond to the published NERKI sequence [Jakacka et al., 2001] using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) resulting in NERKI-Dual. To create the Cre-dependent expression constructs, ERα was PCR amplified from ERα-Dual with or without the Flag-epitope tag and cloned as an *Nhe*I/*Kpn*I fragment into pCMVflox [Moeller et al., 2005] resulting in ERα-Flox and ERα-Flag-Flox, respectively. NERKI-Flox and NERKI-Flag-Flox were created in an identical manner but using NERKI-Dual as the PCR template. The Cre expression construct, pBS513 EF1alpha-cre, was purchased from Addgene (Cambridge, MA).

RNA ISOLATION AND cDNA SYNTHESIS

Total cellular RNA was harvested from either cortical bone or culture cells using QIAzol Lysis Reagent and RNeasy Mini Columns (Qiagen, Valencia, CA). DNase treatment was performed to degrade potential contaminating genomic DNA using an on-column RNase-free DNase solution (Qiagen). One microgram of total RNA was used in a reverse transcriptase (RT) reaction using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems by Life Technologies, Foster City, CA) according to manufacturer instructions.

SUPERARRAY OSTEOGENIC ARRAY

cDNA prepared from 3-month-old female $ERα^{+/+}$ and $ERα^{-/NERKI}$ cortical bone ($n = 6$) was used in a real-time quantitative PCR (QPCR) assay using the Mouse Osteogenesis RT^2 Profiler™ PCR Array and analyzed using the manufacturer's software. The data are presented as relative expression normalized to the $ERα^{+/+}$ expression level.

HISTOLOGY AND β-GALACTOSIDASE (β-GAL) STAINING

Non-decalcified femurs from 6-week-old female $ERα^{+/+}$//TOPGAL and $ERα^{-/NERKI}$//TOPGAL mice were fixed, frozen, and sectioned using the CryoJane tap system (Leica Microsystems, Wetzlar, Germany) as previously described [Salie et al., 2008]. The sections were stained using the β-Galactosidase Reporter Gene Staining Kit to detect differences in β-gal activity according to manufacturer instructions.

CELL CULTURE, ADENOVIRAL PRODUCTION, AND INFECTION

U2OS and U2OS-Wnt10b cells were cultured as previously described [Modder et al., 2011a]. ERα- and NERKI-Dual constructs were used to produce Type 5 (dE1/E3) adenovirus (Vector Biolabs) resulting in Ad-ERα and Ad-NERKI. A multiplicity of infection (MOI) of 12.5, which was previously demonstrated to result in ~100% infection rates and equal protein levels for ERα and NERKI (data not shown), was used for infection of both adenoviruses into U2OS cells in the presence of 8 μg/ml hexadimethrine bromide (polybrene) to enhance adenoviral infection.

LENTIVIRAL LUCIFERASE REPORTER ASSAYS

To produce stable Wnt-reporter cell lines, U2OS and U2OS-Wnt10b cells were transduced with the Cignal Lenti TCF/LEF Reporter (luc) Kit for 48 h. Selective media [U2OS-media supplemented with 10 μg/ml puromycin (Invitrogen, Carlsbad, CA)] was then applied to the cells and the resistant population was expanded resulting in the U2OS-TCF/LEF-Luc and U2OS-Wnt10b-TCF/LEF-Luc cell lines. Adenoviral infections in these lines were performed as described in the previous subsection. Following incubation at 37°C for 48 h, cells were harvested in 1× Passive Lysis Buffer and equal quantities of protein extracts were assayed using Luciferase Assay Reagent on a GloMax® 96 Microplate Luminometer (Promega. Protein concentrations were determined using a BCA Protein Assay Kit. The data are presented as mean luciferase values (per μg protein) normalized to U2OS-TCF/LEF-Luc infected with Ad-ERα ($n = 6$).

GENE EXPRESSION ANALYSIS

The RT reactions were diluted 1:5 and 1 μl used in a 10 μl total reaction volume for QPCR using the QuantiTect SYBR Green PCR Kit (Qiagen) and the ABI 7900HT Fast Real-Time PCR System (Applied Biosystems). All primers were designed using Primer Express® Software Version 3.0 (Applied Biosystems) and the sequences are available upon request. The method for data normalization using multiple reference genes and threshold calculations is as previously described [Modder et al., 2011b].

TRANSIENT TRANSFECTION

U2OS cells were plated in 6-well plates at a density of 2.6×10^4 cells/cm^2 the day before transfection. ERα-Flox, ERα-Flag-Flox, NERKI-Flox, or NERKI-Flag-Flox (250 ng) in either the absence or presence of a Cre-expression plasmid (pBS513 EF1alpha-cre; 250 ng) were transiently transfected ($n = 6$) using FuGENE 6 transfection reagent (Roche Diagnostics, Indianapolis, IN). The empty expression vector Dual-CCM was used to normalize transfected DNA levels. Cell lysates were prepared 48 h later and subjected to Western blot analysis.

WESTERN BLOT ANALYSIS

U2OS whole cell extracts were prepared by lysing the cells for 30 min on ice in lysis buffer [20 mM Tris (pH 8.0), 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet-P40] supplemented with Complete Mini EDTA-free Protease Inhibitor tablets and PhosStop Phosphatase Inhibitor tablets (Roche Diagnostics). Protein concentrations were determined and 20 μg of protein were subjected to Western blot analysis as previously described [Monroe et al., 2003]. The blots were incubated with antibodies directed against Flag (1:1,000), β-catenin (1:1,000) or α-tubulin (1:100,000) for 1 h. Species specific anti-IgG secondary antibodies linked to horseradish peroxidase (Sigma) were used at a 1:5,000 dilution for 45 min. Washed blots were visualized using enhanced chemiluminescence (Amersham Pharmacia) and exposed to X-ray film.

IMMUNOFLUORESCENT STAINING AND CONFOCAL MICROSCOPY

U2OS cells were plated in a 4-well Lab-Tek™ Chamber Slide™ system (Thermo Scientific) at a density of 2.6×10^4 cells/cm^2. The next day, the cells were infected with either Ad-ERα or Ad-NERKI as described above and allowed to incubate at 37°C for 24 h in the presence of 100 ng/ml Wnt10b (R&D Systems, Minneapolis, MN). The cells were fixed for 15 min with 2% paraformadehyde (v/v) and 0.5% (v/v) Triton X-100 in PBS and blocked for 30 min with 5% BSA in PBS. The cells were then incubated with antibodies against β-catenin (1:50) or Flag (1:100) for 1 h. Following two PBS washes, the cells were incubated with the appropriate secondary antibody (Texas Red-conjugated goat anti-rabbit IgG at 1:500 and Fluorescein-conjugated horse anti-mouse IgG at 1:1,000) for 1 h and washed with PBS. The cells were incubated with the nuclear stain 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen) for 5 min and washed with PBS twice. The slides were kept at 4°C overnight for confocal microscopy. The images were collected and stored using the manufacturer provided software. All images were collected using identical background and image capture settings.

STATISTICAL ANALYSIS

Calculations and statistical analyses were performed using Microsoft® Office Excel 2003 (Microsoft Corporation, Redmond, WA). The data are presented as the mean ± standard error (SE). All *P*-values ≤ 0.05 were considered statistically significant using Student's *t*-test. The O'Brien statistical test was performed as previously described [O'Brien, 1984].

RESULTS

Lef1 EXPRESSION IS SUPPRESSED IN ERα$^{-/NERKI}$ MICE

Previous studies have demonstrated that mice harboring the NERKI mutation (ERα$^{-/NERKI}$) exhibit osteopenia [Syed et al., 2005, 2007], indicating that elimination of direct ERα-dependent ERE binding has significant deleterious effects on bone formation. As a basis to understand the molecular mechanisms of this observation we isolated RNA from flushed cortical bone of 3-month-old wild-type (ERα$^{+/+}$) and ERα$^{-/NERKI}$ mice and assayed known osteogenic regulators using the Mouse Osteogenesis RT^2 Profiler™ PCR Array (Fig. 1A). Expression of the Wnt target gene, *lymphoid enhancer factor-1* (*Lef1*), was significantly reduced 2.5-fold in the cortical bone of ERα$^{-/NERKI}$ as compared to ERα$^{+/+}$ mice (Fig. 1B), suggesting that alterations in Wnt signaling may be a possible cause of the impaired bone formation observed in ERα$^{-/NERKI}$ mice.

Wnt SIGNALING IS SUPPRESSED IN ERα$^{-/NERKI}$ MICE IN VIVO

To investigate whether reduced expression of *Lef1* in cortical bone of ERα$^{-/NERKI}$ mice leads to suppression of the Wnt signaling pathway in vivo, we crossed ERα$^{+/+}$ or ERα$^{-/NERKI}$ mice with TOPGAL transgenic mice (Fig. 2A). This widely used reporter strain was made by fusing three LEF/TCF binding sites to *c-fos* minimal promoter and expresses β-galactosidase (β-gal) in response to *Lef1*-mediated Wnt signaling [DasGupta and Fuchs, 1999]. Analysis of β-gal stained bone sections revealed significantly less β-gal expression in ERα$^{-/NERKI}$ mice as compared to ERα$^{+/+}$ (Fig. 2B), confirming suppression of Wnt activity in vivo.

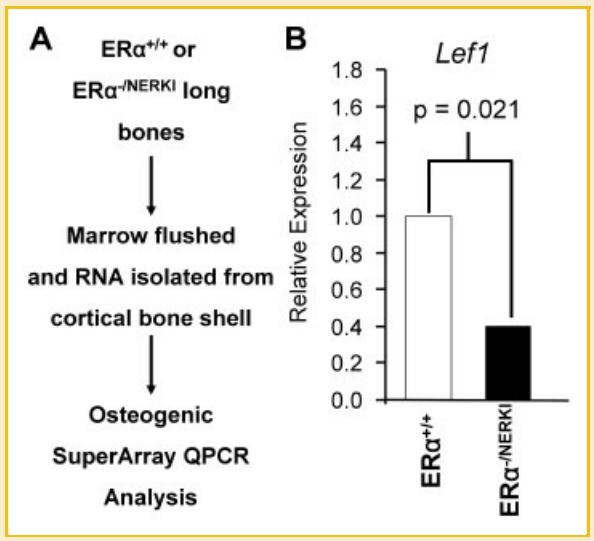

Fig. 1. *Lef1* expression levels are suppressed in cortical bone from ERα$^{-/NERKI}$ mice. A: Long bones from 3-month-old female ERα$^{+/+}$ or ERα$^{-/NERKI}$ mice were excised and the bone marrow flushed. RNA was isolated from the resulting cortical shell and was used in QPCR analyses using the Mouse Osteogenesis RT^2 Profiler^{TM} PCR Array (SABiociences). B: QPCR analysis revealed that *Lef1* was suppressed in ERα$^{-/NERKI}$ cortical bone when compared to ERα$^{+/+}$. The bars represent relative expression levels and a statistically significant difference of $P = 0.021$ (Student's *t*-test) is indicated for *Lef1* expression.

NERKI ALTERS EXPRESSION PATTERNS OF GENES INVOLVED IN Wnt SIGNALING

To further explore the transcriptional effects of NERKI in a more controlled cell model system (without the confounding effects of the presence of ERβ), we introduced ERα or NERKI into the ER-negative cell line U2OS using adenoviral transduction. QPCR analysis was performed to assess the gene expression patterns from the NERKI-transduced versus ERα-transduced cells using primers specific for genes in pathways with known importance in bone. Table I lists the genes evaluated in pathways for osteoblast differentiation, Wnt targets, Wnt inhibitors, Bmps, Bmp targets, Notch targets, apoptosis, proliferation, estrogen targets, NFkB targets, and endothelin-1 targets. The O'Brien Umbrella test was used to evaluate whether these pre-specified groups of genes were altered as a group and the *P*-values are shown in Table I. This analysis revealed that the NERKI receptor significantly upregulated Wnt inhibitor genes but significantly downregulated genes involved in proliferation, apoptosis, as well as Notch and estrogen target genes. The significant upregulation of the Wnt inhibitor genes was of particular interest since our previous data demonstrated suppression of the Wnt pathway in ERα$^{-/NERKI}$ mice (Figs. 1 and 2). In particular, the Wnt inhibitor genes *AXIN2*, *DKK1*, *SFRP4*, and *SOST*, all of which have important roles in bone [Monroe et al., 2012], were highly regulated (Fig. 3). It is important to note that other than the estrogen targets, few genes were significantly regulated by 17-β-estradiol (data not shown) demonstrating that these transcriptional differences are independent of estrogen.

NERKI SUPPRESSES Wnt SIGNALING THROUGH THE DESTABILIZATION OF CELLULAR β-CATENIN PROTEIN LEVELS

To confirm the NERKI-dependent suppression of the Wnt pathway in vitro, we produced two cell models in which a Wnt-responsive luciferase cassette (TCF/LEF-Luc) was stably introduced into either control U2OS cells or U2OS-Wnt10b cells [Modder et al., 2011a]. These models allow for determination of the overall level of Wnt signaling using a simple luciferase assay in control U2OS cells (U2OS-TCF/LEF-Luc) or in cells in which Wnt signaling is elevated (U2OS-Wnt10b-TCF/LEF-Luc), in the presence of either adeno-

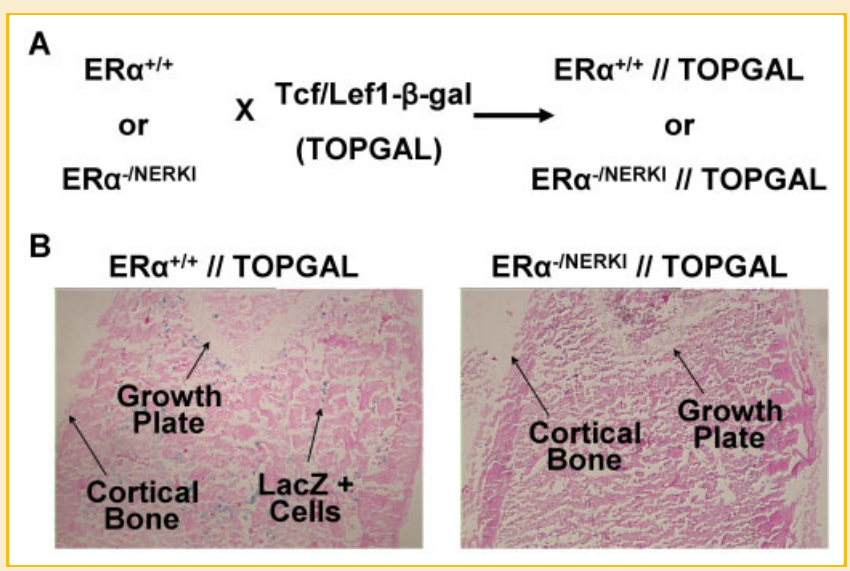

Fig. 2. Wnt activity is suppressed in vivo in ERα$^{-/NERKI}$ mice. A: ERα$^{+/+}$ and ERα$^{-/NERKI}$ mice were crossed with the TOPGAL Wnt-reporter strain and described in Materials and Methods Section. B: Non-decalcified femurs isolated from 6-week-old ERα$^{+/+}$//TOPGAL and ERα$^{-/NERKI}$//TOPGAL mice were fixed, frozen, sectioned and stained for β-galactosidase activity. Arrows indicate the growth plate, cortical bone, and LacZ positive (blue) cells.

TABLE I. Genes Examined in Each of the Pathways Analyzed Along With the Results of the O'Brien Umbrella Statistical Test, Which Measures Changes in All the Genes in Each Pathway as a Group

Pathway	Genes	O'Brien umbrella *P*-value	Direction of change
Osteoblast differentiation	Alkaline phosphatase, osteocalcin, osteonectin, osteopontin, bone sialoprotein, Col1α1, Col1α2, Runx2, osterix, Msx2, Dlx5	0.1081	
Wnt targets	*Lef1*, Tcf7, Axin2, EphrinB4, Cyr61, connexin 43, cyclin D1, periostin, survivin, versican, Sost-dc1	0.2454	
Wnt inhibitors	DKK1–4, SFRP1, SFRP3–5, Sost, Kremen1/2, Axin2	**0.0433**	Up in $ERα^{-/NERKI}$
Bmps	BMPR1A, BMPRII, Noggin, BMP1–7, CDKN1A	0.4678	
Bmp targets	Id1/2, Areb6, Lysyl Oxidase, Sox4, Smad6/7, TIEG	0.5614	
Notch Targets	Hey1, PPARG, NFkB1/2, CD44, NR4A2, Fra1	**0.0209**	Up in $ERα^{-/NERKI}$
Apoptosis	Fas, Fasl, Bad, Bax, Bcl2, Bcl-xL, Caspase 3, Caspase 8, p53	**0.0202**	Up in $ERα^{-/NERKI}$
Proliferation	CyclinA1, CyclinB1, CyclinB2, CyclinC, CyclinD1, CyclinD2, CyclinE1, CDK2, CDK6, E2F1	**0.0294**	Up in $ERα^{-/NERKI}$
Estrogen targets	PR, vWF, NFKB1A, Col18a1, Snai1, DHCR7, ARID4A, GPER, CyclinD1	**0.0194**	Down in $ERα^{-/NERKI}$
NFκB targets	IL1α, IL1β, TNFα, TNFβ, IL6, IL8 ICAM1, Angiotensinogen, IFNγ, CCL2	0.2454	
Endothelin-1 targets	ET1, IL11, CTGF, Nov, SGK, Timp3	0.6631	

Significant *P*-values for pathways differing between the ERα-transduced versus NERKI-transduced are highlighted in bold.

virally transduced ERα or NERKI (Fig. 4A). As expected, in the presence of ERα, Wnt signaling is stimulated 1.8-fold in the U2OS-Wnt10b cell line (Fig. 4B). Interestingly, the presence of the NERKI receptor completely abolished Wnt10b-dependent Wnt pathway activation. This demonstrates that NERKI blocks Wnt-dependent activation of TCF/LEF transcription factor binding sites, suggesting that NERKI may influence β-catenin function.

To test this possibility, we employed a Cre-dependent transfection system to activate ERα or NERKI expression in U2OS cells and total β-catenin protein levels were measured. Since the ERα and NERKI protein products in our adenoviral systems contained N-terminal Flag epitope tags, we tested both the untagged and tagged versions to verify that the tag did not influence receptor activity. Although the presence of ERα did not affect β-catenin protein levels, NERKI expression drastically destabilized β-catenin (Fig. 5A). The data were similar for both the untagged and tagged versions of the ERs, confirming no non-specific effects of the Flag-tag were evident.

Given previous reports that ERα and β-catenin proteins interact in vitro [Armstrong et al., 2007], we examined the colocalization patterns of both ERα and NERKI with β-catenin using immunofluoresence confocal microscopy. A FITC-conjugated secondary antibody was used for either ERα or NERKI (green), whereas a Texas Red-conjugated secondary antibody was used for β-catenin. Therefore colocalization would produce a yellowish/brown signal. We observed significant ERα and β-catenin colocalization in both the cytoplasm and nucleus (Fig. 5B, Upper Merge Panel). Strikingly, in the presence of the NERKI receptor the overall amount of β-catenin staining was decreased and little colocalization was observed. These data suggest that NERKI may antagonize Wnt signaling through the destabilization of β-catenin. Furthermore, since previous reports indicate that ERα and β-catenin function together to regulate transcription of estrogen- and Wnt-responsive genes [Kouzmenko et al., 2004], these data also suggest the NERKI suppresses Wnt signaling through its inability to properly interact with β-catenin.

DISCUSSION

Although considerable recent research has focused on the role of ERα in bone biology [Windahl et al., 1999, 2001; Sims et al., 2002;

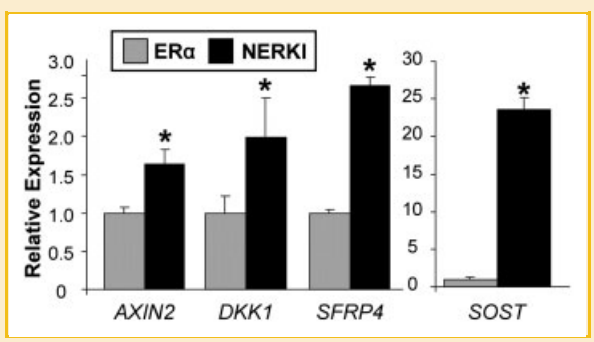

Fig. 3. NERKI expression modulates Wnt inhibitor gene expression. ER-negative U2OS cells were transduced with an adenovirus for either ERα or NERKI and QPCR analysis for Wnt-repressor genes was performed. The data are presented as the mean ± SE and an asterisk (*) represents statistical significance of $P \leq 0.05$ (Student's *t*-test).

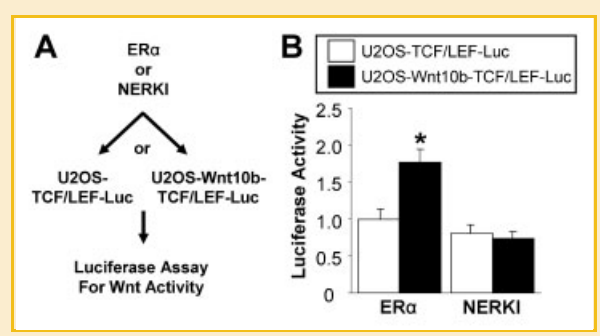

Fig. 4. NERKI expression suppresses Wnt10b-dependent activation of a stable Wnt-reporter construct. A: U2OS or U2OS-Wnt10b cells, which stably harbor a Wnt-responsive luciferase cassette (U2OS-TCF/LEF-Luc and U2OS-Wnt10b-TCF/LEF-Luc, respectively), were transduced with an adenovirus for either ERα or NERKI. B: Protein lysates were harvested 24 h later and assayed for luciferase activity. The data are presented as the mean ± SE and an asterisk (*) represents statistical significance of $P \leq 0.05$ (Student's *t*-test).

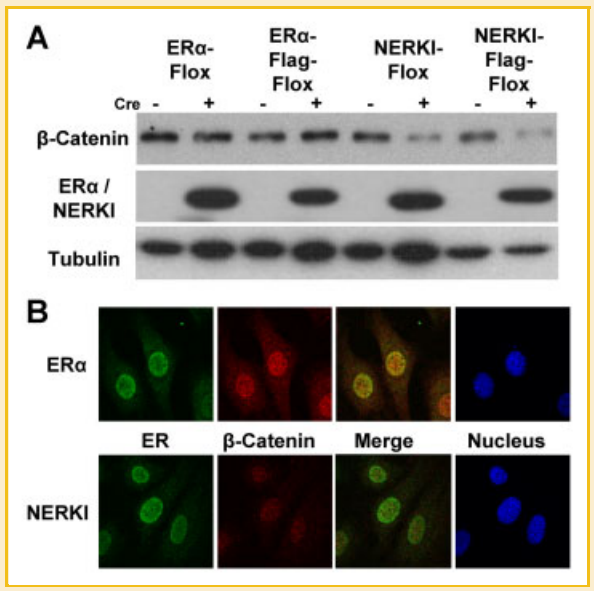

Fig. 5. NERKI destabilizes cellular β-catenin levels. A: U2OS cells were transiently transfected with the indicated Cre-dependent constructs in either the absence or presence of Cre to control expression of the construct. Equal amounts of protein lysates were subject to Western blot analysis using antibodies directed against β-catenin, Flag (to monitor ERα/NERKI gene activation), and tubulin. B: Cells were infected with adenoviruses for either ERα or NERKI in the presence of Wnt10b and subjected to immunofluorescent staining as described in Materials and Methods Section. DAPI counterstain was used to identify the nucleus.

Bonnelye and Aubin, 2005; Imai et al., 2009], the molecular mechanisms of how ERα influences the activity of bone cells are just beginning to be understood. Previously, we have demonstrated that mice expressing an ERα mutation which cannot bind DNA directly ($ERα^{-/NERKI}$) have impaired bone formation [Syed et al., 2005, 2007]. The present work provides evidence that the osteoporotic phenotype observed in $ERα^{-/NERKI}$ mice may be due to suppression of the Wnt pathway, which is widely accepted as a major anabolic regulator of bone. This contention is based the observation that $ERα^{-/NERKI}$ mice exhibit reduced levels of *Lef1*, a downstream transcriptional regulator of the canonical Wnt signaling pathway which functions in concert with β-catenin to regulate Wnt-dependent gene expression. Further evidence demonstrates that expression of NERKI increases expression of various secreted Wnt antagonists (*Axin2*, *Dkk1*, *Sfrp4*, and *Sost*), destabilizes β-catenin protein levels, and alters its colocalization with β-catenin.

Examination of the role of *Lef1* in bone metabolism in vivo has been hampered by the fact that $Lef1^{-/-}$ mice perish 1 week after birth due to multiple tissue abnormalities [van Genderen et al., 1994]. To circumvent this limitation, Noh et al. [2009] examined female $Lef1^{-/+}$ mice and observed both trabecular and cortical bone deficits. This phenotype is strikingly similar to the osteoporotic $ERα^{-/NERKI}$ phenotype where a 2.5-fold decrease in *Lef1* expression is observed. Furthermore, $ERα^{-/NERKI}$ mice bred into a Wnt-reporter genetic background exhibited decreased Wnt activity in vivo, as compared to wild-type mice bred into the same background. Collectively, these data suggest that impairments in Wnt signaling through misregulation of *Lef1* may explain, at least in part, the observed osteopenia in the $ERα^{-/NERKI}$ mouse model.

Modulation of the Wnt pathway is accomplished through the actions of specific Wnt activators and Wnt antagonists [reviewed in Monroe et al., 2012]. Several families of secreted Wnt inhibitors exist, which include the dickkopf (Dkk) family, secreted frizzled-related protein (Sfrp) family and sclerostin (Sost). Sfrp proteins antagonize Wnt signaling through the direct interference of Wnt-Frizzled (Fzd) interactions; whereas Dkk proteins and Sost bind and sequester Lrp5/6 and inhibit Wnts from associating with the Fzd/Lrp coreceptor complex. Either mechanism leads to a suppression of the Wnt signaling pathway. Our data demonstrate that expression of the Wnt antagonists *Axin2*, *Dkk1*, *Sfrp4*, and *Sost* is increased in cells expressing the NERKI receptor as compared to the wild-type ERα. Genetic ablation models (e.g., knockouts) of these genes generally exhibit increased bone mass [Balemans et al., 2002; MacDonald et al., 2004, 2007; Yu et al., 2005; Morvan et al., 2006; Krause et al., 2010] and transgenic overexpression of Dkk1, Sfrp4, and Sost exhibit decreased bone mass [Winkler et al., 2003; Loots et al., 2005; Li et al., 2006; Fleming et al., 2008; Nakanishi et al., 2008; Cho et al., 2010; Guo et al., 2010; Yao et al., 2011]. We also demonstrate that NERKI inhibits Wnt10b-dependent activation of the Wnt pathway. It is tempting to speculate that NERKI, through an unknown mechanism, stimulates the production of these potent Wnt inhibitors which in turn suppresses the Wnt signaling pathway in an autocrine or paracrine fashion. These data also suggest that this mechanism may account for the osteopenia observed in the $ERα^{-/NERKI}$ mouse model, however further experimentation is needed to directly test the effects of these Wnt inhibitors in vivo.

Activation of the canonical Wnt pathway involves β-catenin activation and nuclear translocation, leading to regulation of *Lef1*-dependent transcription. Therefore β-catenin represents an important control point for regulation of global Wnt pathway activity. Recent reports have demonstrated an interplay between the β-catenin and ER pathway since ERα is necessary for the proper nuclear translocation of β-catenin [Armstrong et al., 2007] and that ERα and β-catenin serve as reciprocal transcriptional coactivators [Kouzmenko et al., 2004]. More specific mechanistic details of ERα and β-catenin are lacking, however our data suggest that disruption of classical ERα signaling (e.g., direct ERα binding to estrogen response elements) drastically affects both the level of β-catenin protein and its colocalization with ERα within the nucleus. These observations could be possibly due to an improper (or non-existant) interaction between the NERKI and β-catenin proteins, and interference with the transcriptional potential of each protein on the other.

In summary, we provide evidence that the osteoporotic phenotype of the $ERα^{-/NERKI}$ mice is associated with suppression of the Wnt signaling cascade. The evidence supporting this model includes suppression of *Lef1* expression and global Wnt activity in the cortical bone of $ERα^{-/NERKI}$ mice, enhanced expression of several secreted Wnt antagonists, destabilization of β-catenin protein and mislocalization of the NERKI receptor and β-catenin. Collectively, these observations strongly suggest that alterations in the Wnt pathway contribute to the $ERα^{-/NERKI}$ skeletal phenotype

and ERα DNA binding is important for maintaining normal bone formation.

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