*Original Article*

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# Platelet-derived growth factor receptor kinase inhibitor AG-1295 promotes osteoblast differentiation in MC3T3-E1 cells *via* the Erk pathway

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**Summary** **Previous studies have conflicting views on the effect of platelet-derived growth factor (PDGF)/PDGF receptor (PDGFR) signaling on osteogenesis. The current study investigated the effect of PDGF receptor-beta (PDGFR-β) inhibition by AG-1295 on the osteogenic differentiation of the mouse pre-osteoblastic cell line MC3T3-E1. Osteogenic differentiation was induced by treatment with β-glycerophosphate, ascorbic acid, and dexamethasone along with or absent AG-1295. Results showed that AG-1295 significantly increased alkaline phosphatase (ALP) activity and enhanced the formation of mineralized nodules in a dose-dependent manner. Furthermore, treatment with AG-1295 resulted in up-regulated mRNA expression of the osteogenic marker genes collagen type I (*Col1A*), runt-related transcription factor 2 (*Runx2*), osterix (*Osx*), tissue-nonspecific alkaline phosphatase (*Tnap*), and osteocalcin (*Ocn*). Consistent with its effect on osteoblast differentiation, AG-1295 also significantly suppressed the phosphorylation of Erk1/2 in MC3T3-E1 cells. In conclusion, findings suggest that blocking the PDGFR-β pathway with AG1295 markedly promotes osteoblast differentiation and matrix mineralization in mouse osteoblastic MC3T3-E1 cells and that the Erk1/2 pathway might participate in this process.**

***Keywords:*** Platelet-derived growth factor receptor-beta, AG-1295, extracellular signal-regulated kinases 1 and 2, matrix mineralization

## 1. Introduction

Platelet-derived growth factor receptors (PDGFR) are cell surface tyrosine kinase receptors for members of the PDGF family (*1*). PDGF/PDGFR signaling is reported to be involved in the regulation of various cell functions by activating three major signal transduction pathways including MAPK/Erk, PI3K, and PLC-γ (*2-5*).

Accumulating evidence suggests that PDGFR signaling plays an important role in the regulation of osteoblasts or mesenchymal stem cells (MSCs). Recent experiments suggest that activation of PDGFR signaling by PDGF factor BB plays a positive role in bone formation (*6,7*). Fierro *et al.* (*8*) showed that inhibition of PDGFR activity by imatinib *in vitro* partially suppressed osteogenesis of MSCs. That said, some literature has suggested that PDGFR signaling suppresses osteoblast differentiation (*9-11*). For example, Tokunaga *et al.* (*12*) found that knockout of the PDGFR-β gene in murine MSCs enhanced osteogenic differentiation. Clinically, long-term inhibition of PDGFR signaling by imatinib therapy has been reported to promote bone formation in patients with chronic myeloid leukemia (CML) (*11,13*). In particular, a recent study indicated that PDGFR signaling inhibition by AG-1296 does not significantly contribute to the osteogenic differentiation of MSC cells, as indicated by alkaline phosphatase (ALP)

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activity, osteogenic marker gene expression, and matrix calcium deposition staining (*14*).

Some previous studies, however, have reported that PDGFR signaling inhibits osteogenesis. Furthermore, the mechanisms by which PDGFR signaling inhibits osteogenic differentiation remain unclear. Therefore, the present study examined the effect of PDGFR-β inhibition by Tyrphostin AG-1295, a potent PDGFR-β blocker (*15*) on matrix mineralization in MC3T3-E1 cells. Findings suggest that blocking of PDGFR signaling by Tyrphostin AG-1295 promotes osteoblast differentiation and mineralization and that activation of the Erk1/2 pathway might be involved in this process.

## 2. Materials and Methods

### 2.1. *Chemicals and antibodies*

PDGFR inhibitor AG-1295 was purchased from Calbiochem (San Diego, CA, USA). Alpha-modified minimum essential medium (α-MEM), Dulbecco's modified Eagle's medium (DMEM), and Fetal bovine serum (FBS) were purchased from Gibco (Rockville, MD, USA). β-Glycerophosphate, ascorbic acid, and dexamethasone were purchased from Sigma (St. Louis, MO, USA). Rabbit monoclonal antibodies for Erk1/2 and p-Erk1/2 were obtained from Cell Signaling Technology (Beverly, MA, USA).

### 2.2. *Cells and osteoblast differentiation induction*

The osteoblastic cell line MC3T3-E1 (subclone 14), which was established from normal mouse calvaria (*16*), was purchased from the Cell Bank of the Type Culture Collection of the Chinese Academy of Sciences. MC3T3-E1 cells were cultured in α-MEM supplemented with 10% FBS and 1% penicillin-streptomycin. To stimulate mineralization, MC3T3-E1 cells at $1 \times 10^4$ cells/well were first cultured in 24 or 6-well plates to 80% to 90% confluence, and then cells were placed in osteogenic media (DMEM with 10% FBS, 1% penicillin-streptomycin, 10 mmol/L β-glycerophosphate, 50 μg/mL ascorbic acid, and 10 nmol/L dexamethasone) in the absence (the positive control group) or presence of AG-1295 (1, 10, and 20 μmol/L) for the times indicated. The negative control group was placed in basic medium (DMEM with 10% FBS, 1% penicillin-streptomycin) alone.

### 2.3. *ALP activity*

MC3T3-E1 cells were cultured in 24-well ($1 \times 10^4$ cells/well) plates during osteogenic induction as described above. After treatment, cell lysates were prepared with 100 μL assay buffer containing 25 mM Tris-HCl (pH 7.4) and 0.5% Triton X-100. Fifty μL of each sample was mixed with 100 μL *p*-nitrophenyl phosphate (PNPP) substrate (Sigma) and cells were incubated at 37°C for 30 min. The absorbance of the colored product was detected at 405 nm. Cellular ALP activity was normalized to total protein content using the bicin-choninic acid (BCA) method.

### 2.4. *Analysis of mineralization*

After osteogenic induction, mineral deposition was assessed by staining with Alizarin-Red on days 21, 25, and 30. The cells were fixed in formalin for 20 min at room temperature and washed with distilled water. A 2% Alizarin Red solution was added to the fixed cells and cells were incubated for 10-20 min. Culture plates were rinsing with distilled water and then imaged.

### 2.5. *Quantitative real-time polymerase chain reaction (RT-qPCR)*

Total RNA was prepared at the indicated time points using Total RNA Kit I (Omega, Bio-Tek, Norcross, GA, USA) in accordance with the manufacturer's instructions. Complementary DNA (cDNA) was synthesized using ReverTra Ace qPCR RT Kit (Toyobo). Real-time PCR was performed as usual with SYBR Green mix (Toyobo, Osaka, Japan) and a Lightcycler 480 (Roche Applied Science, Mannheim, Germany). Gene-specific primer sequences are listed in Table 1. Gene expression was normalized with the acidic ribosomal phosphoprotein P0 (*36B4*) of the mouse housekeeping gene (*17*). Cycling conditions were 94° C, 15 min, followed by 40 cycles of 94°C, 15 sec; 57°C, 20 sec; and 72°C, 10 sec.

### 2.6. *Western blotting*

After treatment, cells were lysed in lysis buffer (50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1 mmol/L ethylenediaminetetraacetic acid, 0.25% Na-deoxycholate, 1% NP-40, 1 mmol/L phenylmethane-sulfonylfluoride, 1 mmol/L sodium orthovanadate, 1 μg/mL leupeptin, 1 μg/mL aprotinin, and 1 μg/mL pepstatin). Equivalent amounts (25 μg) of protein were separated on 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels and subsequently transferred to Hybond-enhanced chemiluminescence (ECL) nitrocellulose membranes. Membranes were probed with antibodies against total Erk1/2, phospho- Erk1/2, and Actin. Protein bands were observed using ECL and specific bands were detected with X-film.

### 2.7. *Statistical analysis*

Statistically significant differences between groups were determined using an unpaired Student's *t*-test. Statistical significance was defined as $p < 0.05$.

## 3. Results

### 3.1. *PDGFR-β kinase inhibitor Tyrphostin AG1295 increased ALP activity during the early stages of osteoblast differentiation in MC3T3-E1 cells*

ALP activity was assessed as an early indicator of osteoblastic lineage to study the effect of AG-1295 on the osteogenic differentiation of MC3T3-E1 cells. ALP activity was determined on days 3, 6, 9, and 14 with osteogenic induction in the presence or absence of AG-1295 (1, 10, and 20 μmol/L). Figure 1 shows the ALP activity of MC3T3-E1 cells under different conditions. ALP activity in all groups of MC3T3-E1 cells peaked on day 9 and decreased on day 14, which is consistent with the results of previous studies. ALP activity increased significantly by 20 μmol/L AG-1295 on days 3, 6, and 9 ($p < 0.05$). However, there were no significant differences in ALP activity in the groups on day 14. This indicates that AG-1295 increased ALP activity during the early stages of osteoblast differentiation in MC3T3-E1 cells.

### 3.2. *PDGFR-β kinase inhibitor Tyrphostin AG-1295 enhanced matrix mineralization in MC3T3-E1 cells*

MC3T3-E1 cells were stained with Alizarin Red to detect nodule mineralization on days 21, 25, and 30. As shown in Figure 2, AG-1295 markedly enhanced nodule mineralization in a concentration-dependent manner. Cells treated with 10 μmol/L and 20 μmol/L AG-1295 had obvious mineralized nodules on day 21, while MC3T3-E1 cells cultured in osteogenic media had obvious mineralized nodules prior to day 30 (Figure 2).

### 3.3. *Effect of AG-1295 on the expression of osteoblast-specific marker genes in MC3T3-E1 cells during osteoblast induction*

Real-time PCR results for osteogenic marker genes collagen type I (*Col1A*), runt-related transcription factor 2 (*Runx2*), osterix (*Osx*), tissue-nonspecific alkaline phosphatase (*Tnap*), osteocalcin (*Ocn*), and progressive ankylosis (*AnK*) are shown in Figure 3. According to the measured level of ALP activity and Alizarin Red S staining, blocking PDGFR-β with AG-1295 could elevate the mRNA expression of most of the osteogenic markers investigated. After AG-1295 treatment, significantly up-regulated mRNA levels of *Col1A*, *Runx2*, *Osx*, *Tnap*, and *Ocn* were identified on days 9 and 14. However, AG-1295 down-regulated the expression of *Ank*.

### 3.4. *Effect of PDGF-β inhibition by AG-1295 on the Erk1/2 pathway in MC3T3-E1 cells*

To evaluate whether the Erk1/2 pathway is involved in the regulation of AG-1295 as part of mineralization in

**Table 1. Primer pairs used for quantitative real-time reverse transcription-polymerase chain reaction**

| Gene | Forward (5' to 3') | Reverse (5' to 3') | Reference |
|---|---|---|---|
| *Col1A* | CACCCCAGCCGCAAAGAGT | CGGGCAGAAAGCACAGCACT | (*23*) |
| *Tnap* | GGGGACATGCAGTATGAGTT | GGCCTGGTAGTTGTTGTGAG | (*23*) |
| *Runx2* | CTCAGTGATTTAGGGCGCATT | AGGGGTAAGACTGGTCATAGG | (*24*) |
| *Ocn* | TGCTTGTGACGAGCTATCAG | GAGGACAGGGAGGATCAAGT | (*24*) |
| *Osx* | GGAGGTTTCACTCCATTCCA | TAGAAGGAGCAAGGGGACAGA | (*25*) |
| *Ank* | GAACTATCTGCCGCAC | AGGCGAGTAAACGCAA | (*23*) |
| *36B4* | AAGCGCGTCCTGGCATTGTCT | CCGCAGGGGCAGCAGTGGT | (*24*) |

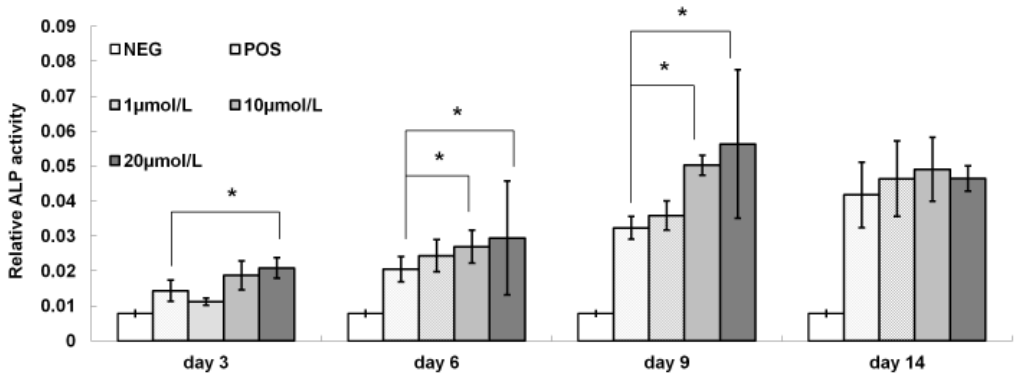


**Figure 1. The effect of Tyrphostin AG1295 treatment on alkaline phosphatase (ALP) activity in MC3T3-E1 cells.** Cells were incubated in osteogenic media with 0.1% DMSO (POS) and with 1, 10, and 20 μmol/L of Tyrphostin AG1295. ALP activity was determined on days 3, 6, 9, and 14. Cells cultured in basic medium containing 0.1% DMSO alone served as a negative control (NEG). Values are expressed as the mean ± S.D. ($n = 4$ per each group). Asterisks indicate statistically significant differences between groups (* $p < 0.05$).

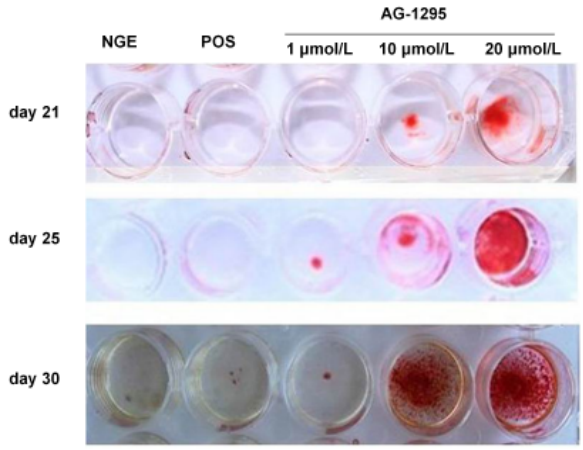


**Figure 2. The effect of Tyrphostin AG1295 treatment on the formation of mineralized nodules in MC3T3-E1 cells.** Cells were cultured in osteogenic media for 21, 25, and 30 days in the presence of 0.1% DMSO (POS) or 20 μmol/L Tyrphostin AG1295 and subjected to alizarin red staining. For the negative control (NEG), MC3T3-E1 cells were cultured in basic medium containing 0.1% DMSO alone.

MC3T3-E1, the expression of total and phosphorylated Erk1/2 protein was detected with Western blotting. As shown in Figure 4, a marked level of phosphorylated Erk1/2 protein was detected on 10 day of osteogenic induction, while no significant changes in the level of total Erk1/2 were noted. Furthermore, the phosphorylation activation of Erk1/2 was significantly suppressed by 20 μmol/L Tyrphostin AG-1295 in MC3T3-E1 cells cultured in osteogenic media on day 10.

## 4. Discussion

The current study demonstrated that blocking PDGFR signaling with Tyrphostin AG-1295 significantly promoted osteoblast mineralization. AG-1295 increased ALP activity in the early stages of osteoblast differentiation in MC3T3-E1 cells and enhanced matrix mineralization in the later stages of osteoblast differentiation. Like osteoblast phenotype induction,

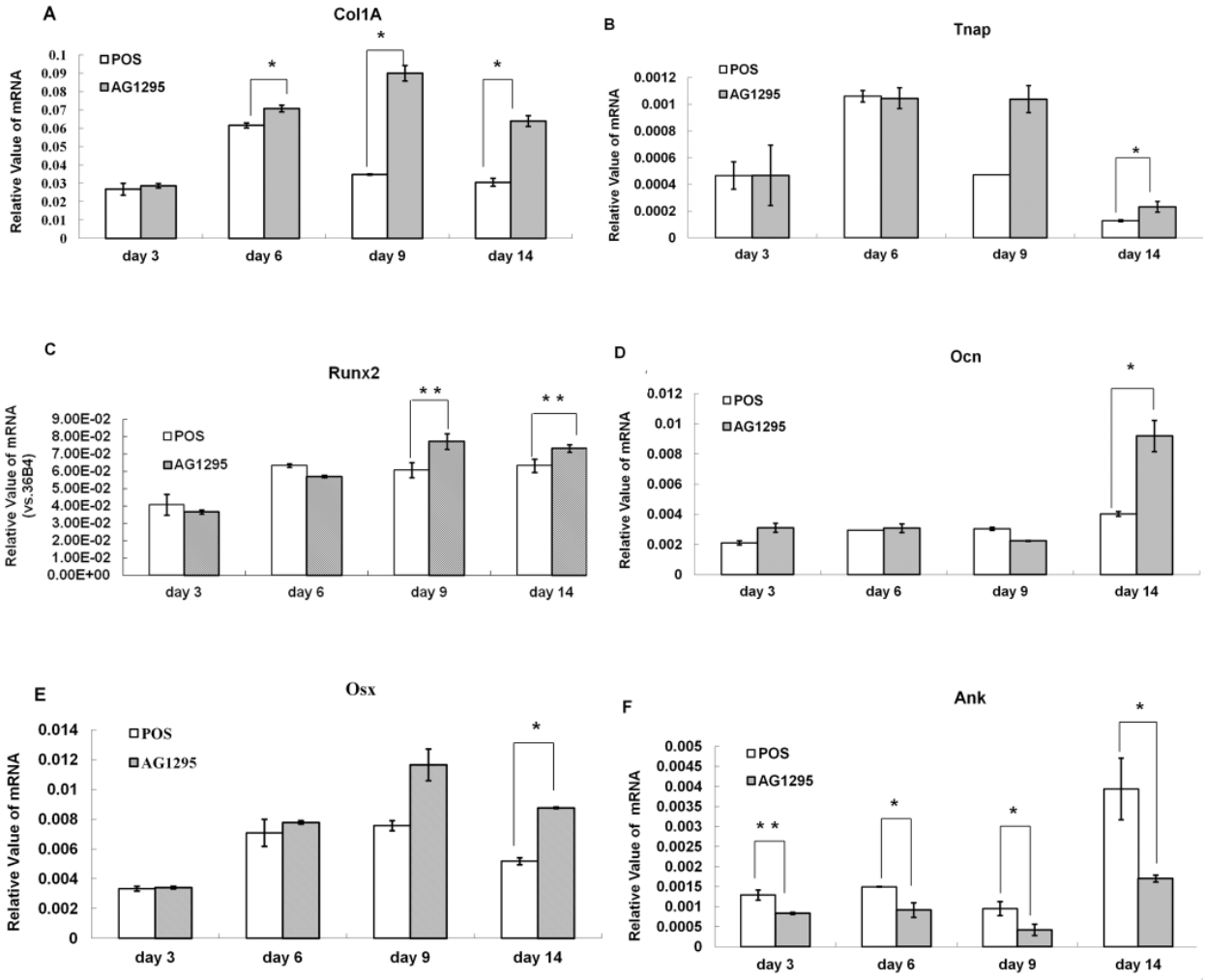


**Figure 3. Effect of PDGF receptor inhibition on osteogenic marker gene expression assessed by quantitative real-time reverse transcription-polymerase chain reaction.** Cells were grown in osteogenic media containing 0.1% DMSO (POS.) or 20 μmol/L Tyrphostin AG1295 for 3, 6, 9, and 14 days. Total RNA was extracted and measured by quantitative real-time polymerase chain reaction for *Col1A*, *Tnap*, *Runx2*, *Ocn*, *Osx*, and *Ank* mRNA expression. mRNA expression levels were normalized to 36B4. Results are expressed as mean arbitrary units ± S.D. ($n = 3$). Asterisks indicate statistically significant differences between groups ($^{*}\ p < 0.05$; $^{**}\ p < 0.01$).

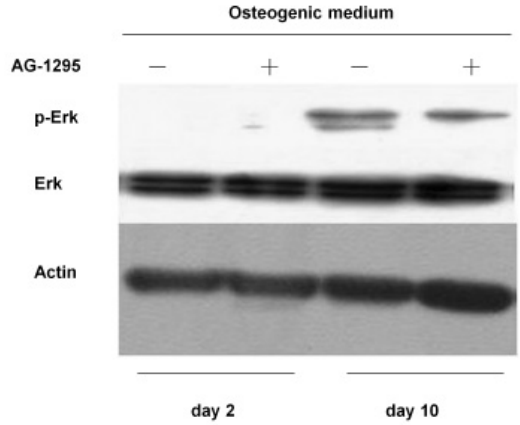


**Figure 4. Inhibition of Erk1/2 by MC3T3-E1 cells treated with Tyrphostin AG1295.** Cells were cultured in osteogenic media for 2 and 10 days in the presence of 0.1% DMSO or 20 μmol/L Tyrphostin AG1295, and cells were cultured in basic medium containing 0.1% DMSO alone as a negative control. At the appointed time, protein extraction and Western blotting were performed.

AG-1295 also increased the expression of most osteogenic markers. These findings suggest that PDGFR signaling suppresses osteoblast differentiation. The current findings differ from those of a previous study by Kumar *et al.*, in which another PDGFR inhibitor, AG-1296, failed to affect the osteogenic differentiation of human mesenchymal stem cells (MSCs). Differences may have arisen because of the different cell lines and PDGFR inhibitors used. Human MSCs give rise to osteoblastic lineages when cultured in specific differentiation media. Both MC3T3-E1 and human MSCs are commonly used in studies of osteogenic differentiation. They also have a similar mineralization process. Differences in results may be due primarily to different PDGFR inhibitors. AG-1296 has been found to inhibit PDGFR-α longer and greater than AG-1295, while AG-1295 is a specific inhibitor of PDGFR-β (*18,19*). Nemoto *et al.* (*20*) indicated that during formation of mineralized nodules PDGFR-β remained slightly elevated while PDGFR-α remained slightly depressed. Therefore, the current findings also suggest that PDGFR-β rather than PDGFR-α signaling is involved in the regulation of osteoblast differentiation.

The detailed mechanism for regulation of mineralization by PDGFR signaling is not clear at present. Several previous studies have shown that the Erk pathway, a downstream effector of PDGFR signaling, is essentially involved in regulation of matrix mineralization. Higuchi *et al.* (*21*) demonstrated that Erk inhibitor PD98059 promoted osteoblastic differentiation induced by BMP-2 in C2C12 pluripotent mesenchymal cells. Chaudhary and Avioli reported that Erk activation by PDGF factor BB or fibroblast growth factor-2 suppressed type I collagen expression in MC3T3-E1 cells (*22*). These findings suggest that the Erk pathway is a negative regulator of osteoblastic differentiation. To explore the possible role AG1295 plays in osteogenesis, its effect on the Erk1/2 pathway was investigated in MC3T3-E1 cells. As expected, the phosphorylation activation of Erk1/2 was significantly suppressed by 20 μmol/L Tyrphostin AG-1295 in MC3T3-E1 cells at day 10, which suggests that AG-1295 might promote osteoblast differentiation by suppressing the activation of Erk1/2, a downstream factor of the PDGFR pathway.

In summary, the present study has demonstrated that inhibition of PDGFR signaling by Tyrphostin AG-1295 significantly promoted osteogenesis *via* the Erk1/2 pathway. Findings suggest that PDGFR-β signaling might play an important role in osteoblast differentiation.

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