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ANATOMICAL PATHOLOGY

Pathogenetic implications of early growth response 1 in Ewing sarcoma

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Summary

Ewing sarcoma (ES) is the second most common primary malignant bone tumour, mainly occurs in children and adolescents, and has an overwhelming mortality. Despite extensive studies, few effective oncogenic signals have been described. Therefore, it is crucial to exploit novel pathognomonic factors and targetable biomarkers for ES patients. Based on previous studies, we speculate that insulin-like growth factor 1 receptor (IGF1R), which is upregulated by early growth response 1 (EGR1), may play a pivotal role in strengthening the downward transmission of IGF1 cascades. Therefore, in this study, we concentrated on determining the pathogenetic contribution of EGR1 in diverse ES cells. This report is the first to study the pathogenic role of EGR1 in ES.

ES cells were cultured and transfected with Stealth RNAi human EGR1 small interfering RNA (siRNA) or negative control. Cell proliferation and invasion potential were measured. mRNA and protein expression of EGR1, IGF1R, and EWS-FLI1 also were assessed.

In all EGR1 siRNA-transfected cells (SK-ES-1, RD-ES, and HS863.T), cell proliferation and invasive potential decreased significantly in EGR1 siRNA-transfected ES cells. mRNA and protein expression for EGR1, IGF1R, and EWS-FLI1 were also significantly reduced.

In conclusion, EGR1 upregulated IGF1R expression and enhanced the expression of the oncogenic fusion protein EWS-FLI1. The EWS-FLI1/EGR1/IGF1R cascade combined with the previously confirmed pathways can form a speculative circuit, implicating positive feedback for tumourigenesis in ES. Therefore, EGR1 inhibitors are expected to be useful for the treatment of ES by preventing oncogenic IGF1/IGF1R expression.

Key words: Ewing sarcoma; EGR1; IGF1R; EWS-FLI1.

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INTRODUCTION

Ewing sarcoma (ES), the second-most common primary malignant bone tumour, mainly occurs in children and adolescents with a proclivity to white males.[1] It is also defined as Ewing sarcoma family tumour (ESFT), which includes extraskeletal ES and primitive neuroectodermal tumours (PNET). ES is overwhelmingly aggressive, with a rate of metastasis of 27% at the time of diagnosis.[1] Chemotherapy combined with intercalated locoregional treatments such as surgery and radiation is the accepted management strategy.[2] Recently, the survival rate of ES patients has improved due to the better elucidation of pathogenetic tumour biology and its application to treatment options. The 5-year survival rate increased from approximately 15% to 39% for patients with metastatic lesions, and from 44% to 68% for patients with localised lesions.[1] Therefore, it is essential to continue developing biomarkers and targetable biological markers in ES. Despite extensive studies, to date only a few useful oncogenic signals, including the insulin-like growth factor1 (IGF1) pathway, insulin-like growth factor-binding protein 3 (IGFBP3) downregulation, sonic hedgehog (SHH) signalling, and microsatellite-related signalling have been identified.[3] One of the most potent pathways in ES is the IGF1 pathway, which is activated by the EWS-FLI1 fusion protein. However, the exact mechanism underlying enhanced IGF1 signalling has not been elucidated.[4]

Based on previous studies, we speculated that IGF1R, which is upregulated by early growth response 1 (EGR1), may play a pivotal role in strengthening the downward transmission of IGF1 cascades. This was based on three findings: (1) the oncogenic EWS-FLI1 fusion protein contributes to *EGR1* gene expression by binding to its promoter;[5] (2) upregulated EGR1 directly adheres to a promoter site in the 5′-untranslated region (UTR) of *IGF1R* gene and increases IGF1R protein;[6,7] and (3) enhanced IGF1R can help transmit downward cascades for IGF1 more effectively through RAS or AKT/mTOR, promoting tumour cell survival and proliferation.[4]

Based on this evidence, EGR1 expression may be related to the pathogenesis of ES by connecting the oncogenic EWS-FLI1 protein and IGF1R overexpression, leading to increased downstream signalling after IGF1 stimulation. Here, we focused on determining the pathogenetic contribution of EGR1 in diverse ES cells. This report is the first to investigate the pathogenetic role of EGR1 in ES.

MATERIALS AND METHODS

Cell culture

The cells (fibroblasts, ATCC CCL-110; SK-ES-1, ATCC HTB-86; RD-ES, ATCC HTB-166; HS863.T, ATCC CRL-7598; American Type Culture Collection, USA) were cultured in RPMI1640 medium (Gibco BRL, USA) supplemented with antibiotics (100 U/mL penicillin A and 100 U/mL streptomycin) and 10% heat-inactivated fetal bovine serum (FBS; Sigma Chemical, USA), and were maintained at 37°C in 5% CO_2 humidified air.

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Table 1 Primer sequences

Genes	Accession no.	Forward primer	Reverse primer	Size (bp)
GAPDH	NM_002046.3	5′-GCT CTC TGC TCC TCC TGT TC-3′	5′-ACG ACC AAA TCC GTT GAC TC-3′	115
EGR1	NM_001964.2	5′-AGCCCTACGAGCACCTGAC-3′	5′-GGGCAGTCGAGTGGTTTG-3′	104
IGF1R	NM_000875.3	5′-GGGAGAGAGCCTCCTGTGA-3′	5′-TGATGATGCGATTCTTCGAC-3′	72
EWS-FLI1	AF327066.1	5′-GGGCACAAACGATCAGTAAGA-3′	5′-GCTAGGCGACTGCTGGTC-3′	85

siRNA transfection

Three Stealth small interfering RNAs (siRNAs) for human EGR1 and Control Stealth RNAi were obtained through the BLOCK-iT RNAi design program (Invitrogen, USA). Transfection was performed using a Gene Pulser Xcell Electroporation System (Bio-Rad Laboratories, USA). The sequences used for siRNA targeting of EGR1 were 5′-UCUCCCAGGACAAUU-GAAAUUUGCU-3′ and 5′-AGCAAAUUUCAAUUGUCCUGGGAGA-3′.

Cell viability assay

Viability of EGR1 siRNA-transfected cells was quantified using the Cell Titer 96 Aqueous One solution cell proliferation assay kit (Promega Corporation, USA). Cells were plated in 96-well tissue culture plates at a density of 1×10^4 cells per well and cultured for 1, 2, 3, or 4 days. After the cells were cultured, Cell Titer 96 Aqueous One solution (Promega) was added and absorbance was measured at 490 nm after 1 h.

Cell invasion assay

For invasion assays, cells were transfected with EGR1 siRNA or negative control and seeded in 24-well BD BioCoat Matrigel Invasion Chambers (BD Biosciences Pharmingen, USA) according to the manufacturer's protocol. The cells were placed in the insert of the chamber, and medium containing 10% FBS was dispensed into the lower chamber. After 24 h, the cells that adhered to the lower surface of the insert were stained (Diff Quik; Polysciences, USA) and counted.

Real-time PCR

Total RNA was isolated from cells using the NucleoSpin RNA isolation kit (Macherey-Nagel, Germany). cDNA was synthesised using a Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Germany) and stored at −70°C until further use. Real-time polymerase chain reaction (PCR) was performed to verify the differential expression of selected genes using a Roche LightCycler 480 system and the TaqMan method using a Roche Universal Probe Library (UPL) kit. Relative gene expression was determined by employing the comparative CT method. All reactions were carried out in a total reaction volume of 20 µL, which contained 10.0 µL 2 × UPL master mix, 1.0 µL 5′ primer (10 pmol/µL), 1.0 µL 3′ primer (10 pmol/mL), 0.2 µL UPL probe, 1.0 µL cDNA, and 6.8 µL sterile water. The thermal cycling conditions for PCR were an initial denaturation for 10 min at 95°C, followed by 40 cycles of 94°C for 10 s and 60°C for 30 s. The primers summarised in Table 1 were designed using the Roche ProbeFinder assay tool.

Western blot analysis

Transfected cells were lysed in a RIPA buffer (Rockland, USA). After homogenising the cells with sonication, cells were centrifuged at 13,000 × g, and the supernatants were used for western blot analysis. Target proteins are EGR1 (rabbit mAb, cat. no. 4153, 1:1,000; Cell Signaling Technology, USA), IGF1 (rabbit mAb, cat. no. 3027, 1:1,000; Cell Signaling), EWS-FLI1 (rabbit mAb, cat. no. 35980, 1:1,000; Cell Signaling) and α-tubulin (rabbit Ab, cat. no. 600-401-880, 1:3,000; Rockland) and after the primary antibody is bound to the target protein, a complex with HRP-linked secondary antibody (rabbit IgG, HRP-linked antibody, cat. no. 7074, 1:1,000; Cell Signaling Technology) is formed.

RESULTS

Cell viability assay

To examine the effect of EGR1 siRNA on cell viability (proliferation), three ES cell lines (SK-ES-1, RD-ES, and HS863.T) were transiently transfected with EGR1 siRNA. On the 1st, 2nd, 3rd, and 4th days, proliferation was significantly inhibited in EGR1 siRNA-transfected fibroblast and HS863.T cells compared with negative controls (fibroblast 15.8% reduction, $p<0.001$; HS863.T 11.8% reduction, $p<0.001$) (Fig. 1). Although there was a trend toward

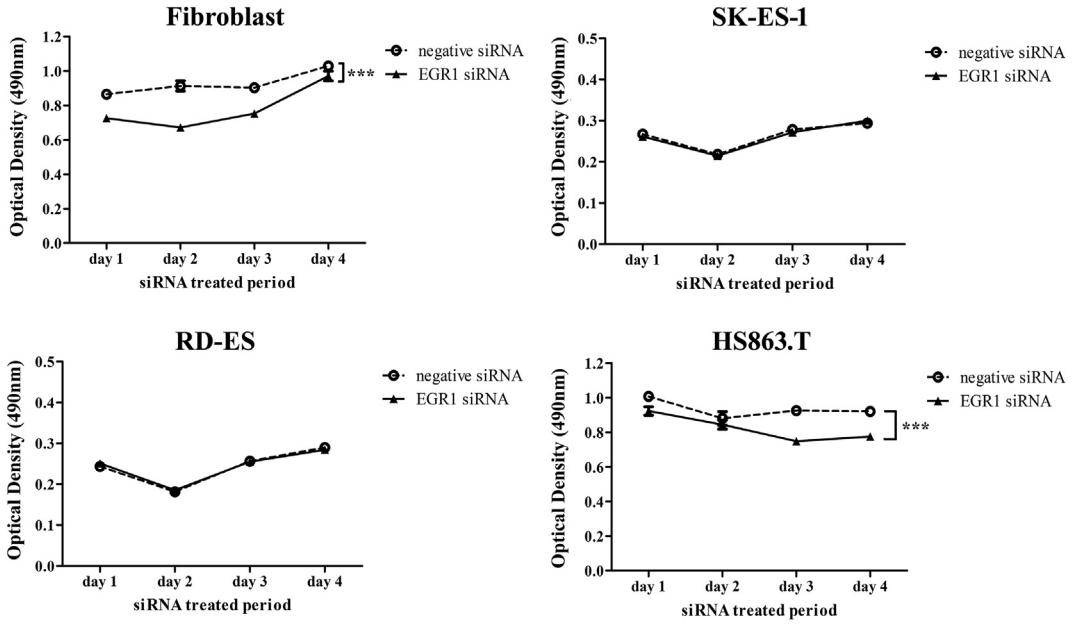

Fig. 1 Cell viability assay. In EGR1 siRNA-transfected fibroblast and HS863.T, cell viability decreases significantly compared with negative controls (fibroblast 15.8% reduction, $p<0.001$; HS863.T 11.8% reduction, $p<0.001$). There was no significant difference in the other two cell lines (SK-ES-1; RD-ES).

decreased proliferation in the other two cell lines (SK-ES-1; RD-ES), this difference was not statistically significant.

Cell invasion assay

To clarify the role of EGR1 regarding the invasive behaviour of ES, we performed migration and invasion assays with ES cell lines transfected with EGR1 siRNA. Three EGR1 siRNA-transfected ES cell lines showed reduced migration (SK-ES-1 32.7%; RD-ES 30.0%; HS863.T 25.8%; cell numbers were corrected for differences in proliferation upon EGR1 siRNA transfection) in two independent experiments (Fig. 2), and significantly reduced invasion was observed in SK-ES-1 (p=0.008) and HS863.T (p=0.013) cells compared with cells transfected with the negative control. There was no significant difference in invasiveness in the fibroblast cell line.

mRNA expression of *EGR1*, *IGF1R*, and *EWS-FLI1* genes

To identify differentially expressed mRNAs of EGR1 siRNA-transfected ES cell lines (SK-ES-1, RD-ES, and HS863.T), total RNA was isolated, and cDNA was synthesised, followed by real-time PCR. By employing the comparative CT method (Fig. 3), EGR1 mRNA expression was significantly reduced in all three EGR1 siRNA-transfected cell lines compared to the cells transfected with the negative control (fibroblast 25.5%, p=0.013; SK-ES-1 50.0%, p<0.001; RD-ES 52.5%, p<0.001; HS863.T 71.5%, p<0.001). IGF1R mRNA expression was also substantially lower in siRNA-transfected cells compared with negative controls (fibroblast 25.5%, p=0.012; SK-ES-1 80.5%, p=0.018; RD-ES 27.1%, p<0.001; HS863.T 60.5%, p<0.001). Notably, a significant abatement of EWS-FLI1 mRNA was indicated (fibroblast 30%, p=0.006; SK-ES-1 54.5%, p=0.015; RD-ES 98.9%, p<0.001; HS863.T 69.4%, p<0.001).

Western blotting of EGR1, IGF1R, and EWS-FLI1 proteins

Expression of the EGR1, IGF1R, and EWS-FLI1 fusion proteins was assessed by western blot analysis. All of the proteins were readily detected 36 h after EGR1 siRNA transfection. Densitometric analysis revealed that EGR1 and EWS-FLI1 were significantly decreased (EGR1 64.2%, p=0.05; EWS-FLI1 62.8%, p=0.016) in all three EGR1

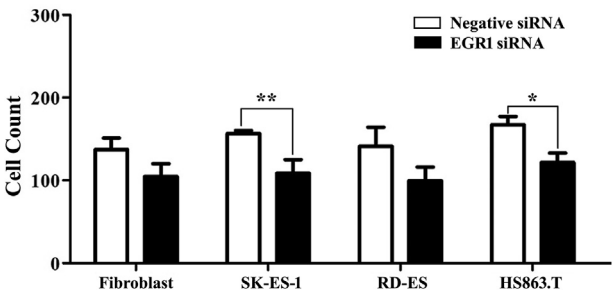

Fig. 2 Cell invasion assay. All four early growth response 1 (EGR1) small interfering RNA (siRNA)-transfected cell lines showed reduced migration and invasion potential in two independent experiments. Statistically significant reductions were observed in SK-ES-1 and HS863.T cells transfected with EGR1 siRNA compared with the cells transfected with negative control siRNA.

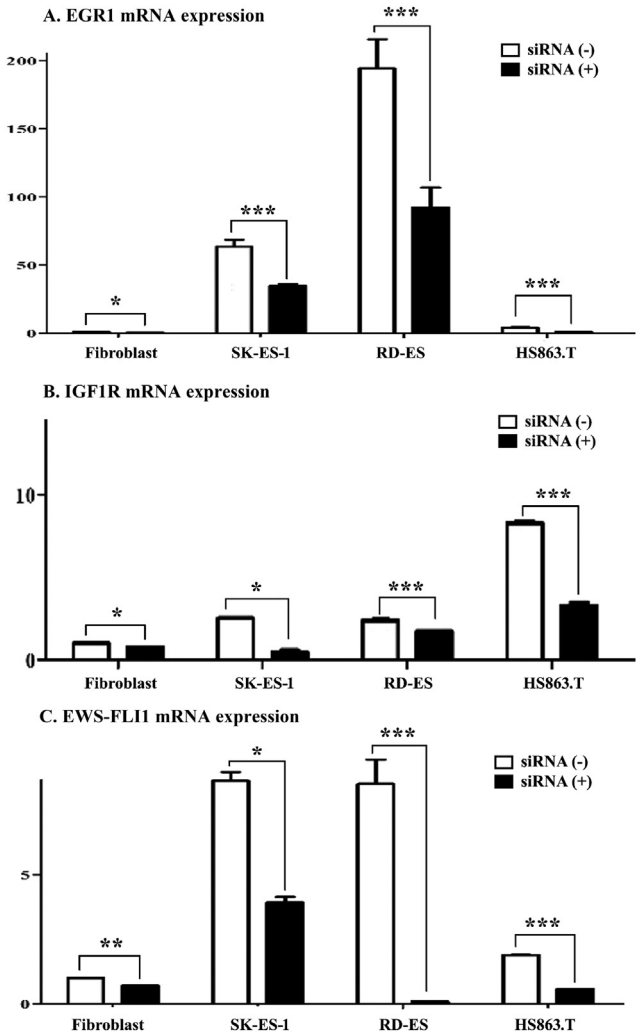

Fig. 3 mRNA expression. The comparative CT method demonstrated that EGR1, insulin-like growth factor 1 receptor (IGF1R), and EWS-FLI1 mRNA expression was significantly decreased in all three EGR1 siRNA-transfected cell lines compared to the cells transfected with negative control siRNA.

siRNA-transfected cell lines, whereas IGF1R showed a tendency toward decreased expression (70.8%, p=0.078) (Fig. 4).

Statistical analysis

Results are presented as the reduction rate (%) in EGR1 siRNA-transfected cells compared to negative controls. Statistical analyses were conducted using SPSS version 12.0. *In vitro* and cell biology data were analysed by one- or two-way analysis of variance (ANOVA) for comparison between groups. Statistical significance was defined as a p value less than 0.05 (***p<0.001; **p<0.01; *p<0.05).

DISCUSSION

The *early growth response 1 (EGR1)* gene, which is located on chromosome 5q31.2 and encodes a protein containing 543 amino acids (predicted molecular weight 57.5 kDa), is an early response transcription factor that activates or suppresses diverse genes depending on the tumour cell type and stimuli. EGR1 expression is regulated by growth factors, cytokines, and DNA damage, as well as environmental and mechanical

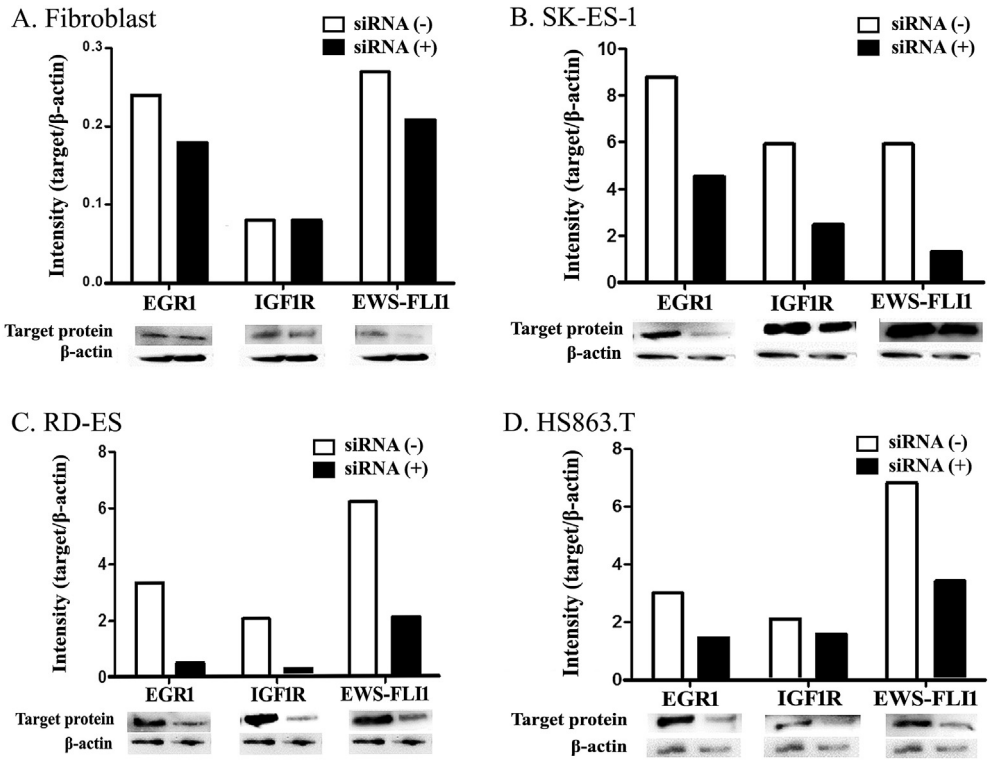

Fig. 4 Western blotting. Densitometric analysis revealed that EGR1 and EWS-FLI1 protein expression were significantly reduced in all three EGR1 siRNA-transfected cell lines, whereas IGF1R expression showed a tendency toward decrease.

stimuli. EGR1 expression also enhances or suppresses the expression of diverse growth factors, growth factor receptors, and cell growth- or differentiation-related proteins, as well as proteins involved in cell apoptosis, growth arrest, and stress responses.[8,9]

In tumour cells, accumulating evidence suggests that EGR1 can function as a tumour suppressor and a tumour promoter, depending on the context. EGR1 achieves a delicate balance by directly binding to promoters of tumour suppressor genes such as TGF-β1, PTEN, and p53, as well as tumour growth-related genes such as fibronectin.[10] The described pathogenic mechanisms by which EGR1 reinforces tumour cell growth include disrupting tumour suppressor function or enhancing tumour promoter function of EGR1 as follows in detail: (1) the tumour suppressor function of EGR1 is impeded by inactivation of PTEN or TP53, which frequently occur in malignant cells, culminating in prolonged tumour cell survival; (2) ELK-1 or mitogen-mediated EGR1 induction can be prevented through the mutational activation of WNT-1 or phosphoinositide 3-kinase (PI3K) activation, circumventing the tumour suppressor function of EGR1;[11,12] and (3) EGR1 overexpression via constitutive activation of ERK promotes tumour growth and progression by canonically augmenting the expression and secretion of diverse growth factors.[13–15]

The regulatory promoter sites of *EGR1* gene include serum response elements (SREs), E26 transformation-specific (ETS)-binding sites (EBSs), AP1-binding sites, and cAMP-response elements (CREs), which are directly bound by serum response factors (SRFs), ETS family proteins, activator protein 1 (AP-1) complex, and cAMP-response element binding protein (CREB), respectively.[5] Watson *et al.* demonstrated that FLI1 and EWS-FLI1 canonically adhered to the EBSs of the *EGR1* promoters as ternary complex factors.[16] Several previous studies have shown that EGR1 expression plays a pivotal role in IGF1R expression during prostatic tumour growth and vascular neointima formation by directly binding to the 5′-UTR of the *IGF1R* gene.[6,7] Toretsky *et al.* showed that the expression of the EWS-FLI1 fusion protein strengthened the IGFIR signalling pathway to be more oncogenic.[17] Following this logic, we hypothesised that the EWS-FLI1/EGR1/IGF1R pathway may be important in ES, and verified the presence of this signal in ES cell lines. Our study is the first to investigate EGR1 in ES. We showed that EGR1 upregulates IGF1R and EWS-FLI1 expression.

After EGR1 siRNA transfection, less effective decreases for cell viability were demonstrated compared with significant reductions for cell invasion and mRNA and protein expression. This discrepancy can reveal that EGR1 expression plays a more effective role in reducing biological aggressiveness rather than tumour cell survival. Patent pathogenetic diversity for ES tumour cells can give rise to different cell viability between ES cell lines. EGR1 siRNA transfection significantly reduced EWS-FLI1 mRNA and protein expression. Although the exact mechanism remains to be further elucidated, speculative explanations are as follows: (1) reduced positive feedback mechanism interrupts ternary complex formation which binds to the EGR1 promoter (Fig. 5); (2) decreased expression of EGR1 elicits loss of EWS-FLI1-binding sites; (3) diminished ternary complex formation and loss of EWS-FLI1-binding EGR1 promoter can increase isolation of EWS-FLI1 protein, which is more susceptible to protein degradation.

The EWS-ETS fusion protein provides an oncogenic stimulus that transforms primary cells, thus initiating ES

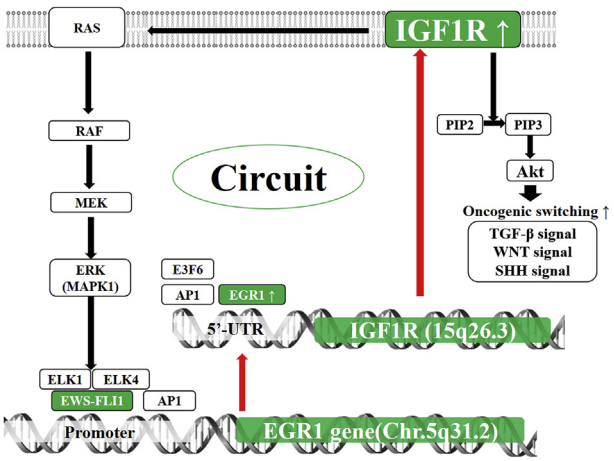

Fig. 5 A speculative relationship diagram for EGR1, IGF1R, and EWS-FLI1 fusion. A circuit combining the EWS-FLI1/EGR1/IFG1R cascade (red arrows) with the previously confirmed pathways (black arrows; not included in our study), representing EWS-FLI1, EGR1, and IGF1R interactions, and its pathogenetic implications in ES.

pathogenesis. Despite extensive characterisation of the EWS-ETS chimaeric protein function in ES, only a few pathogenetic signals are known in ES.[18] One of these signals is IGF1, which is a common target of the EWS-ETS fusion protein. IGF1 is a key growth factor for tumour cell growth in ES.[4] IGF1 binds to IGF1R and boosts downstream RAS and AKT/mTOR, which are implicated in tumour cell survival and proliferation. Clues gleaned from some studies demonstrated that AKT/mTOR switches TGF-β, WNT, and sonic hedgehog (SHH) signals to become more oncogenic.[19–21] Therefore, the IGF1 signal plays a fundamental role in tumourigenesis in ES. Pioneering targetable inhibitors for this IGF1 signal is critically important and helpful in the management of ES patients. In the future, EGR1 inhibitors may be candidate targets to block oncogenic IGF1/IGF1R signalling in ES.

The speculative relationship in ES is illustrated in Fig. 5. Combining the EWS-FLI1/EGR1/IFG1R cascade with the previously confirmed IGF1R/RAS/RAF/MEK/ERK pathways that were not included for our research, one circuit was found that speculates on an association with tumourigenesis in ES. This study was an initial attempt to determine the pathogenic role of EGR1 in ES cells lines. Additional *in vitro* and *in vivo* experiments will be required to elucidate the protein interactions in detail.

The summarised clinicopathological implications for EGR1 in ES include: (1) EGR1 can upregulate IGF1R expression, increasing oncogenic cascades by means of augmenting IGF1 downstream signalling (upregulated RAS and AKT/mTOR) and promoting AKT-related oncogenic switching of TGF-β, WNT, and SHH pathways; (2) EGR1 can enhance oncogenic fusion protein EWS-FLI1 expression, although the exact mechanism remains to be elucidated; (3) the discovered EWS-FLI1/EGR1/IGF1R cascade and previously confirmed pathways can form a speculative circuit, implicating positive feedback for tumourigenesis in ES; and (4) EGR1 inhibitors may be a useful target for ES treatment due to their potential in preventing oncogenic IGF1/IGF1R signalling.

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