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The Evolutionarily Conserved Cassette Exon 7b Drives ERG's Oncogenic Properties

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Abstract

The oncogene *ERG* encodes an ETS family transcription factor and is implicated in blood, vascular, and bone development and in prostate, blood, and bone cancer. The *ERG* gene is alternatively spliced; of particular interest is its cassette exon 7b which adds 24 amino acids, in frame, to the transcriptional activation domain. Higher exon 7b inclusion rates are associated with increased cell proliferation and advanced prostate cancer. The 24 amino acids encoded by exon 7b show evolutionary conservation from humans to echinoderms, highlighting their functional importance. Throughout evolution, these 24 amino acids are encoded by a distinct short exon. Splice-switching oligonucleotides based on morpholino chemistry were designed to induce skipping of *ERG* exon 7b in MG63 osteosarcoma and VCaP prostate cancer cells. Induction of exon 7b skipping reduced cell proliferation and invasion, increased apoptosis *in vitro*, and reduced xenograft growth *in vivo*. We also show that *ERG*'s exon 7b is required for the induction of tissue nonspecific alkaline phosphatase. Together, these findings show that the evolutionarily conserved cassette exon 7b is central to *ERG*'s oncogenic properties.

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Introduction

The ETS-related gene (*ERG*), discovered in 1987, also known as the v-ets avian erythroblastosis virus E26 oncogene homolog, is a member of the highly conserved ETS family of transcription factors [1]. *ERG* is of fundamental importance in several developmental processes including hematopoiesis, chondrocyte maturation, and bone development and in apoptosis and cell migration [2]. The *ERG* gene is located on the q arm of chromosome 21 and expresses at least 30 splice variants [3,4]. Of particular interest are its cassette exons 7 and 7b; they are included in *ERG* transcripts at a higher rate in advanced prostate cancer [5,6]. Exon 7 encodes 27 amino acids and exon 7b encodes 24 amino acids, both in frame. These extra amino acids are added to the transcriptional activation domain (TAD) thought to influence the interaction of ERG with protein partners involved in transcriptional regulation [4]. Therefore, it is reasonable to assume that *ERG* splice isoforms that include or exclude the amino acids encoded by these cassette exons exhibit modified transcriptional activities and distinct biological functions.

Tissue nonspecific alkaline phosphatase (TNSALP) is key to securing an adequately mineralized bone matrix. Loss-of-function mutations in the *TNSALP* gene result in hypophosphatasia (HPP), and variants of the condition including perinatal HPP are lethal. A paucity of calcified collagen is a striking feature of HPP, similar to rickets and osteomalacia [7]. The clear similarities in the phenotypic presentation of HPP, rickets, and osteomalacia suggest a role for vitamin D3 in the regulation of *TNSALP* expression. The active metabolite of vitamin D3, calcitriol (1,25D), promotes the development of mature bone-forming osteoblasts in which *TNSALP* expression is increased. How 1,25D serves to control *TNSALP* expression by osteoblasts is poorly understood, but it is becoming clear that 1,25D requires signaling cooperation from selected growth factors including TGFβ [8], EGF [9], and the pleiotropic lipid

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mediator lysophosphatidic acid (LPA) and selected LPA analogues [10–12]. When the immature MG63 human osteoblast-like osteosarcoma cell line is co-stimulated with 1,25D and LPA, there is a demonstrable synergistic effect resulting in increased *TNSALP* expression. The fact that LPA and 1,25D can together enhance osteoblast maturation explains the effect of 1,25D on osteoblasts cultured in growth medium supplemented with serum, a rich source of LPA bound to the albumin fraction [13]. The potential role of ERG in TNSALP induction, and therefore in bone mineralization, of relevance to osteosarcoma and other cancers, has not yet been examined.

A critical role of *ERG* in bone and cartilage development and bone-related pathologies including bone cancer is rapidly emerging. A study carried out on chicken *ERG* reported that exon 7 is required for chondrocyte development. Skipping of this exon is prevalent in developing articular chondrocytes [14]. *ERG* lacking exon 7 maintains cells in an immature state and prevents maturation into hypertrophic cells and replacement of cartilage with bone, whereas expression of full-length ERG with exon 7 included promotes chondrocyte maturation. The role of the adjoining cassette exon 7b in chondrocyte development is not known. The *ERG* gene is also implicated in osteoarthritis. A murine model of osteoarthritis shows increased expression of *ERG* in articular cartilage, and treatment with a 1,25D analogue eldecalcitol increased its expression levels further [15]. Four weeks of histological assessment indicated a reduction in the progression of osteoarthritis correlated with increased *ERG* expression, suggesting that *ERG* contributes to resistance to osteoarthritis in the early stages of disease. The relevance of cassette exons 7 and 7b in the pathobiology of osteoarthritis is also not known.

In this study, splice-switching oligonucleotides (SSOs) were used to induce exon 7b skipping in the MG63 osteoblast-like cell line and for comparison also in the VCaP prostate cancer cell line. We show that ERG's exon 7b is involved in regulating cell proliferation and apoptosis and in invasion, consistent with its proposed oncogenic role [5,6]. Given the importance of bone formation in the progression of osteosarcoma (osteogenic sarcoma), we also set out to ascertain the potential involvement of the cassette exon 7b of the *ERG* oncogene in osteoblast maturation. *TNSALP* expression was induced in response to co-treatment with 1,25D and a phosphatase resistant analogue of LPA, (3S)1-fluoro-3-hydroxy-4-(oleoyloxy)butyl-1-phosphonate (FHBP), which we have previously shown enhances *TNSALP* expression [12,16]. We show that skipping of *ERG* exon 7b inhibits *TNSALP* induction, ascribing a new function to *ERG* and suggesting that *ERG* is also involved in bone mineralization control in a splice isoform-specific manner.

Materials and Methods

All materials were obtained from Sigma-Aldrich unless otherwise stated.

Cell Lines

MG63 (ECACC, human osteoblast-like osteosarcoma cells, catalogue no. 86051601) and VCaP cells (ECACC, human prostate cancer vertebral metastasis, catalogue no. 06020201) were grown in DMEM with 10% fetal bovine serum and 2 mM glutamine at 37°C in 5% CO_2 in a humidified incubator. Where serum starvation was required, cells were cultured in phenol red-free DMEM:F12 supplemented with 100× stock of essential amino acids (5 mL per 500 ml medium) and glutamine (final concentration 2 mM).

Vivo-Morpholinos

All vivo-morpholino SSOs were purchased from Gene Tools, LLC, USA. An SSO was designed against both the 5′ and 3′ splice sites of *ERG* exon 7b. The antisense sequence of the ERG exon 7b 5′ splice site SSO (E7b5) was 5′-TCCGGTCCATGCTTTTGTGGGGACA-3′, and for the ERG exon 7b 3′ splice site (E7b3), it was 5′-AAGGAAAACA GACGTCCCCCACGUC-3′. The sequence for the control SSO, targeting an intron in the β-globin gene variant associated with β-thalassemia, was 5′-CCTCTTACCTCATTACAATTTATA-3′. Stocks of each vivo-morpholino were prepared in sterile PBS at a concentration of 0.5 mM. Each SSO has an octaguanidine dendrimer moiety to facilitate delivery for cellular uptake and was added directly to media. For experiments with VCaPs, the transfection reagent endoporter (Gene Tools, LLC, USA) was used at 10 μM to facilitate uptake of SSOs.

RNA Extraction and cDNA Synthesis

Total RNA was extracted using the total RNA isolation mini kit (Agilent Technologies Ltd.). All samples were treated with DNAse on the columns using RNase-free DNase I provided in the kit. cDNA was synthesized from 0.2-1 μg of total RNA using 200 U MuLV reverse transcriptase (New England Biolabs), 40 U RNase inhibitor (human placenta) (New England Biolabs), 0.5 mM dNTP, 25 μM oligo-dT primers, and 10× reverse transcriptase buffer (500 mM Tris–HCl pH 8.3, 750 mM KCl, 30 mM $MgCl_2$, 100 mM DTT) (New England Biolabs) in a final reaction volume 20 μl with added nuclease-free water as required (Qiagen).

Semiquantitative Standard PCR and Gel Electrophoresis

Hot Start Taq 2× master mix (New England Biolabs) was used for standard PCR. Reactions were set up at room temperature in a final volume of 25 μl. The expression of *ERG* was measured using the primers listed in Table 1. The final concentration for each primer in the reaction was 0.4 μM. PCRs were run as follows: initial denaturation at 95°C for 30 seconds, then 30 cycles of 95°C for 30 seconds, 54°C for 1 minute, 68°C for 1 minute, and a final extension at 68°C for 5 minutes.

Gel electrophoresis was carried out using 1.5% agarose gels stained with 5 μl Midori Green Advance DNA stain (Geneflow) for every 100 ml of TAE. PCR products were loaded using purple 6× gel loading dye (New England Biolabs) and run at 150 V for the first 10 minutes then at 100 V until adequate migration was achieved. Gels were imaged on the Licor Odyssey Fc imaging system (Licor Limited). Splice isoform ratios were determined by measuring the relative brightness of PCR bands compared to each other using gel Image Studio Lite software (Licor Limited). Percent spliced in (PSI, ψ) was determined as a ratio of the intensity of the top band (exon included) to the total signal of both bands.

Table 1. Primers Used for RT-PCR and RT-qPCR

	Forward Primer (5′-3′)	Reverse Primer (5′-3′)
ERG	GAATATGGCCTTCCAGACGTCAAC	GGTGGCCGTGACCGGTCCAGGCTG
Primers for RT-qPCR are listed below		
TNSALP	CGTCGATTGCATCTCTGGGC	GTCTCTTGCGCTTGGTCTCG
U13397 (spike)	ACTCCGCTCAAGTGTTGAAG	GGTGGCTTGTAGGCAATGAA

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