

5) Endothelin (ET)/ET receptor signaling

The binding of ET to the ET receptors exerts pleiotropic biological effects that influence cell survival, proliferation, invasion, metastasis, as well as angiogenesis (65). In a preclinical model of spontaneous melanoma brain metastasis, Cruz-Muñoz *et al.* have identified the alterations in the expression of ET receptor as a potential factor that influences brain metastatic potential. Induced overexpression of this gene mediated enhanced overall metastatic disease, and resulted in an increased incidence of spontaneous brain metastases (66). We recently demonstrated that the blockade of ET receptor significantly inhibited experimental brain metastases of human NSCLC cells (67).

7. MicroRNA (miRNA)

Mounting evidence indicates that miRNA may be key players in the regulation of tumor cell invasion and metastasis. Chen *et al.* (68) found miR-378 was significantly differentially expressed in the matched NSCLC surgical specimens from 8 patients with brain metastases and 21 without brain metastases. Arora *et al.* (69) reported that miR-328 had a role in conferring migratory potential to NSCLC cells, which might be incorporated into clinical treatment decision making to stratify NSCLC patients at higher risk for brain metastases.

8. Single nucleotide polymorphisms (SNPs)

Studying multiple SNPs in signaling pathways may be useful for pinpointing the genes and polymorphisms involved in conferring risk of brain metastases (70). Multivariate analyses of 33 SNPs from 13 genes in the transforming growth factor- β (TGF- β) signaling pathway have revealed that the GG genotype of SMAD6 : rs12913975 and TT genotype of INHBC : rs4760259 were associated with a significantly higher incidence of brain metastasis in patients with NSCLC at 24 months follow-up, compared with the GA or CT/CC genotypes, respectively (71). In melanoma, TGF- β 2 was reported to be crucial, since its expression is indispensable for the metastasis formation in the brain parenchyma (72).

9. Circulating markers

1) Tumor markers

Tumor markers may be helpful in the prediction of brain metastases. Among them, carcinoembryonic antigen (CEA) is the most widely studied. Lee *et al.* found that the pretreatment serum CEA level was

significantly correlated with brain metastases in 227 advanced NSCLC patients (73). Arrieta *et al.* also reported that high serum CEA level at diagnosis is an independent prognostic factor of CNS metastasis development and survival in patients with advanced NSCLC (74). They considered that surface expression of CEA in tumor cells could be a mechanism of invasion to CNS through immunoglobulin-related transport in BBB.

Pro-gastrin-releasing peptide (ProGRP) is a widely used tumor marker for the screening of SCLC. Yonemori *et al.* retrospectively analyzed the characteristics of the first failure event due to brain metastasis in SCLC patients treated with prophylactic cranial irradiation (PCI). Elevation of ProGRP level before PCI was found to be a significant predictive factor for brain metastasis on multivariate analysis (75).

2) Indicators of CNS injury in the blood

Elevated levels of certain proteins or neurotransmitters in the blood may be indicators of CNS damage caused by invasion of brain metastases (70). S100 β is a nervous system specific cytoplasmic protein found in astrocytes and is released into serum when the BBB is breached (76). However, a confounding factor was the presence of BBB changes due to cerebrovascular disease. Therefore, patients who are found to have evidence of chronic cerebrovascular disease will likely receive no further benefit from routine screening of their serum S100 β level. More recently, proapolipoprotein A1, the precursor of the cholesterol-binding protein apolipoprotein A1, was reported to be significantly increased in patients with CNS disease compared with those affected only by vascular diseases (77).

10. Role of astrocytes in brain metastasis formation

Astrocytes have an indispensable role in the maintenance of BBB properties of cerebral endothelial cells. Therefore, they support endothelial cells in impeding tumor cells from penetrating into the brain. On the other hand, astrocytes have a protective role for brain metastases. Reactive astrocytes induce the protection of tumor cells from chemotherapy through sequestration of calcium from the cytoplasm of tumor cells and by up-regulating survival genes in tumor cells (78, 79). Moreover, astrocytes secrete soluble factors that stimulate the proliferation of tumor cells in the brain microenvironment. In addition, astrocytes were shown to induce proliferation of lung and breast cancer cells by producing interleukin (IL)-1 β , tumor necrosis factor- α

(TNF- α) and/or IL-6 (80, 81). These proinflammatory cytokines secreted by astrocytes might not only induce proliferation of tumor cells, but also support the transendothelial migration and formation of new metastatic colonies in the brain.

IV. TARGETED THERAPIES FOR LUNG CANCER BRAIN METASTASIS

In the study by Kienast *et al.*, the VEGF-A inhibitor bevacizumab blocked angiogenesis and resulted in dormancy of brain metastasis derived from lung cancer cells (82), indicating that anti-angiogenic agents might be promising to inhibit brain metastasis. In early clinical studies, hemorrhagic episodes were reported after treatment with bevacizumab in patients with metastatic spread, including brain metastasis (83). In consequence, guidelines prohibited the use of bevacizumab in this cohort and patients with brain metastasis have been excluded from participating in clinical trials that investigated anti-angiogenic drugs (84, 85). However, recent large meta-analyses performed in over 10,000 patients that received anti-angiogenic agents revealed that these drugs do not increase the risk of intracranial bleeding compared to the untreated population with brain metastasis (0.8-3.3% bleeding risk) (85). These reports have prompted to change the guidelines and allow administration of bevacizumab in patients with brain metastasis from non-squamous NSCLC (84). Based on the new guidelines, the decision to use anti-angiogenics in this patient cohort should be made upon careful assessment of the potential benefits and risks for individual patients (85).

The EGFR tyrosine kinase inhibitors (EGFR-TKIs), gefitinib and erlotinib, have been tested in patients with NSCLC and brain metastasis (50, 86, 87). Similar to primary tumors, the response of brain metastasis to EGFR inhibitors is better in patients with activating EGFR mutations while the activity of these drugs in individuals with wild-type EGFR metastatic disease is very modest (50, 86, 87). Interestingly, the response of chemotherapy-naïve, never-smoker patients with brain metastases after treatment with erlotinib and gefitinib was 74%, and maybe inhibition of EGFR in this patient subgroup is more effective compared to other cohorts (88). The BBB penetrability of erlotinib might be better than gefitinib as small but measurable penetration of erlotinib into cerebrospinal fluid (CSF) has been documented (89). Notably, the incidence of CNS

progression after treatment with gefitinib or erlotinib was lower in NSCLC patients with EGFR mutations compared to patients with wild-type EGFR and therefore these targeted agents might also have a value as prophylactic agents (52). Furthermore, the resistance EGFR mutation T790M occurs also in CNS metastasis. Whether afatinib, a second generation EGFR inhibitor that inhibits T790M also has activity in CNS metastasis with this mutation remains to be determined. In one report, encouragingly, dose escalation of afatinib resulted in remission of a brain metastasis (90). The question whether erlotinib or gefitinib should be combined with other WBRT or stereotactic radiosurgery is subject of current studies.

Activation of ALK oncogene leads to fusion of ALK and the echinoderm microtubule-associated protein-like 4 (EML4) that is encountered in approximately 4% of patients with NSCLC. The targeted agent crizotinib inhibits this oncogenic fusion and can lead to effective local tumor control (91). In a recent case report of a patient with EML4-ALK fusion, though, brain metastasis developed despite control of extracerebral metastases (92). Very low levels of crizotinib were detected in plasma and CSF of the patient, suggesting insufficient BBB penetrability for this agent (92). However, another recent case report demonstrated a twelve-month progression-free survival after treatment with crizotinib in a patient with lung adenocarcinoma with EML4-ALK fusion that developed metachronous miliary lung metastases and brain metastasis (93).

V. CONCLUSION

Brain metastasis has become an increasingly challenging clinical problem, largely due to the recently improved clinical control of systemic metastatic diseases. While the biology of brain metastasis is still poorly understood, it is encouraging to see more efforts are beginning to be directed toward the study of brain metastasis.

The biomarkers mentioned in this review would be promising tools for the prediction of brain metastases. However, currently none of them can predict occurrence of brain metastases alone and much challenge remain for their translation into practice. In this article, the mechanistic basis of lung cancer metastasis to the brain is described (Table 1). The better understanding of molecular biology of lung cancer brain metastasis, including heterogeneous

Table 1. Risk factors for the development of lung cancer brain metastasis.

Risk factors	Authors	Reference No.
Genes		
high CDH2 expression	Grinberg-Rashi H <i>et al.</i>	20
high KIFC1 expression	Grinberg-Rashi H <i>et al.</i>	20
low FALZ gene expression	Grinberg-Rashi H <i>et al.</i>	20
high DCUN1D1 expression	Yoo <i>et al.</i>	23
Cell surface molecules		
high $\alpha 3\beta 1$ integrin expression	Yoshimasu T <i>et al.</i>	28
high ICAM-1 expression	Sipos E <i>et al.</i>	30
high VCAM-1 expression	Sipos E <i>et al.</i>	30
low E-cadherin expression	Yoo JY <i>et al.</i>	32
	Saad AG <i>et al.</i>	9
high N-cadherin expression	Grinberg-Rashi H <i>et al.</i>	20
Soluble factors		
high VEGF expression	Saad AG <i>et al.</i>	9
	Yano S <i>et al.</i>	33
	Hu L <i>et al.</i>	43
	Kienast Y <i>et al.</i>	82
high PlGF expression	Li B <i>et al.</i>	35
high CXCR4 expression	Salmaggi A <i>et al.</i>	40
	Paratore S <i>et al.</i>	41
high CXCL12 expression	Salmaggi A <i>et al.</i>	40
	Paratore S <i>et al.</i>	41
Proteases		
high MMP-9 expression	Hu L <i>et al.</i>	43
high ADAM9 expression	Shintani Y <i>et al.</i>	44
Driver mutations		
EGFR mutation	Li Z <i>et al.</i>	49
	Eichler AF <i>et al.</i>	50
ALK translocation	Yang P <i>et al.</i>	55
Signaling pathways		
activation of WNT/TCF signaling	Nguyen DX <i>et al.</i>	57
activation of Rho/ROCK signaling	Li B <i>et al.</i>	61
activation of HGF/Met signaling	Navab R <i>et al.</i>	62
	Benedettini E <i>et al.</i>	63
Circulating markers		
CEA elevation	Lee DS <i>et al.</i>	73
	Arrieta O <i>et al.</i>	74
ProGRP elevation	Yonemori K <i>et al.</i>	75
Others		
high Amphiregulin expression	Sun M <i>et al.</i>	
low Caspase-3 expression	Saad AG <i>et al.</i>	
high Caveolin-1 expression	Cassoni P <i>et al.</i>	
low CD44 expression	Kergi HA <i>et al.</i>	
high EGF expression	Sun M <i>et al.</i>	
high ERCC1 expression	Gomez-Roca C <i>et al.</i>	
high IGF-1 expression	Hwang CC <i>et al.</i>	
high Ki-67 levels	Saad AG <i>et al.</i>	
low Neuregulin1 expression	Sun M <i>et al.</i>	
inactivating mutation of PTEN	Hahn M <i>et al.</i>	
high S100A7 expression	Zhang H <i>et al.</i>	
high phosphorylated Her3 expression	Sun M <i>et al.</i>	
high phospho-S6 expression	McDonald JM <i>et al.</i>	
low TGF- α expression	Sun M <i>et al.</i>	

genetic profiles, is essential to find appropriate targets of prevention of brain metastasis formation. In addition, prospective randomized clinical studies are

needed to further assess the utility of these biological markers.

It is indisputable that the microenvironment cells

in the tumor stroma contribute significantly to the outgrowth of cancer cells both at the primary site and in distant metastatic organs. The occurrence of brain metastasis reflects the culmination of such tumor-microenvironment interactions. Particularly, the specialized physiology of the brain not only contributes to the colonization of metastatic tumor lesions but also significantly affects the efficacy and outcome of therapeutic interventions. Future clinical interventions to treat patients with brain metastasis must take into consideration the impact of these important microenvironmental determinants.

DISCLOSURE OF CONFLICT OF INTERESTS

All authors have no conflict of interests.

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Early Growth Response 4 Is Involved in Cell Proliferation of Small Cell Lung Cancer through Transcriptional Activation of Its Downstream Genes

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Abstract

Small cell lung cancer (SCLC) is aggressive, with rapid growth and frequent bone metastasis; however, its detailed molecular mechanism remains poorly understood. Here, we report the critical role of early growth factor 4 (EGR4), a DNA-binding, zinc-finger transcription factor, in cell proliferation of SCLC. EGR4 overexpression in HEK293T cells conferred significant upregulation of specific splice variants of the parathyroid hormone-related protein (*PTHrP*) gene, resulting in enhancement of the secretion of PTHrP protein, a known mediator of osteolytic bone metastasis. More importantly, depletion of *EGR4* expression by siRNA significantly suppressed growth of the SCLC cell lines, SBC-5, SBC-3 and NCI-H1048. On the other hand, introduction of *EGR4* into NIH3T3 cells significantly enhanced cell growth. We identified four *EGR4* target genes, *SAMD5*, *RAB15*, *SYNPO* and *DLX5*, which were the most significantly downregulated genes upon depletion of *EGR4* expression in all of the SCLC cells examined, and demonstrated the direct recruitment of EGR4 to their promoters by ChIP and luciferase reporter analysis. Notably, knockdown of the expression of these genes by siRNA remarkably suppressed the growth of all the SCLC cells. Taken together, our findings suggest that EGR4 likely regulates the bone metastasis and proliferation of SCLC cells via transcriptional regulation of several target genes, and may therefore be a promising target for the development of anticancer drugs for SCLC patients.

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Introduction

Lung cancer is one of the most common cancers, and its incidence is rising worldwide [1]. The high mortality and poor prognosis of lung cancer result from difficulties in early diagnosis and its high metastatic potential. Lung cancer is classified into two major types, small-cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), which account for approximately 25% and 75% of cases, respectively. SCLC presents with aggressive clinical behavior characterized by rapid growth and frequent metastases to the brain, lung, liver and bone [2]. In particular, bone metastasis causes severe complications in SCLC and can lead to bone pain, pathological fractures, hypercalcemia, spinal cord compression and other nerve compression syndromes [3,4], and it is often associated with high morbidity and poor prognosis. Current treatments are generally palliative. Therefore, it is highly important to prevent and treat osteolytic bone metastases.

Bone metastasis has been generally classified as osteolytic, leading to bone destruction; osteoblastic, leading to new bone formation; or mixed based on the primary mechanism of interference with normal bone remodeling. The balanced activity of osteolytic and osteoblastic factors is thought to regulate bone metastasis [4,5]. Recently, several molecules have been reported to play important roles as osteoblastic factors involved in osteoformation [4–6]. However, the precise mechanisms responsible for tumor growth in bones remain unexplored.

Comprehensive transcriptomics confer a precise characterization of individual cancers that should help to improve clinical strategies for neoplastic diseases through the development of novel drugs. Hence, “omics” technology approaches are effective for identifying target molecules involved in carcinogenic and metastatic pathways, including bone metastasis. To this end, the genome-wide transcriptomics of human SCLC engaged in organ-preferential metastasis in mice was analyzed, and several genes potentially involved in bone metastasis were found [7]. In this

study, we focused on early growth response 4 (*EGR4*), which is significantly upregulated in bone metastatic tumors compared with other organ metastases (lung, kidney and liver) derived from human SCLC cells [7].

The *EGR4* gene belongs to the early growth response family of immediate early genes encoding four DNA-binding, zinc-finger transcription factors (*EGR1* to *EGR4*) [8]. This gene (*pAT133*, *NGFI-C*) was first identified as a zinc-finger protein immediately induced by mitogenic stimulation in T lymphocytes and fibroblasts [9,10]. It has been reported that *EGR4*-null mice have male infertility because of arrested spermatogenesis but no female infertility is observed [11,12], suggesting that *EGR4* plays a critical role in some types of human idiopathic male infertility. Moreover, *EGR4* is known to have a neural-specific expression pattern in rats [13] and regulate brain-derived neurotrophic factor (BDNF)-mediated neuron-specific potassium chloride cotransporter 2 (*KCC2*) transcription via the ERK1/2 signaling pathway in immature neurons [14]. However, the pathophysiological role of *EGR4* in carcinogenesis in SCLC, has not been elucidated. In this study, we report that *EGR4* acts as a transcriptional activator via regulation of specific downstream genes in SCLC cell proliferation.

Materials and Methods

Cell lines

The human SCLC cell lines SBC-3 and SBC-5 were kindly provided by Drs. M. Tanimoto and K. Kiura of Okayama University [15]. The NSCLC cell line PC14PE6 was kindly provided by Dr. I. J. Fidler of M. D. Anderson Cancer Center [16]. The human SCLC cell line NCI-H1048 and human NSCLC cell lines A549 and NCI-H1048 were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). The human ACC-LC319/bone2 cell line was established as previously described [17]. The MC3T3-E1 murine osteoblastic subclone 4 cell line was kindly provided by Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan). The human small airway epithelial cell line (SAEC) was purchased from Lonza (Walkersville, MD, USA). All cells were cultured under appropriate conditions.

Plasmid constructs

The entire coding sequence of human *EGR4* (NM_001965) was amplified by PCR using KOD plus DNA polymerase (Toyobo, Osaka, Japan). The PCR product was inserted into the *EcoRI* and *XhoI* sites of the pCAGGSn3FH vector which contains an N-terminal FLAG tag. For luciferase reporter plasmids, DNA fragments from the 5'-flanking regions of *PTHrP-V3* and *V4* (NM_198964.1 and NM_198966.1, respectively), *SAMD5* (NM_001030060.2), *RAB15* (NM_198686.2), *SYNPO* (NM_007286.5) and *DLX5* (NM_005221.5), which include potential EGR binding sites as predicted by the MatInspector program (Genomatix, <http://www.genomatix.de/matinspector.html>), were amplified by PCR and inserted into the appropriate restriction enzyme sites in the pGL3-enhancer vector (Promega, Madison, WI, USA). The PCR primer sets used in this study are shown in Table S1. The DNA sequences of all constructs were confirmed by DNA sequencing (ABI 3500xL sequencer; Life Technologies, Foster City, CA, USA).

RNA extraction, reverse transcription, semi-quantitative PCR and real-time PCR

Total RNA extraction, reverse-transcription, semi-quantitative RT-PCR and Real-time PCR experiments were conducted as previously described [18]. The expression levels in each sample

were normalized to the β -actin mRNA content. The sequences of each primer set are listed in Table S2.

Western blot analysis

Western blot analysis was performed as previously described [18]. After SDS-PAGE, membranes blotted with proteins were incubated with anti-FLAG M2 (Sigma-Aldrich, St. Louis, MO, USA, F3165) or anti- β -actin (AC-15, Sigma-Aldrich, A-5441) mouse monoclonal antibodies diluted at 1:5000. The membranes were then incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h, and the protein bands were visualized with enhanced chemiluminescence (ECL) detection reagents (GE Healthcare, Piscataway, NJ, USA).

Measurement of PTHrP secretion

HEK293T cells (1.5×10^5 cells/12-well plate) were transfected with the pCAGGSn3FH-EGR4 or mock (no insert) plasmids using FuGENE 6 (Promega). At 48 h after transfection, the culture medium was collected and centrifuged at 4°C at 15,000 rpm. The PTHrP protein concentration in the conditioned media was determined by an immunoradiometric (IRMA) assay (SRL Inc., Tokyo, Japan).

Effect of conditioned medium derived from EGR4-overexpressing HEK293T cells on *RANLK*, *IL-6* and *IL-8* expression

HEK293T cells (2.6×10^6 cells/10 cm plate) were transiently transfected with the pCAGGSn3FH-EGR4 or mock vector for 48 h, and the culture media was then replaced with DMEM plus 0.1% FBS for an additional 48 h. The culture medium was subsequently collected, and the conditioned medium was transferred to murine MC3T3-E1 osteoblast cells that were pre-cultured with differentiation medium containing ascorbic acid (100 μ g/ml) for 5 days. After 48 h, the expression levels of murine *RANLK*, *IL-6*, and *IL-8* was analyzed by real-time PCR as described above.

Chromatin immunoprecipitation (ChIP) assay

HEK293T cells (2.5×10^6 cells/10 cm dish) were transfected with 8 μ g of the pCAGGSn3FH-EGR4 or mock vector for 48 h and then ChIP assays were performed using the EZ-ChIP kit (Millipore, Billerica, MA, USA) as previously described [19]. The PCR primer sets to detect the EGR-binding sites used are listed in Table S3.

Luciferase assay

HEK293T cells (2.5×10^4 cells/48-well dish) were co-transfected with either 100 ng of the pGL3-enhancer promoter vector as described above or the mock vector in combination with 100 ng of the pCAGGSn3FH-EGR4 or mock vector (100 ng). pRL-TK was used as an internal control. After 48 h, the cells were harvested and analyzed for *Firefly* luciferase and *Renilla* luciferase activity using the dual luciferase reporter assay (Promega) as previously described [19]. Data were expressed as the fold increase over mock-transfected cells (set at 1.0) and represented as the mean \pm SE of two independent experiments.

NIH3T3 cell proliferation assay

NIH3T3 cells (0.5×10^5 cells/6-well dish) were transiently transfected with 3 μ g of pCAGGSn3FH-EGR4 or mock vector using FuGENE 6 (Promega). Cell proliferation assays were performed at 48, 72 and 96 h after transfection, respectively, using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) as

previously described [18]. These experiments were performed in triplicate. Western blot analysis was performed as described above.

Gene silencing effects by siRNA treatment

We used siRNA oligonucleotides (Sigma-Aldrich Japan KK, Japan) to knock down *EGR4*, *DLX5*, *SYNPO*, *SAMD5* and *RAB15* expression in SBC-5, SBC-3, NCI-H1048 or PC14PE6. The sequences targeting each gene are listed in Table S4. Cells were plated in 12-well dishes (SBC-5 and PC14PE6; 1.5×10^4 cells/well, SBC-3; 2.5×10^4 cells/well, NCI-H1048; 5.0×10^4 cells/well). Transfection of 100 nM siRNA to SBC-5 and PC14PE6 cells was performed using Lipofectamine 2000 reagent (Life Technologies) as previously described [20]. SBC-3 and NCI-H1048 cells were transfected with 50 nM siRNAs using Lipofectamine RNAi Max transfection reagent (Life Technologies) according to the manufacturer's instructions. At 48, 96 or 120 h after transfection, total RNA extraction, real-time PCR and cell proliferation assays were performed as described above.

Identification of EGR4 downstream genes by DNA microarray

SBC-5 cells (1×10^6 cells/35 mm dish for 24 h) were transfected with 10 nM siRNA directed against EGR4 (EGR4-2) or EGFP (siEGFP; a control) using Lipofectamine RNAi Max transfection reagent (Life Technologies). Total RNA was extracted from each sample at 48 and 72 h after transfection of siRNA. The DNA microarray and data analyses were performed using the Agilent Whole Human Genome Microarray (4 \times 44K, G4110F; Agilent Technologies, Santa Clara, CA, USA) and GeneSpring software (version 11.5; Agilent Technologies) as previously described [21]. A corrected *P* value was calculated with Benjamini Hochberg false discovery rate (FDR) analysis, and $P < 0.05$ was considered significant. The extent and direction of the differential expression between time points (48 and 72 h) were determined by calculating fold change values. The DNA microarray analysis data have been submitted to the NCBI Gene Expression Omnibus (GEO) database as series GSE40558.

RNAseq data analysis of lung cancers

Publicly available gene expression data (normalized values from Illumina RNAseq v2, level 3, LUAD and LUSC) from The Cancer Genome Atlas (TCGA; <http://cancergenome.nih.gov/>) were downloaded from TCGA matrix. The differential expression (by fold change value) between cancer tissues and the adjacent normal lung was calculated according to the normalized gene expression value of each sample.

Statistical analysis

Statistical analysis was performed using Student's *t*-test. $P < 0.05$ was considered significant.

Results

EGR4 directly regulates the transcriptional activity of the *PTHrP* gene

Analysis of the genome-wide gene expression profile of the organ-preferential metastasis of the human SCLC cell line SBC-5 in mice identified early growth response 4 (*EGR4*), which was significantly upregulated in bone metastatic tumors ($p < 0.001$, ratio; 2.22) compared with other organ metastases (lung, kidney and liver) [7]. First, to clarify the role of EGR4 as a transcription factor involved in bone metastasis, we focused on the parathyroid hormone-related protein (*PTHrP*) gene as a candidate down-

stream target of EGR4 because the *PTHrP* gene is known to be a potent activator of osteoclastic bone resorption [4] and encodes a protein secreted from SBC-5 cells [22,23]. Moreover, it has been reported that treatment with an anti-PTHrP neutralizing antibody inhibits the production of SBC5 cell bone metastasis in the SCID mouse model [22,23].

In the National Center for Biotechnology Information (NCBI) database, the *PTHrP* gene is reported to possess four transcriptional variants, designated *PTHrP* variant 1 (PTHrP-V1, GenBank accession no. NM_198965.1), variant 2 (PTHrP-V2, NM_002820.2), variant 3 (PTHrP-V3, NM_198964.1) and variant 4 (PTHrP-V4, NM_198966.1). The full-length cDNAs of *PTHrP-V1*, *V2*, *V3* and *V4* consist of 1331, 1881, 1862 and 1312 nucleotides that encode 177, 175, 175 and 177 amino acids, respectively, and consist of 5, 4, 3, and 4 exons, respectively. The V1 variant lacks exon 3, and the V2 variant lacks exon 3 and possesses exon 5b, which is 1,027 bp longer at the 3' end than exon 5a. The V3 and V4 variants commonly lack exons 1 and 2 and possess exon 3, which is located within intron 2 with a length of 281 bp. The V3 variant further lacks exon 6, and possesses exon 5b and V2 variant. The V4 variant possesses exon 5a and exon 6, indicating that the *PTHrP-V1/V2* and *V3/V4* variants have different promoter regions (Figure 1A). Subsequent real-time RT-PCR analysis confirmed that the *PTHrP-V3* and *V4* splicing variants were predominately upregulated at the transcriptional level in EGR4-overexpressing HEK293T cells compared with mock-transfected cells (Figure 1B). Accordingly, to obtain direct evidence for the upregulation of *PTHrP-V3* and *V4* by EGR4, we first searched for putative EGR DNA binding motifs with the MatInspector program (described above) because it has been reported that the EGR family, including the EGR4 protein, preferentially binds to an EGR consensus motif (5'-GCGG/TGGGCG-3') [24–27]. We found a potential EGR DNA binding motif within the *PTHrP-V3* and *V4* promoter region (–515 to –499). Subsequently, we examined the transcriptional activity of EGR4 by a luciferase reporter assay using a pGL3 luciferase plasmid containing the EGR4 binding motif in the *PTHrP-V3/V4* promoter. A significant increase in luciferase activity was observed with FLAG-EGR4 transfection compared with the mock control vector in HEK293T cells (Figure 1C). To further investigate whether EGR4 could bind to a potential *PTHrP-V3* and *V4* EGR binding motif, we performed a ChIP assay. The genomic fragment including the potential EGR binding motif (–515 to –499) of *PTHrP-V3* and *V4* was specifically bound by EGR4 protein in products immunoprecipitated with an anti-FLAG antibody, suggesting that EGR4 directly bound to the promoter region of the *PTHrP-V3* and *V4* variants (Figure 1D). Taken together, these findings suggest that the EGR4 might directly upregulate the *PTHrP-V3* and *V4* variants in SCLC cells.

Paracrine effects of PTHrP secreted from EGR4-overexpressing cells

It has been reported that PTHrP protein secreted from cancer cells regulates the expression of the *RANKL*, *IL-6* and *IL-8* genes, which have been implicated as factors that enhance osteoclast formation and bone destruction in malignant diseases [28–30] in osteoblast cells. According to these data and our findings as shown in Figure 1, we hypothesized that PTHrP protein is secreted from EGR4-overexpressing cells. Our results showed that the PTHrP protein concentration was significantly increased in media conditioned from EGR4-overexpressing HEK293T cells (14.43 ± 1.04 pmol/L) compared with conditioned media from mock-transfected cells (11.83 ± 0.15 pmol/L, $P < 0.05$; Figure 2A).

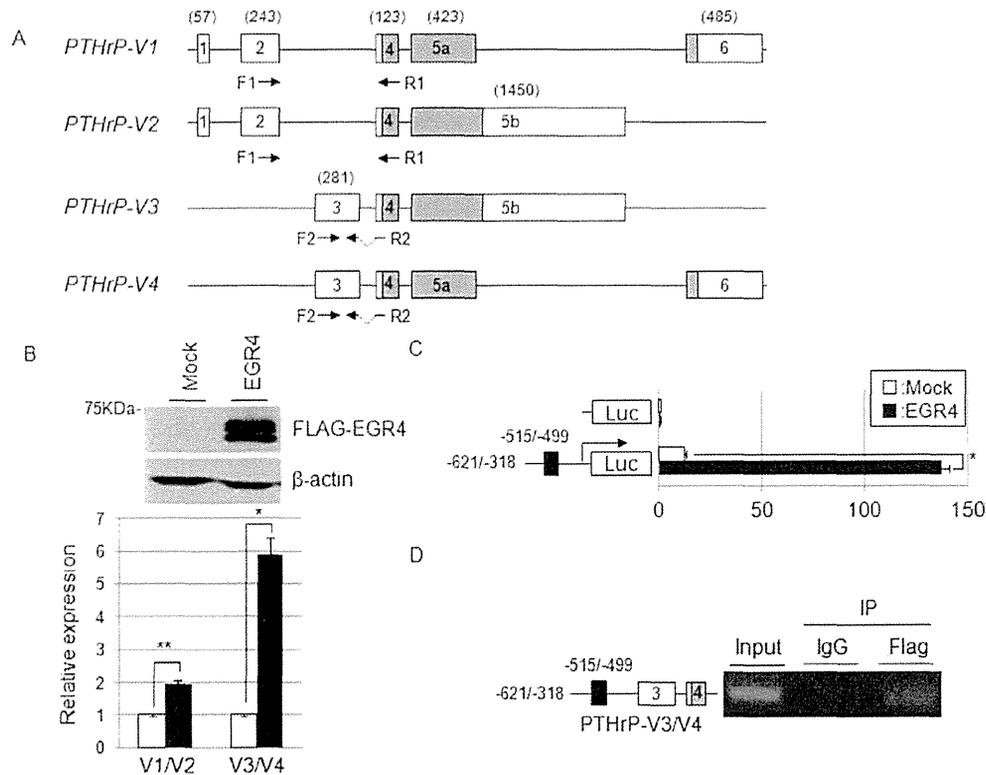


Figure 1. EGR4 directly transactivates specific splice variants of the *PTHrP* gene. A: Genomic structures of the four splice variants of *PTHrP*. The gray and white boxes indicate coding and non-coding regions, respectively. The arrows indicate the primer sets used to perform RT-PCR for each transcript. The numbers in parentheses indicate the length of each exon. B: Upper panel: western blot analysis of HEK293T cells expressing exogenous FLAG-tagged EGR4 (FLAG-EGR4) or cells transfected with the mock vector. Lower panel: real-time RT-PCR analysis of *PTHrP* splice variants (V1/V2 and V3/V4) in EGR4-overexpressing HEK293T cells. C: Luciferase assay of the *PTHrP*-V3 and -V4 (V3/V4) promoter regions (n = 2, *P < 0.05). This experiment was performed using a part of the lysates from cells expressing exogenous FLAG-EGR4 or those transfected with the mock vector used in B. D: ChIP assay of the *PTHrP*-V3/V4 promoter region. ChIP assays were used to determine direct EGR4 binding to the *PTHrP*-V3/V4 promoter. The PCR product was from -620 to -318 of the region upstream of the 5' end of exon 3 of *PTHrP*-V3/V4, which was designated as the +1 position. doi:10.1371/journal.pone.0113606.g001

Next, we evaluated the paracrine effects of conditioned medium from EGR4-overexpressing HEK293T cells on osteoblast cells. As shown in Figure 2B, we transferred conditioned medium from HEK293T cells transfected with the FLAG-EGR4 construct to MC3T3-E1 murine osteoblast cells and then performed real-time PCR to examine the effects of the conditioned medium on the expression level of the *RANKL*, *IL-6* and *IL-8* genes. All three genes were significantly upregulated in osteoblast cells treated with conditioned medium from HEK293T cells ectopically expressing FLAG-EGR4 compared with mock-transfected cells (Figure 2C). Collectively, these findings suggest that the increase in PTHrP secretion from EGR4-overexpressing cells may enhance the expression of the *RANKL*, *IL-6* and *IL-8* genes in osteoblast cells.

Effect of *EGR4* on cell growth

We first examined *EGR4* expression in SCLC cells by semi-quantitative RT-PCR and found that *EGR4* was highly expressed in SBC-3, SBC-5 and NCI-H1048 cells but not in the small airway epithelial cell line SAEC (Figure 3A). Next, to assess whether *EGR4* is essential for the growth of SBC-5 cells, we used an RNA interference approach with two different siRNA oligonucleotides. Real-time PCR analysis showed that *EGR4*-specific siRNAs (siEGR4-1 and siEGR4-2) significantly suppressed the expression of *EGR4* compared with siEGFP as a control (Figure 3B). MTT

assay showed that the introduction of siEGR4s (siEGR4-1 and siEGR4-2) significantly suppressed the growth of SBC-5 cells (Figure 3C), which is in accordance with the *EGR4* knockdown results. We also confirmed significant growth inhibitory effects of *EGR4* knockdown in other SCLC cell lines SBC-3 and NCI-H1048 overexpressing *EGR4* (Figure S1). To further confirm the growth promoting effect of *EGR4*, FLAG-EGR4 construct or mock vector was transiently transfected into NIH3T3 cells, and MTT assay was performed as described above. As shown in Figure 3D, FLAG-EGR4-transfected cells grew significantly faster than those transfected with mock vector. These findings suggest that overexpression of *EGR4* might be involved in the growth of SCLC cells.

Identification of *EGR4* target genes

To obtain further insight into the biological role of *EGR4* on cell growth, we attempted to identify downstream genes specifically regulated by *EGR4* in SCLC cells. siEGR4 or siEGFP (control siRNA) was transfected into SBC-5 cells in which *EGR4* was highly expressed (Figure 3A), and alterations in gene expression at two time points were monitored by DNA microarray analysis. To identify the genes putatively regulated by *EGR4*, we selected genes with the following two criteria: (i) expression level was decreased by more than two-fold at 48 and 72 h in cells

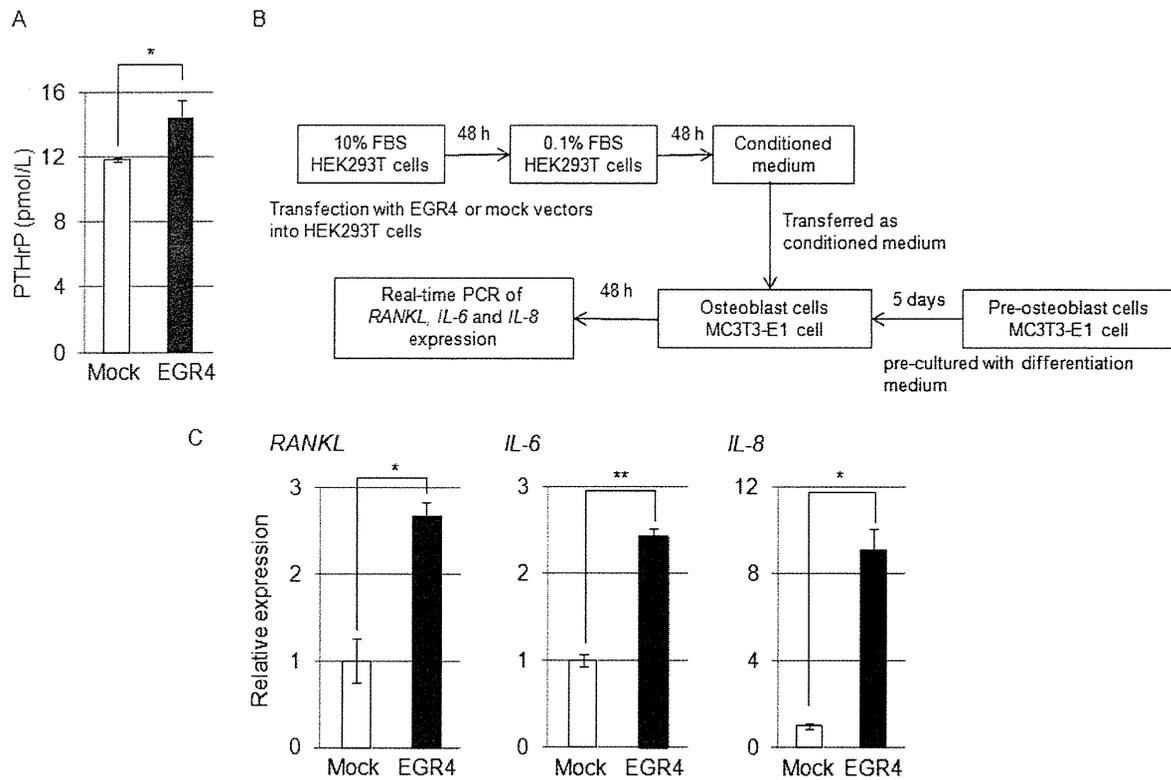


Figure 2. PTHrP secretion leads to transactivation of specific PTHrP splice variants. A: Secretion of the PTHrP protein from EGR4-overexpressing HEK293T cells ($n=3$, $*P<0.05$). B: Measurement scheme for the paracrine effects of conditioned media from EGR4-overexpressing cells. C: Real-time PCR analysis of the paracrine effects on the expression of the *RANKL*, *IL-6* and *IL-8* genes when medium from EGR4-overexpressing HEK293T cells was cultured with mouse MC3T3-E1 osteoblast cells ($n=2$, $*P<0.05$, $**P<0.01$). doi:10.1371/journal.pone.0113606.g002

transfected with siEGR4 compared with cells transfected with the control siEGFP, and (ii) a putative EGR binding motif was predicted to exist within 500 bp of the transcription start site by the MatInspector program (described above). We identified 13 genes that were downregulated upon knockdown of EGR4 expression (Table S5). Real-time PCR analysis confirmed that seven transcripts were significantly downregulated at both time points in EGR4-knockdown cells (Figure 4A). Subsequently, we also evaluated the upregulation of these genes upon exogenous EGR4 expression in HEK293T cells and ultimately selected four EGR4 candidate target genes, including distal-less homeobox 5 (*DLX5*), synaptopodin (*SYNPO*), sterile alpha motif domain containing 5 (*SAMD5*), and RAB15, a member of the RAS oncogene family (*RAB15*), which were significantly upregulated by EGR4 overexpression (Figure 4B). We confirmed significant downregulation of *DLX5*, *SYNPO* and *SAMD5* genes by EGR4 knockdown in SBC-3 cells (Figure S2).

To obtain direct evidence for the transactivation of four EGR4 candidate target genes, we measured the transcriptional activity of EGR4 by a luciferase reporter assay. FLAG-EGR4-transfected cells had significantly higher luciferase activity than mock-transfected cells (Figure 4C). Next, we investigated the recruitment of EGR4 to each EGR4-binding site by ChIP assay. EGR4 was shown to bind to the predicted EGR-binding motif within the promoter regions of all target genes (Figure 4D). These results suggest that EGR4 directly transactivates *SAMD5*, *RAB15*, *SYNPO* and *DLX5*. Subsequently, we investigated the biological role of the

four EGR4 target genes in the proliferation of SCLC cells. Introduction of the siRNAs into SBC-5, SBC-3 and NCI-H1048 cells resulted in a significant reduction in the expression of the target genes accompanied by significant suppression of cell proliferation (Figure 5A–D, Figure S3), suggesting that these genes are also likely to play a crucial role in the proliferation of SCLC cells via EGR4 transcriptional activation.

Discussion

In this study, our aim was to identify and characterize molecules or pathways potentially involved in cancer metastasis, particularly bone metastasis. Through a genome-wide transcriptomic analysis of the organ-preferential metastasis of human SCLC cells in mice, we found that *EGR4*, a member of a family of four related zinc-finger Cys₂-His₂ type proteins (*EGR1* to *EGR4*), is significantly upregulated in bone metastatic tumors compared with other organs i.e., the lung, kidney and liver [7]. EGR4 was initially identified as a zinc-finger transcription factor immediately induced by mitogenic stimulation in T lymphocytes and fibroblasts [31]. Gene targeting studies in mice have shown that EGR4 regulates several critical genes involved in the early stages of meiosis and plays an indispensable role in male murine fertility [11,12]. Furthermore, it has been reported that EGR4 binds to nuclear factor activated T cells (NFAT) or nuclear factor kappa B (NFκB) to enhance the transcription of downstream genes encoding inflammatory cytokines, such as IL-2, TNF-α and ICAM-1 [32,33]. A previous report described that the expression level of

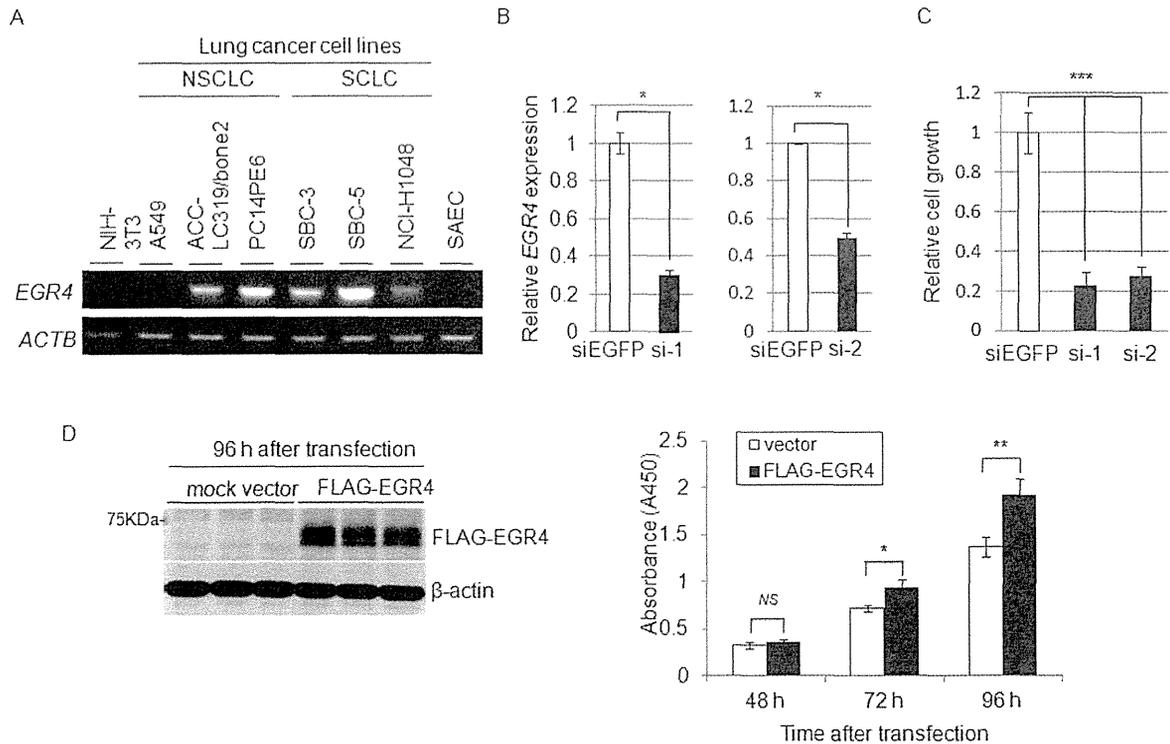


Figure 3. Effects of *EGR4* gene on cell growth. A: Expression of *EGR4* in SCLC and NSCLC cell lines was determined by semi-quantitative RT-PCR. B: Effects of *EGR4* knockdown on cell proliferation in SBC-5 cells. Real-time PCR of *EGR4* in siEGFP- or siEGFP4 (siEGFP4-1, siEGFP4-2)-treated cells at 5 days after siRNA treatment ($n=2$, $*P<0.05$). *ACTB* was used as a quantitative control for real-time RT-PCR. C: Cell proliferation was determined by MTT assay at 5 days after siRNA treatment ($n=3$, $***P<0.005$). (si-1; siEGFP4-1, si-2; siEGFP4-2). D: Growth-promoting effect of exogenous *EGR4* on NIH3T3 cells ($*P<0.05$, $**P<0.01$, NS, no significance). Western blot analysis was performed at 96 h after transfection (left panel). MTT assay was performed at 48, 72 and 96 h after transfection with FLAG-EGR4 (black) or mock vector (white) (right panel). These experiments were performed in triplicate. doi:10.1371/journal.pone.0113606.g003

PTHrP, a potent activator of osteoclastic bone resorption, in bone metastases tends to be higher than that in metastases to the kidneys, livers, and lungs using a genome-wide transcriptomics of human SCLC cells in mice [7]. Accordingly, in this study, we focused on *PTHrP*, a potent activator of osteoclastic bone resorption, as an *EGR4*-downstream gene to clarify the pathophysiological role of *EGR4* as a transcription factor in SCLC bone metastases.

PTHrP is known to be a key mediator of humoral hypercalcemia malignancies and osteolytic lung cancer metastases [22,23,34]. Approximately 80% of patients with solid tumors and hypercalcemia have increased *PTHrP* concentrations in their plasma [35]. It has been reported that *PTHrP* protein secreted from cancer cells regulates the expression of the *RANKL*, *IL-6* and *IL-8* genes, which have been implicated as factors that enhance osteoclast formation and bone destruction in malignant diseases [28–30] in osteoblast cells. We found that *EGR4* directly transactivates specific variants (*V3* and *V4*) of the *PTHrP* gene, thereby possibly promoting the secretion of the *PTHrP* protein in *EGR4*-overexpressing cells, resulting in subsequent transactivation of the *RANKL*, *IL-6* and *IL-8* genes via paracrine action of *PTHrP*. *RANKL* is known to bind the *RANK* receptor on osteoclast precursors and induce osteoclast formation. *IL-6* and *IL-8* have also been reported to be important for osteoclastogenesis and osteoclast activation, respectively [30]. Therefore, these findings suggest that induction of *PTHrP* by *EGR4* overexpression may be responsible for the bone metastasis of SCLC lung cancer

cells. However, we found that *PTHrP* gene expression was not reduced by *EGR4* knockdown in SBC5 cells (data not shown). A possible reason for this result is that several factors are involved in the regulation of *PTHrP* expression in addition to the *EGR4* transcription factor. For example, *EGR4* is reported to functionally cooperate with *NF- κ B* and *NFAT* and induce the expression of cytokine genes [32,33]. Indian hedgehog and *TGF- β* have also been reported to stimulate perichondrial and breast cancer production, respectively [36,37]. Moreover, miR-33a has been reported to repress the *PTHrP*-mediated expression of *PTHrP* in NSCLC [38], and knockdown of zinc-finger E-box binding homeobox 1 (*ZEB1*), a transcriptional repressor, reduces *PTHrP* secretion in SCLC [39]. Therefore, it is necessary to further explore the mechanism of *PTHrP* transactivation via endogenous *EGR4* expression in SBC-5 cells in greater detail.

Notably, we showed that depletion of *EGR4* by siRNA led to a significant reduction in cell proliferation in SBC-5, SBC-3 and NCI-H1048 cells, and that *EGR4* transactivated a set of genes possibly related to lung cancer cell growth including four *EGR4*-downstream genes, *DLX5*, *RAB15*, *SAMD5* and *SYNPO*. Knockdown of the expression of these genes by siRNA led to a significant reduction in cell growth in SCLC cells, suggesting that these genes are involved in the growth of SCLC lung cancer cells. It has been reported that *DLX-5* overexpression in lung cancer cells is associated with tumor size and predictive of poor prognosis and NSCLC cell proliferation [20]. *RAB15* was originally identified as a brain-tissue specific RAB protein within the RAB

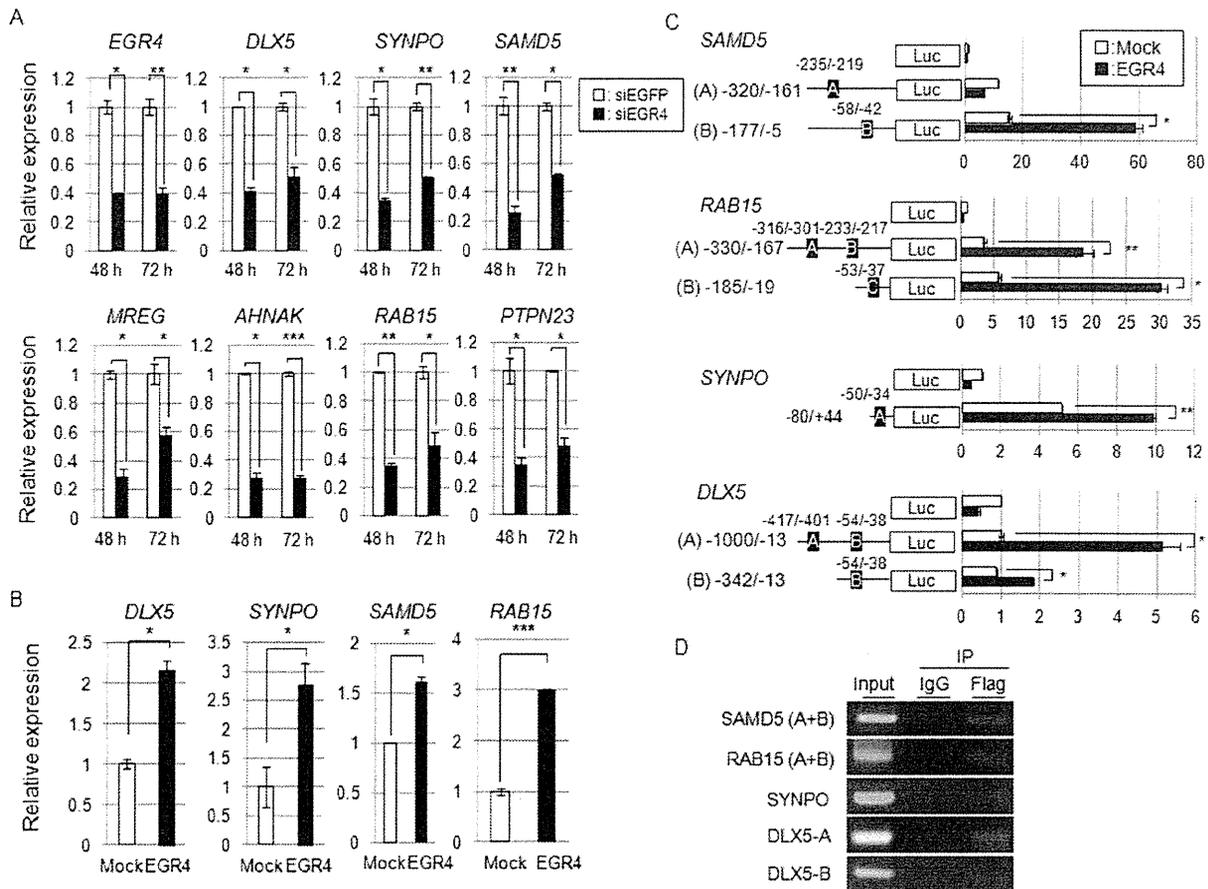


Figure 4. Identification of EGR4-downstream genes involved in the proliferation of SCLC cells. A: Real-time PCR of *EGR4* and seven downstream genes (*DLX5*, *SYNPO*, *SAMD5*, *MREG*, *AHNAK*, *RAB15*, and *PTPN23*) in siEGFP- or siEGR4-treated SBC-5 cells (n = 2, *, P < 0.05, **, P < 0.01, ***, P < 0.005). B: Real-time PCR of the *DLX5*, *SYNPO*, *SAMD5*, and *RAB15* genes in mock- or EGR4-overexpressing HEK293T cells (n = 2, *, P < 0.05, ***, P < 0.005). This experiment was performed using total RNA from cells expressing exogenous FLAG-tagged EGR4 (FLAG-EGR4) or those transfected with the mock vector used in Figure 1B. C: Luciferase assay of the *SAMD5*, *RAB15*, *SYNPO* and *DLX5* genes. (n = 2, *, P < 0.05, **, P < 0.01). D: ChIP assays were used to determine the direct binding of EGR4 to the promoters of the *SAMD5*, *RAB15*, *SYNPO* and *DLX5* genes. doi:10.1371/journal.pone.0113606.g004

family of small G proteins that regulates the endocytic recycling pathway [40] and is associated with the retinoic acid-induced differentiation of neuroblastoma cells [41]. *SAMD5* has been reported to be one of 24 discriminating genes with an expression level that significantly differs between responders and nonresponders to chemoradiotherapy in rectal cancer [42]. *SYNPO* has been reported to be an actin-binding protein that functions in actin dynamics, cell migration, and tumor suppression [43] and is exclusively expressed in highly dynamic cell compartments such as kidney podocyte foot processes [44]. Although the precise function of these genes in lung carcinogenesis remains largely unknown, our findings suggest that EGR4 may be a pivotal regulator that selectively activates the transcription of several target genes in lung cancer cells.

In addition, we demonstrated that *EGR4* was highly expressed in NSCLC and SCLC cell lines (Figure 3A). In addition, analysis of publicly available RNAseq data sets from The Cancer Genome Atlas (TCGA) revealed that *EGR4* was up-regulated (more than 2-fold) in 17 of 39 lung adenocarcinoma cases (Figure S4A), and in 19 of 46 squamous cell carcinoma (SCC) cases (Figure S4B) compared with their corresponding normal lung. Furthermore, we

found that knockdown of *EGR4* by siRNA suppressed the proliferation of PC14PE6 NSCLC cells (Figure S5), but did not find the inhibitory effects of EGR4 knockdown on its downstream genes, *SAMD5*, *RAB15*, *SYNPO* and *DLX5* expression in PC14PE6 cells (data not shown). These findings suggest the possibility that EGR4 may play different roles in NSCLC cell growth. Therefore, it is necessary to further explore the mechanism of *EGR4* transactivation in NSCLC cells.

In summary, we demonstrated that EGR4 directly transactivates specific variants (*V3* and *V4*) of the *PTHrP* gene, thereby possibly enhancing secretion of the PTHrP protein in EGR4-overexpressing cells, resulting in subsequent transactivation of the *RANKL*, *IL-6* and *IL-8* genes via paracrine action of the PTHrP protein, a mediator of osteolytic bone metastasis (Figure 2). Moreover, EGR4 also transactivates *SAMD5*, *RAB15*, *SYNPO* and *DLX5*, which are involved in the proliferation of SCLC cells. Collectively, our findings suggest that EGR4 is likely to play an important role for the promotion of SCLC growth through the up-regulation of its downstream genes, and it could be a novel therapeutic target for the development of anticancer drugs.

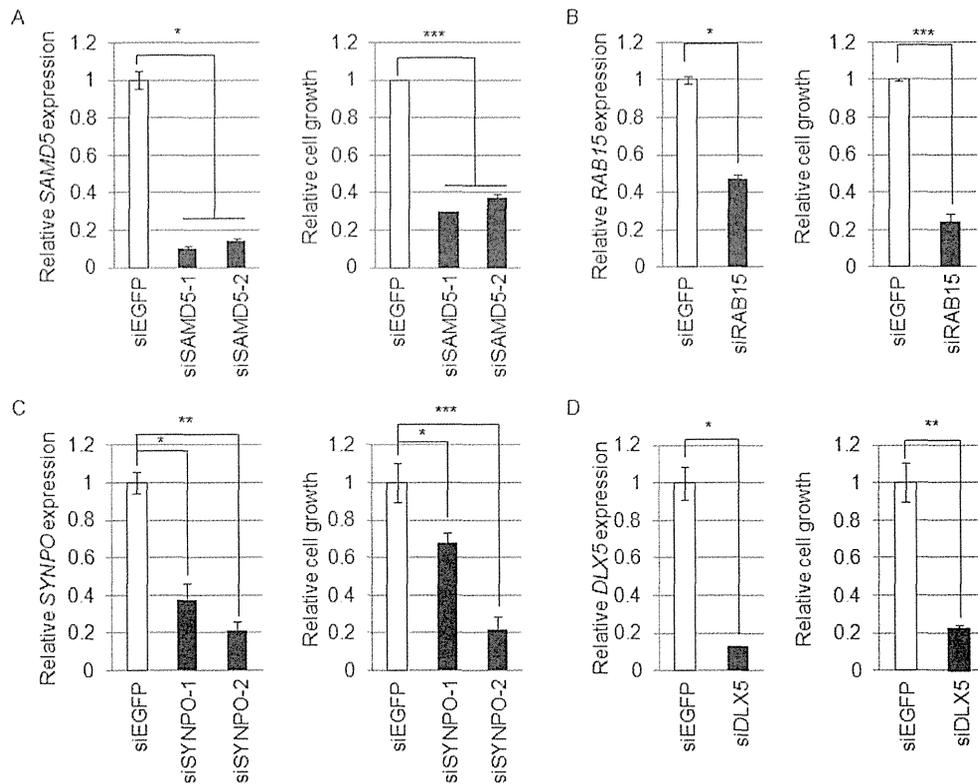


Figure 5. EGR4 downstream target genes regulate the cell proliferation of SBC-5 cells. Effects of the *EGR4* downstream genes *SAMD5* (A), *RAB15* (B), *SYNPO* (C) and *DLX5* (D) on cell proliferation were determined by siRNA knockdown in SBC-5 cells. The left panel shows the real-time PCR results for target genes in siRNA-treated cells (n = 2). The right panel shows results from cell proliferation analyses as measured by MTT assay (*SAMD5* and *RAB15*: n = 2, *DLX5* and *SYNPO*: n = 3, *, P < 0.05, **, P < 0.01, ***, P < 0.005). doi:10.1371/journal.pone.0113606.g005

Supporting Information

Figure S1 Effects of EGR4 gene on SBC-3 and NCI-H1048 cell growth. Effects of *EGR4* knockdown on cell proliferation in SBC-3 and NCI-H1048 cells. Real-time PCR of *EGR4* in siEGFP- or siEGR4 (siEGR4-1, siEGR4-2)-treated SBC-3 cells (A) and NCI-H1048-cells (C) at 5 days after siRNA treatment (n = 2, *P < 0.05, ** P < 0.01). *ACTB* was used as a quantitative control for real-time RT-PCR. Cell proliferation of SBC-3 (B) and NCI-H1048 (D) was determined by MTT assay at 5 days after siRNA treatment (n = 3, *P < 0.05, ** P < 0.01, ***P < 0.005). (si-1; siEGR4-1, si-2; siEGR4-2). (TIFF)

Figure S2 Expression of EGR4-downstream genes in siEGR4-treated SBC-3 cells. Real-time PCR of *EGR4* and 3 downstream genes (*DLX5*, *SYNPO* and *SAMD5*) in SBC-3 cells treated with siEGFP or siEGR4 for 48 h (n = 2, *, P < 0.05, **, P < 0.01, ***, P < 0.005). (TIFF)

Figure S3 EGR4-downstream target genes regulate the cell proliferation of SBC-3 and NCI-H1048 cells. Effects of the *EGR4* downstream genes on cell proliferation were determined in SBC-3 (A–C) and NCI-H1048 cells (D, E). The left panel shows the real-time PCR results for *EGR4*-downstream genes in siRNA-treated cells (n = 2). The right panel shows results from cell

proliferation analyses as measured by MTT assay (n = 3, *, P < 0.05, **, P < 0.01, ***, P < 0.005). (TIFF)

Figure S4 Overexpression of EGR4 in lung cancers. The cases overexpressing *EGR4* (>2 fold compared with the adjacent normal lung) are indicated as black bars in adenocarcinoma (A) and squamous cell carcinoma (SCC) (B). (TIFF)

Figure S5 Effect of EGR4 on cell proliferation in PC14PE6 cells. Knockdown of *EGR4* at the mRNA level was analyzed by real-time PCR (n = 2, **P < 0.01, ***P < 0.005). Cell proliferation was determined by an MTT assay at 4 days after siRNA treatment (n = 3, ***, P < 0.005). (TIFF)

Table S1 Primer sequences for plasmid construction. (DOCX)

Table S2 Primer sequences for real time PCR or RT-PCR. (DOCX)

Table S3 Primer sequences for ChIP assay. (DOCX)

Table S4 siRNA sequences. (DOCX)

Table S5 Putative downstream EGR4 target genes identified by microarray analysis. (DOCX)

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Author Contributions

Conceived and designed the experiments: TK. Performed the experiments: TM LTD TY MK KD. Analyzed the data: TM LTD TY MK KD. Contributed reagents/materials/analysis tools: SS YN. Wrote the paper: TK TM LTD MK.

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Thymidine Phosphorylase Regulates the Expression of CXCL10 in Rheumatoid Arthritis Fibroblast-like Synoviocytes

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Objective. Thymidine phosphorylase (TP) in rheumatoid arthritis (RA) fibroblast-like synoviocytes (FLS) is induced by tumor necrosis factor α (TNF α) and other cytokines that have been reported to be major inflammation mediators in RA. We previously demonstrated that TP plays an important role in angiogenesis and tumor growth, invasion, and metastasis. The aim of this study was to investigate whether the role of TP in the pathogenesis of RA is similar to its role in tumors.

Methods. In FLS obtained from 2 patients with RA, the expression of TP, interferon- γ (IFN γ)-inducible protein 10 (CXCL10), and other cytokines was measured by quantitative real-time polymerase chain reaction, immunoblotting, and enzyme-linked immunosorbent assays. Microarray analysis was performed using FLS transfected with *TYMP* complementary DNA and treated with a TP inhibitor.

Results. The expression of TP in FLS was up-

regulated by TNF α , interleukin-1 β (IL-1 β), IL-17, IFN γ , and lipopolysaccharide. Microarray analysis of FLS overexpressing TP identified *CXCL10* as a thymidine phosphorylase-related gene. The expression of CXCL10 was induced by TNF α , and this induction was suppressed by *TYMP* small interfering RNA and TP inhibitor. Furthermore, the combination of TNF α and IFN γ synergistically augmented the expression of TP and CXCL10. TP-induced CXCL10 expression was suppressed by the antioxidant EUK-8. In the synovial tissue of patients with RA, TP levels were significantly correlated with CXCL10 expression.

Conclusion. The combination of TNF α and IFN γ strongly induced the expression of thymidine phosphorylase in RA FLS. The induction of thymidine phosphorylase enhanced the expression of CXCL10, which may contribute to the Th1 phenotype and bone destruction observed in RA.

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by synovial inflammation and progressive destruction of articular cartilage and bone. Recently, several specific molecules, such as tumor necrosis factor α (TNF α), interleukin-6 (IL-6), and CTLA-4, were demonstrated to be therapeutic targets in RA, and antagonists to these molecules were developed (1). These biologic agents provide a dramatic effect for the therapeutic management of RA and have updated the treatment strategy for RA (1). However, the pathogenesis of RA has not yet been fully clarified.

Meanwhile, the cytokines implicated in RA, such as TNF α , IL-1 α , and interferon- γ (IFN γ), have been shown to induce thymidine phosphorylase (TP) in tumor cells (2). TP is involved in the metabolism of pyrimidine nucleoside and converts thymidine to thymine and 2-deoxy-D-ribose 1-phosphate. We previously showed

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that TP is identical to platelet-derived endothelial cell growth factor, an angiogenic factor, and is expressed in many solid tumors (3). We also demonstrated that in cancers, TP promoted tumor angiogenesis, proliferation, and metastasis (4–6). The overexpression of TP has also been observed in inflammatory diseases such as RA, psoriasis, atherosclerosis, and chronic glomerulonephritis (7). In particular, the expression of TP in RA was significantly correlated with disease activity (8).

It has been reported that TP levels in synovial fluid and serum were higher in patients with RA than in patients with osteoarthritis (OA) and healthy control subjects (9), and that TP was induced by TNF α , IL-1, IL-6, and IL-8 in RA fibroblast-like synoviocytes (FLS) (8). Although the molecular basis for TP induction by these cytokines is not fully understood, previous studies demonstrated that the TP promoter region contains a cluster of 6–9 Sp1-binding motifs (7), and that TNF α induced the expression of TP through the activation of transcription factor Sp1 in RA FLS (10). These findings indicate the putative mechanisms underlying how TNF α stimulates the expression of TP.

RA is characterized as a Th1 disease, and the Th1/Th2 cytokine imbalance with a predominance of Th1 cytokines, including IFN γ , has been suggested to be important in the pathogenesis of RA (11). Th1 cells express particular chemokine receptors such as CXCR3 and CCR5 (12), and IFN γ -inducible protein 10 (CXCL10) is one of the CXCR3-binding ligands. CXCL10 has been detected in the sera, synovial fluid, and synovial tissue of patients with RA (13). CXCL10 in RA has been shown to promote FLS invasion (14) and increase the expression of RANKL in CD4+ T cells; CXCL10 also had a potential role in joint erosion (15). These findings suggest that CXCL10 is implicated in the pathogenesis of RA.

In this study, we investigated whether and how TP is involved in the pathogenesis of RA, using RA FLS. We observed that TP regulated the expression of cytokines, including CXCL10, in RA FLS. TP may contribute to the pathogenesis of RA, such as Th1 cell predominance, invasion of FLS, and bone destruction, through CXCL10 expression.

MATERIALS AND METHODS

Cell culture. FLS were obtained by collagenase digestion of synovial tissue from 2 patients with RA (named KTZRA cells and IORA cells) and 2 patients with OA (named K604OA cells and SNOA cells), as described previously (16,17). The human embryonic lung fibroblast cell line MRC-5 was purchased from ATCC. Cells were grown in Dulbecco's

modified Eagle's medium (Nissui Seiyaku) containing 10% fetal bovine serum, 2 mM glutamine, and 100 units/ml penicillin at 37°C in a humidified atmosphere of 5% CO₂. All experiments were performed using FLS from passages 3–5.

Reagents. Recombinant human TNF α (rhTNF α), rhIL-1 β , rhIL-6, and rhIL-8 were purchased from R&D Systems. Recombinant human IFN γ , rhIL-17, lipopolysaccharide (LPS), and EUK-8 were purchased from PeproTech, HumanZyme, Sigma-Aldrich, and Calbiochem, respectively. The TP inhibitor (TPI) 5-chloro-6-[1-(2-iminopyrrolidignyl) methyl] uracil hydrochloride was supplied by Taiho Pharmaceutical.

Transfection. Full-length *TYMP* complementary DNA (cDNA) was provided by Dr. K. Miyazono and Dr. C-H. Heldin (Ludwig Institute for Cancer Research, Uppsala, Sweden). *TYMP* and scrambled small interfering RNA (siRNA) duplexes were purchased from Sigma. The transfection of *TYMP* cDNA and the transfection of *TYMP* and scrambled siRNA in FLS were carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Real-time polymerase chain reaction (PCR) analysis. Total cellular RNA was extracted from cells, using TRIzol reagent according to the manufacturer's instructions (Invitrogen). One microgram of RNA was reverse transcribed using a ReverTra Ace α First Strand cDNA Synthesis Kit (Toyobo). Quantitative real-time PCR was performed using SYBR Premix Ex Taq (Takara) on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) according to the manufacturer's instructions. Quantitative measurements were determined using the $\Delta\Delta C_t$ method, and the expression of GAPDH was used as an internal control. Melting curve analyses of all real-time PCR products were performed and shown to produce the sole DNA duplex. The primers used were as follows: for *TYMP*, 5'-GCTGGAGTCTATTCCTGGATTC-3' (sense) and 5'-ACTGAGAATGGAGGCTGTGATG-3' (antisense); for *CXCL10*, 5'-TGACTCTAAGTGGCATTCAAG-3' (sense) and 5'-CAGGTACAGCGTACAGTTCT-3' (antisense); for *CXCL11*, 5'-AAGCCTCCATAATGTACCA-3' (sense) and 5'-TATAAGCCTTGCTTGCTTCG-3' (antisense); for CD40, 5'-TTTCTGATACCATCTGCGAGC-3' (sense) and 5'-CAACCAGGTCTTTGGTCTCAC-3' (antisense); for *CCL3*, 5'-AACCAGTTCTCTGCATCACT-3' (sense) and 5'-GCTCGTCTCAAAGTAGTCAG-3' (antisense); for *IL32*, 5'-ACAACAA GGAACACTCTGTG-3' (sense) and 5'-AAACACTCCAGGATCAGTCT-3' (antisense); for *CCL7*, 5'-AATACTTCAACTACCTGCTGC-3' (sense) and 5'-CACAGATCTCCTTGTCAGT-3' (antisense); for *ANGPTL4*, 5'-TGGAGGCTGGACAGTAATTC-3' (sense) and 5'-GTGATGCTATGCACCTTCTC-3' (antisense); for *ADAMTS4*, 5'-ATTGGCTCCAA GAAGAAGTTT-3' (sense) and 5'-ACCACATTGTTGTATCCGTAC-3' (antisense).

Immunoblot analysis. Whole cell extracts were prepared with M-PER reagent (Thermo Scientific) containing phosphatase and protease inhibitor cocktails (Roche). The concentrations of protein were measured using the Bradford method. Fifty micrograms of total cell extract protein was electrophoresed on 4–10% NuPAGE Bis-Tris Mini Gels (200V for 40 minutes). Gel proteins were then electrophoretically transferred onto polyvinylidene difluoride membranes (Millipore) using a Bio-Rad Trans-Blot SD system (18). The membrane was treated with the blocking agent Blocking One

(Nacalai Tesque) for 1 hour and incubated with the mouse monoclonal antibody against human TP, as described previously (19), or with anti- β -actin antibody (1:1,000 dilution; Santa Cruz Biotechnology) or anti- α -tubulin antibody (Calbiochem) overnight at 4°C. Following 4 washes, the membrane was incubated with horseradish peroxidase-conjugated secondary antibodies (Amersham Pharmacia) in the buffer for 1 hour at room temperature. The membrane was then washed and developed using an Enhanced Chemiluminescence Western blotting Detection system (Amersham Pharmacia). Finally, immunoreactive bands were read under an LAS-4000 mini luminescent image analyzer (Fujifilm Medical Systems).

Microarray analysis. Total RNA from FLS was isolated using an RNeasy Mini Kit (Qiagen). The relative purity of the RNA was measured using an Agilent 2100 Bioanalyzer. Total RNA (130 ng) was amplified and labeled using an Agilent Low Input Quick Amp Labeling Kit, One-Color (5190-2305), and labeled RNA was hybridized with a Human Gene Expression 4x44K v2 Microarray Kit (catalog no. G4845A). Agilent Feature Extraction version 10.7.3 image analysis software was used to extract data from raw microarray image files. Data visualization and analysis were performed using GeneSpring GX version 12.1 software.

Enzyme-linked immunosorbent assays (ELISAs) of CXCL10. CXCL10 concentrations in the culture medium were determined by ELISA (R&D Systems) according to the manufacturer's instructions.

Published microarray data sets. Public microarray data sets (the GSE1919 [Ungethuen] data set and the GSE39340 [Chang] data set) were downloaded from the GEO database. Data visualization and analysis were performed using GeneSpring GX version 12.1 software. Monochannel data in the Ungethuen data set and the Chang data set were normalized using the robust multiarray average and the 75th percentile shift, respectively.

Statistical analysis. Results were analyzed using GraphPad Prism version 5.0 software. Statistical analyses for the experiments were carried out using one-way analysis of variance. Statistical analyses for microarray data obtained from GEO were carried out using the Kruskal-Wallis test and Spearman's correlation. Data are presented as the mean \pm SD. *P* values less than 0.05 were considered significant.

RESULTS

Inflammatory cytokine-augmented TP expression in FLS. Previous studies indicated that TNF α induced the expression of TP in RA FLS (8). We observed TP expression in both KTZRA cells (Figure 1A) and IORA cells (results not shown) treated with TNF α . The expression of *TYMP* messenger RNA (mRNA) in RA FLS increased in a time-dependent manner and reached a maximum level 36 hours after TNF α stimulation and also increased with increasing concentrations of TNF α up to 1,000 pg/ml (Figure 1A). The expression of *TYMP* mRNA also increased with increasing concentrations of TNF α up to 1,000 pg/ml in OA FLS but decreased in MRC-5 cells (data not shown).

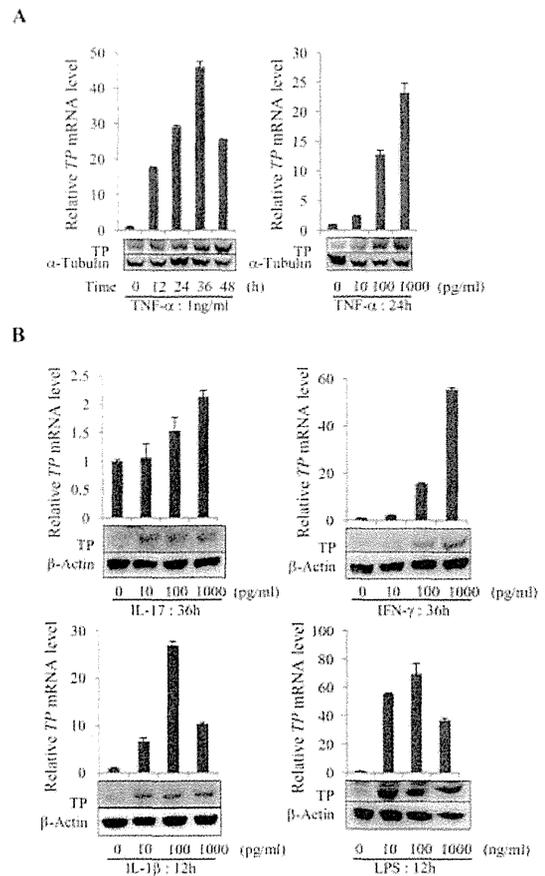


Figure 1. Thymidine phosphorylase gene (*TP* [*TYMP*]) expression in rheumatoid arthritis fibroblast-like synoviocytes. **A**, Expression of *TP* mRNA and protein in KTZRA cells incubated in the presence of tumor necrosis factor α (TNF α) for the indicated periods of time (left) or in the presence of various concentrations of TNF α for 24 hours (right). **B**, Expression of *TP* mRNA and protein in KTZRA cells incubated in the presence of various concentrations of interleukin-17 (IL-17), interferon- γ (IFN γ), IL-1 β , or lipopolysaccharide (LPS). Messenger RNA levels were determined by real-time polymerase chain reaction, and protein levels were assessed by immunoblotting. Values are the mean \pm SD of triplicate experiments.

Additionally, the expression of *TYMP* mRNA in RA FLS increased in a time-dependent manner after treatment with IL-17, IFN γ , IL-1 β , and LPS but not after treatment with IL-6 or IL-8 (data not shown). *TYMP* mRNA expression also increased in a dose-dependent manner following treatment with IL-17 and IFN γ , and also partially with IL-1 β and LPS (Figure 1B). These results suggested that various cytokines that were associated with RA disease activity augmented the expression of TP in FLS.