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# Cyclic Guanosine Monophosphate (cGMP)- Dependent Protein Kinase II Blocks Epidermal Growth Factor (EGF)/Epidermal Growth Factor Receptor (EGFR)-Induced Biological Effects on Osteosarcoma Cells

Authors' Contribution:  
Study Design A  
Data Collection B  
Statistical Analysis C  
Data Interpretation D  
Manuscript Preparation E  
Literature Search F  
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**Background:** The present work was performed to detect the potential inhibitory effect of cyclic guanosine monophosphate (cGMP)-dependent protein kinase II (PKG II) on epidermal growth factor (EGF) receptor-induced biological activity and related signal cascades in osteosarcoma cells.





**Material/Methods:** We transfected the osteosarcoma MG-63 cell line with an adenoviral vector encoding PKG II cDNA (Ad-PKGII) and incubated the transfected cells with 250  $\mu$ M 8-pCPT-cGMP to activate the PKG II. We stimulated the MG-63 cells with 100 ng/ml EGF, and then detected their proliferation using a CCK-8 assay. Transwell assay was used to examine MG-63 cell migration; and Western blot analysis was used to detect expression of matrix metalloproteinase 9 (MMP-9) and activation of ERK and Akt.

**Results:** Stimulating cells by 100 ng/ml EGF promoted MG-63 cell proliferation and migration, ERK and Akt phosphorylation, and MMP-9 expression. These effects of EGF were inhibited in MG-63 cells infected with Ad-PKGII and incubated with 8-pCPT-cGMP.

**Conclusions:** Our results demonstrate that Ad-PKGII infection significantly inhibited EGF-induced proliferation and migration, as well as the associated-signal cascades; which indicates that PKG II might be a potential anti-cancer factor.

**MeSH Keywords:** **Cell Proliferation • Cyclic GMP-Dependent Protein Kinase Type II • Epidermal Growth Factor • Osteosarcoma • Receptor, Epidermal Growth Factor • Transcellular Cell Migration**

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## Background

Osteosarcoma (OS) is the third most common malignant tumor in adolescents, and is the most common primary bone malignant tumor [1]. OS has been proposed to originate from mesenchymal stem cells or osteoblasts, based on different clinical and experimental data [2]. OS occurs predominantly in the long bones, specifically in the metaphyseal areas [3], and exhibits a high degree of malignancy, rapid growth, and aggressive invasion. Hematogenous metastasis occurs early, with high incidence of lung metastasis [4]. The main clinical treatment of OS is definitive surgery with radiotherapy and chemotherapy [5]. However, the mortality rate remains high, especially in patients with metastatic disease.

Epidermal growth factor receptor (EGFR) is a membrane surface receptor with tyrosine kinase activity. EGF binds to EGFR and causes EGFR-specific tyrosine residue phosphorylation, thereby leading to activation of signal transduction pathways, including MAPK/ERK and PI3K/Akt pathways [6–8]. EGFR is highly expressed in a variety of human malignancies [9,10]. In recent years, important advances have been made in the study of EGFR, including indications that EGFR-mediated signal transduction is closely linked to the occurrence, development, and prognosis of malignant tumors [11,12]. EGFR expression in OS is well documented [13–15], and correlates with worse prognosis. EGFR may play an important role in OS development; therefore, the EGFR pathway inhibition could represent a viable treatment option for OS [16].

PKG II, a serine/threonine protein kinase, exists in eukaryotic cells and is abundant in the brain and intestinal mucosa [17]. The function of PKG II was long unclear. However, in recent years, our studies [18–22] have provided data showing that PKG II is closely associated with cell proliferation and migration, and the occurrence and development of tumors. The present work aimed to detect the inhibitory effect of PKG II on OS cell proliferation, migration, and other biological activities induced by activation of EGFR.

## Material and Methods

### Cell lines and reagents

The MG-63 human osteosarcoma cell line was provided by the Institute of Cell Biology (Shanghai, China). Adenoviral vectors containing cDNA encoding  $\beta$ -galactosidase (pAd-LacZ) and PKG II (pAd-PKG II) were kind gifts from Prof. Gerry and Prof. Renate, University of California, San Diego, CA, USA. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco (Grand Island, NY, USA). The following primary antibodies were used in this study: anti-PKG

II (Abgent, San Diego, CA, USA), goat anti- $\beta$ -actin (Santa Cruz, CA, USA), mouse anti-phosphorylated EGFR (tyrosine 1068); (Cell Signaling, Danvers, MA, USA), rabbit anti-EGFR (Cell Signaling), rabbit anti-p-ERK (thr202/tyr204; Cell Signaling), and rabbit anti-ERK (Cell Signaling). HRP-conjugated secondary antibodies were obtained from Santa Cruz. EGF was purchased from Sigma (St. Louis, MO, USA); electrochemiluminescence reagents were obtained from Millipore (Billerica, MA, USA); and the Transwell system was purchased from Corning (Tewksbury, MA, USA).

### Cell culture and adenoviral vector infection

MG-63 cells were cultured in DMEM with 10% FBS at 37°C and 5% CO<sub>2</sub>. The cells were sub-cultured when they reached 90% confluence. The cells were freshly plated at 70–80% confluence, and the infection was subsequently performed following our lab's protocol [19].

### Cell-counting kit (CCK)-8 assay

MG-63 cells were plated in 96-well plates (5×10<sup>3</sup> cells per well). After the cells were treated, 10  $\mu$ l CCK-8 was added to every well, and then incubated for 2 h. The optical density at 450 nm was detected using a microplate reader. The data are presented as a percentage of the value obtained for the control group.

### Cell migration assay

We plated 5×10<sup>3</sup> cells into the upper chamber of a Transwell system containing DMEM without FBS. Cells that migrated to the bottom side of the membrane were induced by DMEM containing 10% FBS in the lower chamber for 12 h at 37°C. The cells were then fixed with 4% paraformaldehyde solution for 30 min and stained with Giemsa solution for 10 min. The stained cells were examined with a microscope. The cells that migrated were counted in 5 randomly selected fields per insert.

### Western blotting

MG-63 cells were lysed in radioimmunoprecipitation assay buffer supplemented with proteinase inhibitors. Equal amounts of total protein were loaded onto and separated on a 10% polyacrylamide gel by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Following electrophoresis, the proteins were transferred to a polyvinylidene difluoride membrane (PVDF), which was then blocked in 5% (w/v) non-fat milk and incubated with primary antibodies overnight at 4°C. The membrane was washed 3 times with Tris-buffered saline/Tween, then incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at 37°C. Electrochemiluminescence was used to visualize positive bands on the membrane.

## Statistical analysis

Statistical analyses were performed using the Statistical Package for Social Sciences System Version 14.0 (SPSS, Chicago, IL, USA). Data are shown as means  $\pm$  standard deviation (SD). Statistical significance was assessed using ANOVA, and  $P < 0.05$  was considered statistical significance.

## Results

### PKG II inhibits EGF-induced cell proliferation

CCK-8 assays were performed to assess the effect of PKG II on EGF-stimulated proliferation of MG-63 cells. EGF treatment resulted in 75–90% more cells than control, which was abrogated by cGMP in Ad-PKG II transduced MG-63 cells ( $P < 0.05$  Ad-PKG II + cGMP + EGF vs. Ad-PKG II + EGF and Ad-PKG II + cGMP + EGF vs. Ad-LacZ + cGMP + EGF) but not in parental MG-63 or Ad-LacZ transduced MG-63 cells (Figure 1).

### PKG II inhibits EGF-induced cell migration

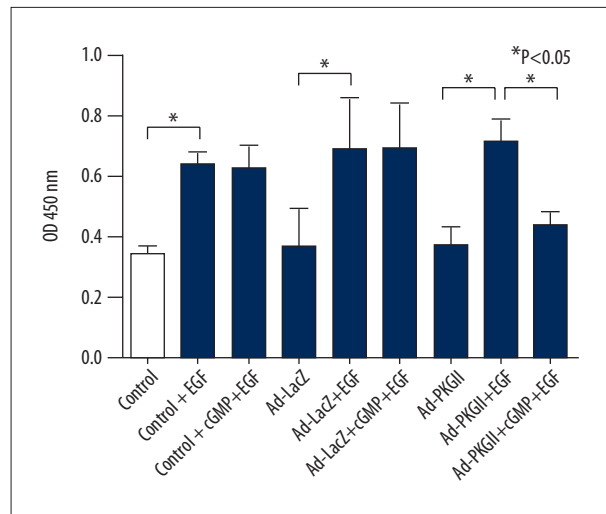
To investigate whether PKG II influenced MG-63 migration, Transwell migration assay was performed. EGF-treated MG-63 cells had more than 40% more migrating cells, which was abolished by cGMP in PKG II-overexpressed MG-63 cells ( $P < 0.05$  Ad-PKG II + cGMP + EGF vs. Ad-PKG II + EGF and Ad-PKG II + cGMP + EGF vs. Ad-LacZ + cGMP + EGF) but not in control Ad-LacZ MG-63 cells (Figure 2).

### PKG II inhibits EGF-induced MMP-9 expression

Because EGFR-induced MMP-9 played significant roles in osteosarcoma invasion [23], we then evaluated the effect of PKG II on EGFR-induced MMP-9 expression in MG-63 cells. Western blot analysis demonstrated that treating both Ad-LacZ- and Ad-PKG II-infected cells with 100 ng/ml EGF increased the expression of MMP-9. Moreover, Ad-PKG II infection facilitated the inhibition of EGF-induced MMP-9 expression (Ad-PKG II + cGMP + EGF group vs. Ad-PKG II + EGF group) (Figure 3).

### PKG II inhibits EGF-induced tyrosine 1068 phosphorylation of EGFR and its downstream pathways

To determine how activated PKG II inhibited EGFR-induced proliferation and migration of MG-63 osteosarcoma cells, we performed Western blot analysis with specific antibodies to monitor the changes of the phosphorylation of EGFR, ERK, and Akt. Treating both Ad-LacZ- and Ad-PKG II-infected cells with 100 ng/ml EGF promoted EGFR, ERK, and Akt phosphorylation (Ad-LacZ + EGF group vs. Ad-LacZ group; Ad-PKG II + EGF group vs. Ad-PKG II group) (Figure 4). EGF-induced EGFR activation and



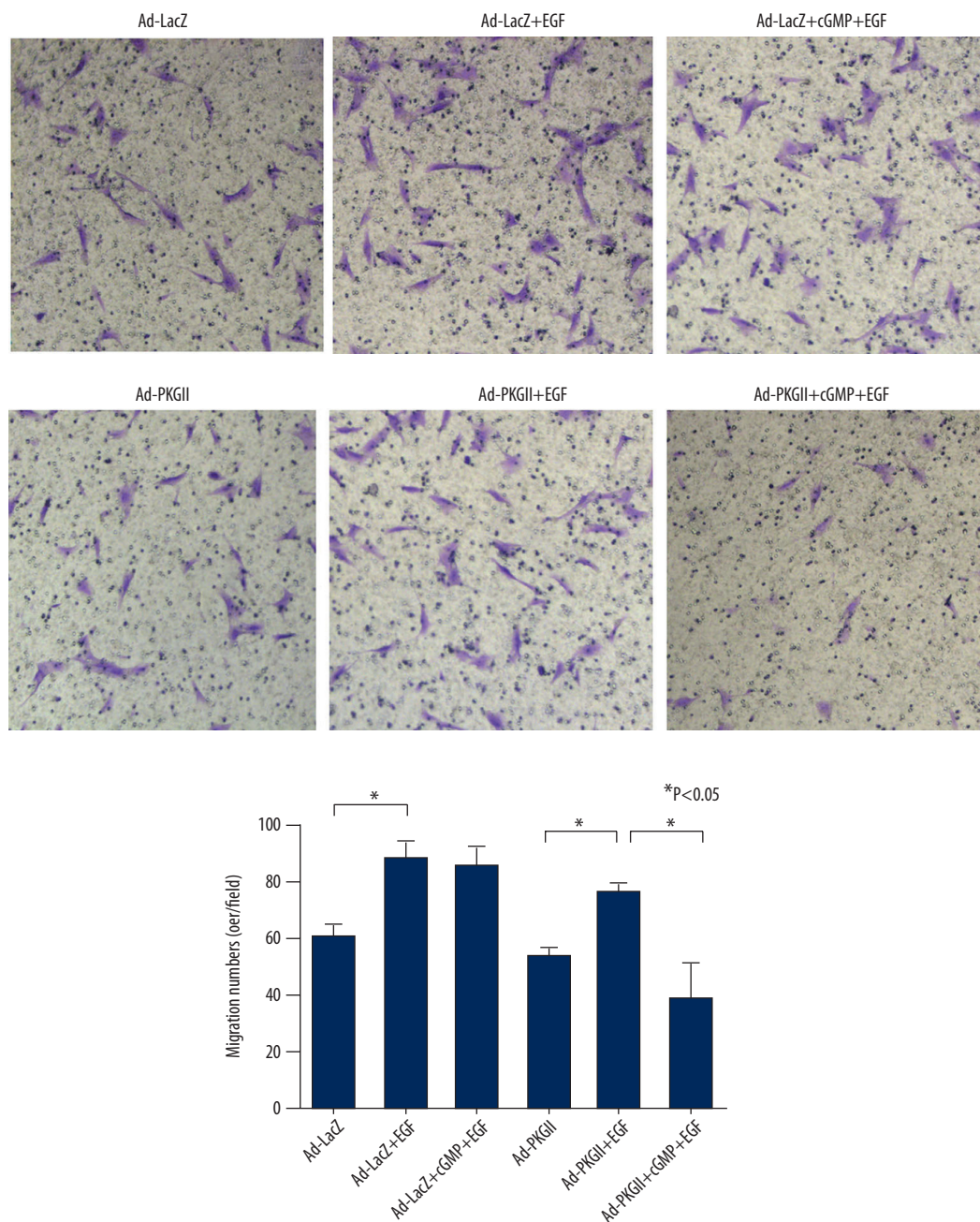
**Figure 1.** PKG II inhibits EGF-induced cell proliferation. The MG-63 cells were infected with Ad-LacZ or Ad-PKG II for 24 h and then placed in serum-free overnight. Next, the cells were incubated with 8-pCPT-cGMP (250  $\mu$ M, 1 h), and then treated with 100 ng/ml EGF for 24 h. Data are means  $\pm$ SD from 3 independent experiments.

phosphorylation of ERK and Akt were inhibited by cGMP only when PKG II was presented (Ad-PKG II + cGMP + EGF group vs. Ad-PKG II + EGF group) (Figure 4).

## Discussion

EGF treatment caused activation of EGFR and its downstream ERK and Akt, which in turn induced overexpression of MMP-9 and proliferation and migration of MG-63 osteosarcoma cells, which was abolished by cGMP-activated PKG II.

EGFR, a product of the proto-oncogene *c-erbB1*, is widely distributed on the surface of mammalian epithelial cells, fibroblasts, glial cells, keratinocytes, and other cell types. EGFR is a 170-kDa protein consisting of 3 domains: an extracellular domain that binds ligands, a transmembrane domain, and an intracellular domain with tyrosine kinase activity [24]. EGF binds with EGFR and induces tyrosine kinase phosphorylation of its intracellular domain [25], which then recruits effector proteins to its phosphorylated C-terminal domain and activates effector protein-mediated signal pathways that regulate proliferation, survival, differentiation, migration, invasion, and injury repair of cells [26]. EGFR is expressed in many malignant tumor cell types [27–29]. Overexpression of EGFR is associated with metastasis, invasion, and poor prognosis of cancers [30]. The present study indicated that EGFR activation induced proliferation and migration of MG-63 osteosarcoma cells.

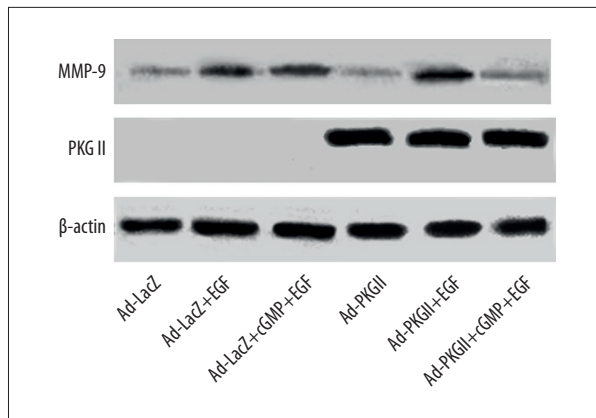


**Figure 2.** PKG II inhibits EGF-induced cell migration. The MG-63 cells were infected with Ad-LacZ or Ad-PKG II for 24 h and then seeded into the Transwell assay system. Treatment with 100 ng/ml EGF for 12 h can induce MG-63 cell migration (Ad-LacZ+EGF vs. Ad-LacZ, Ad-PKG II+EGF vs. Ad-PKG II). However, activated PKG II can inhibit EGF-induced cell migration (Ad-PKG II+cGMP+EGF vs. Ad-PKG II+EGF). Data are means ±SD from 3 independent experiments.

EGF, a ligand of EGFR, is an active polypeptide, that plays a critical role in intercellular interactions [7,31,32]. EGF binds with EGFR to induce cell growth, migration, and other biological activities [33]. EGF and EGFR also are involved in neoplastic

transformation. Up-regulated EGF or EGFR has been detected in various malignancies, including OS [34].

MAPK/ERK and the PI3K/Akt pathway are the most important signal transduction pathways downstream of EGFR [35,36].



**Figure 3.** PKG II inhibits expression of MMP-9 induced by EGF. The MG-63 cells were infected with Ad-LacZ or Ad-PKG II for 24 h and then placed on serum-free medium overnight. Next, the cells were incubated with 250  $\mu$ M 8-pCPT-cGMP for 1 h, and then treated with 100 ng/ml EGF for 48 h. Data are means  $\pm$ SD from 3 independent experiments.

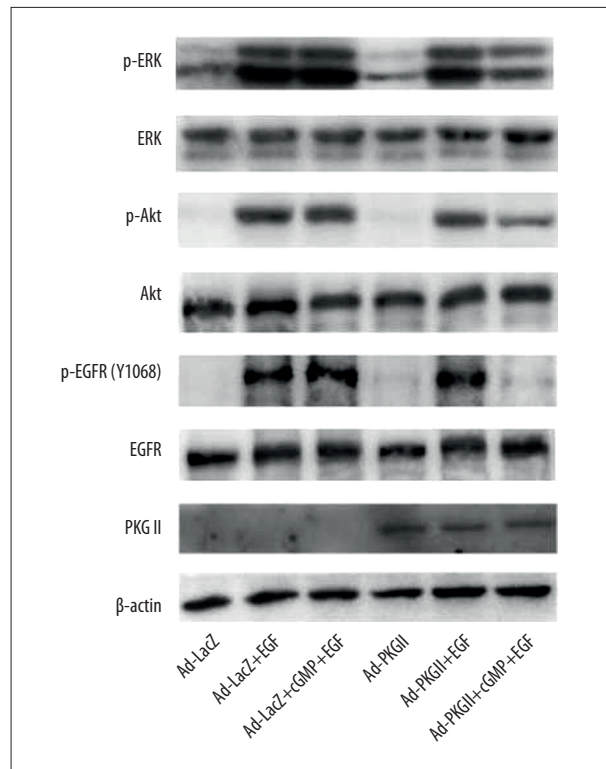
They are both related to cell proliferation, anti-apoptosis, angiogenesis, migration, adhesion, and invasion. Here, Western blot analyses revealed that EGF promoted the EGFR phosphorylation, and activated the ERK and Akt cascades.

## Conclusions

PKG II is a major downstream effector of cGMP in chondrocytes [37], and PKG II-deficient mice show dwarfism due to impaired endochondral ossification. Our previous results indicated that PKG II may be a potential cancer suppressor by blocking proliferation and migration and inducing apoptosis of tumor cells [22,38]. In the present study, we demonstrated that activated PKG II inhibited the proliferation and migration of MG-63 cells induced by EGFR activation. Furthermore, we found that activated PKG II inhibited EGF-induced activation

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**Figure 4.** PKG II inhibits activation of ERK and Akt induced by EGF. The MG-63 cells were infected with Ad-LacZ or Ad-PKG II for 24 h and then placed on serum-free medium overnight. Cells were incubated with 8-pCPT-cGMP (250  $\mu$ M, 1 h), and then treated with 100 ng/ml EGF for 5 h. Data are means  $\pm$ SD from 3 independent experiments.

of the PI3K/Akt and ERK pathways in MG-63 cells, indicating that PKG II is a potential cancer suppressor of OS.

## Conflict of interests

None.

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