CHAPTER 4

The Effect of Platelet-Derived Growth Factor on Osteoprotegerin Production in Osteoblastic Cells

4.1 The effect of PDGF-AB on OPG and DKK-1 production in osteoblastic cells

Whilst investigating the effect of FCS on the production of OPG and DKK-1 production in MG63 cells, it was observed that PDGF-AB could stimulate the production of OPG. PDGF-AB is a potent growth factor, therefore the effect of PDGF-AB on the proliferation of osteoblastic cells was examined. Sub-confluent and confluent MG63 and Saos-2 cells were treated with 0-200ng/ml PDGF-AB for 24 hours. Estimation of cell number with the PI assay showed that in sub-confluent cells PDGF-AB significantly stimulated proliferation in both MG63 and Saos-2 cells ($P<0.001$, Fig 4.1). PDGF-AB did not effect proliferation in either MG63 or Saos-2 cells when confluent. All subsequent experiments therefore were performed on confluent cells.

MG63 and Saos-2 cells were incubated for 24 hours with 0-200ng/ml PDGF-AB in either complete medium or FCS-free medium. Culture media was assayed for OPG and DKK-1. In MG63 cells, OPG production was significantly increased in a dose dependent manner in response to PDGF-AB both in the presence and absence of FCS ($P<0.001$, Fig 4.2A). The lowest effective concentration of PDGF-AB to exhibit a significant response was 10ng/ml in the presence of FCS and 50ng/ml in the absence of FCS. In the presence of FCS, a maximal effect was observed around 100ng/ml where OPG production was significantly increased in MG63 cells by 183% compared to control ($P<0.001$). In the absence of FCS a maximal effect was observed around 50ng/ml where OPG production was significantly increased in MG63 cells by 528% compared to control ($P<0.001$).

DKK-1 production in MG63 cells was significantly increased in response to PDGF-AB in a dose dependent manner in the absence of FCS ($P<0.001$, Fig 4.2B). The lowest effective concentration of PDGF-AB to exhibit a significant response was 50ng/ml. A maximal effect was also observed around 100ng/ml where DKK-1 production was significantly increased in MG63 cells by 154% compared to control ($P<0.01$). PDGF-AB did not have a significant effect on the production of DKK-1 in MG63 cells in the presence of FCS.

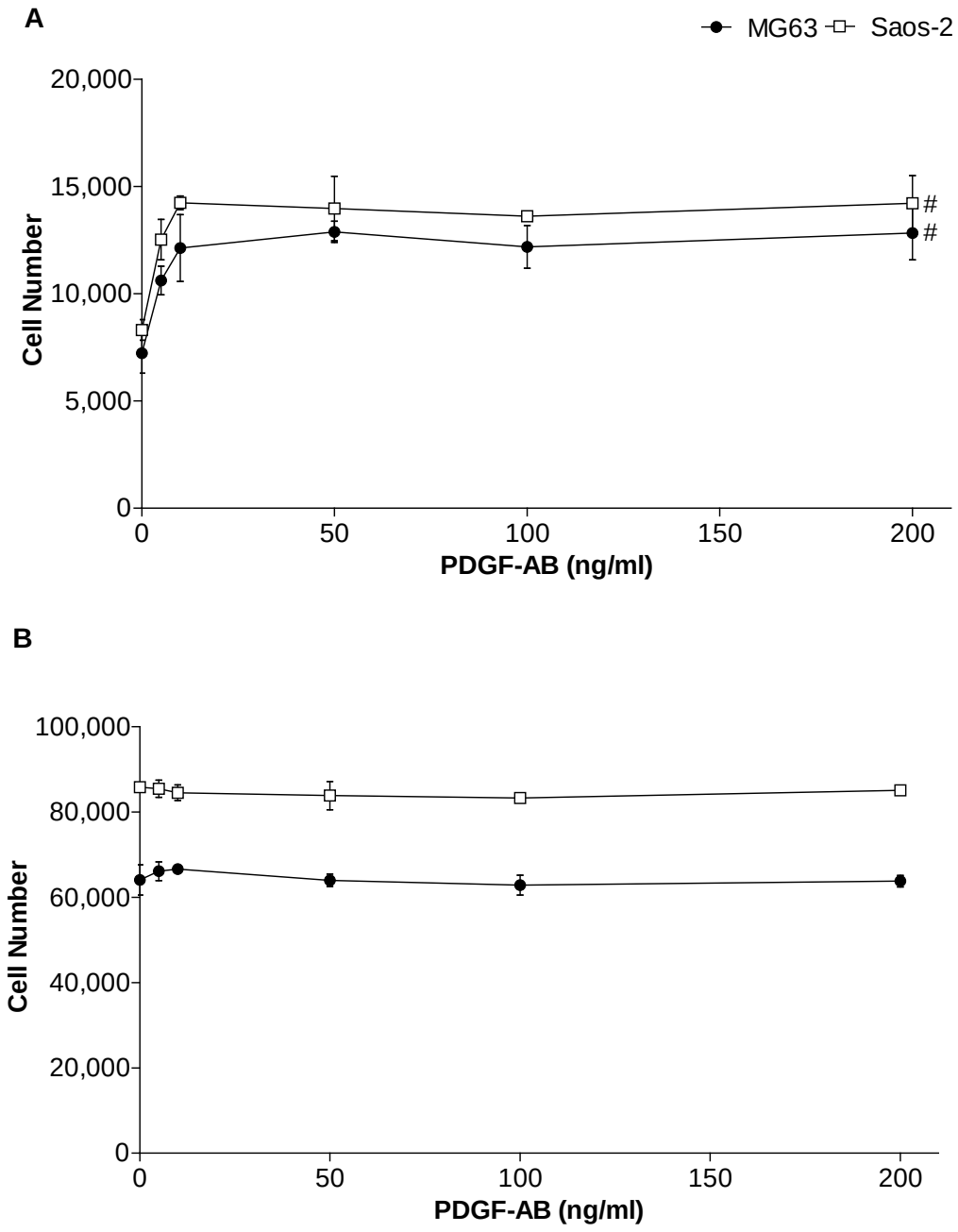

Fig 4.1 The effect of PGDF-AB on osteoblastic cell proliferation. A) Sub-confluent and B) confluent MG63 and Saos-2 cells were stimulated with various concentrations of PDGF-AB for 24 hours. Cell number was estimated using PI. Data shown are mean ± SD, n=4. #P<0.001 versus control (0ng/ml PDGF-AB, one-way ANOVA using Dunnett's multiple comparison test).

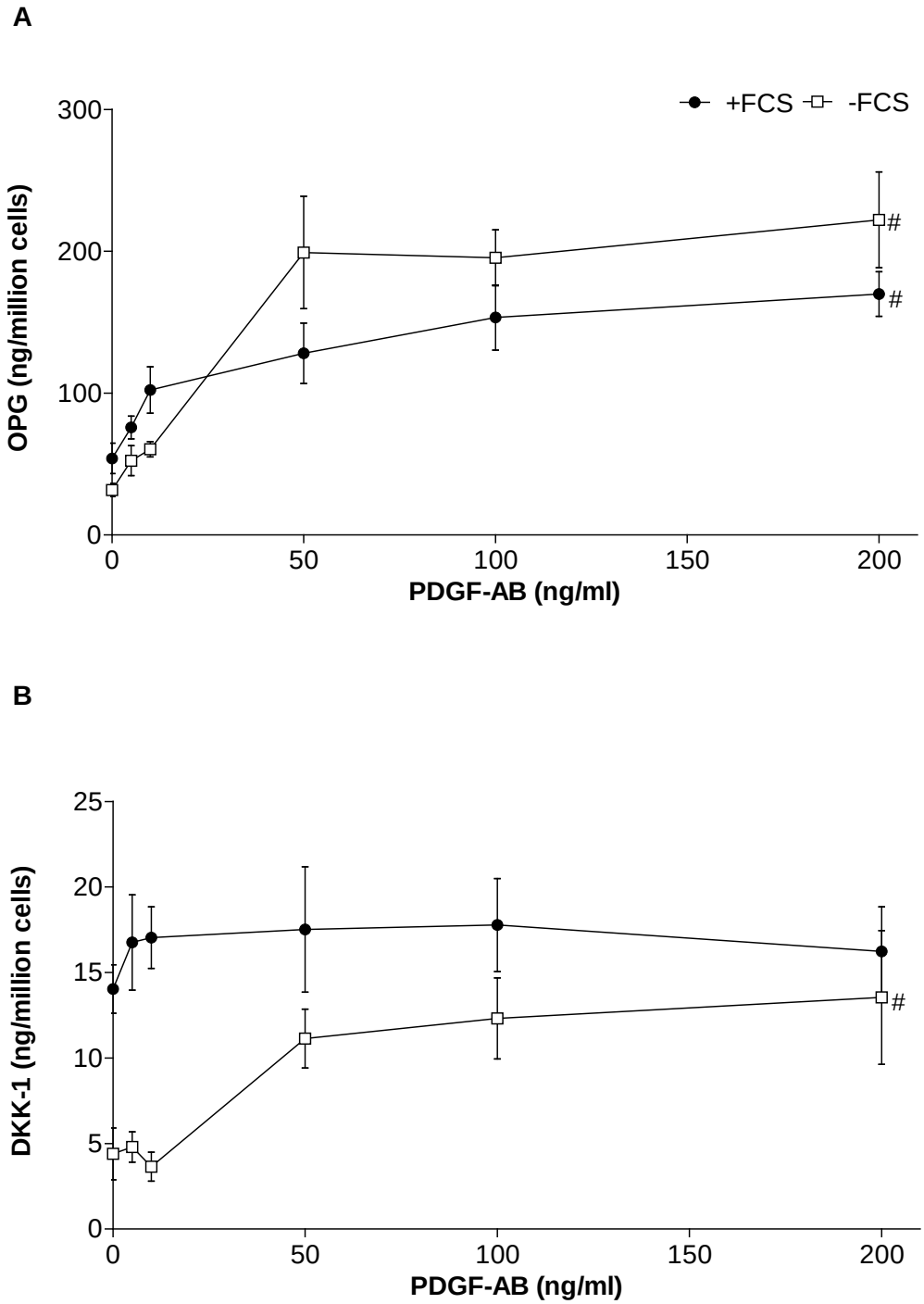

Fig 4.2 The effect of PDGF-AB on OPG and DKK-1 production in MG63 cells. MG63 cells were stimulated with 0-200ng/ml PDGF-AB for 24 hours with and without FCS. Culture medium was assayed for A) OPG and B) DKK-1. Data shown are mean ± SD, n=4. #P<0.001 versus 0ng/ml PDGF-AB (one-way ANOVA using Dunnett's multiple comparison test).

In Saos-2 cells, OPG production was significantly increased in a dose dependent manner in response to PDGF-AB both in the presence and absence of FCS ($P<0.001$, Fig 4.3A). The lowest effective concentration of PDGF-AB to exhibit a significant response was 10ng/ml in the presence of FCS and 50ng/ml in the absence of FCS. A maximal effect was observed around 100ng/ml where OPG production was significantly increased in Saos-2 cells by 218% and 187% compared to control in the presence and absence of FCS respectively ($P<0.001$).

DKK-1 production in Saos-2 cells was significantly decreased in response to PDGF-AB in a dose dependent manner in the absence of FCS ($P<0.001$, Fig 4.3B). The lowest effective concentration of PDGF-AB to exhibit a significant response was 5ng/ml. A maximal effect was observed around 50ng/ml where DKK-1 production was significantly decreased in Saos-2 cells by 73% compared to control ($P<0.01$). PDGF-AB did not have a significant effect on the production of DKK-1 in Saos-2 cells in the presence of FCS.

Despite PDGF-AB having significant effects on the production of both OPG and DKK-1 in MG63 and Saos-2 cells, this line of investigation was not pursued. There are likely to be many consequences for cellular metabolism of growing cells in the absence of FCS. Therefore, the remainder of these results will focus on cells cultured in complete medium.

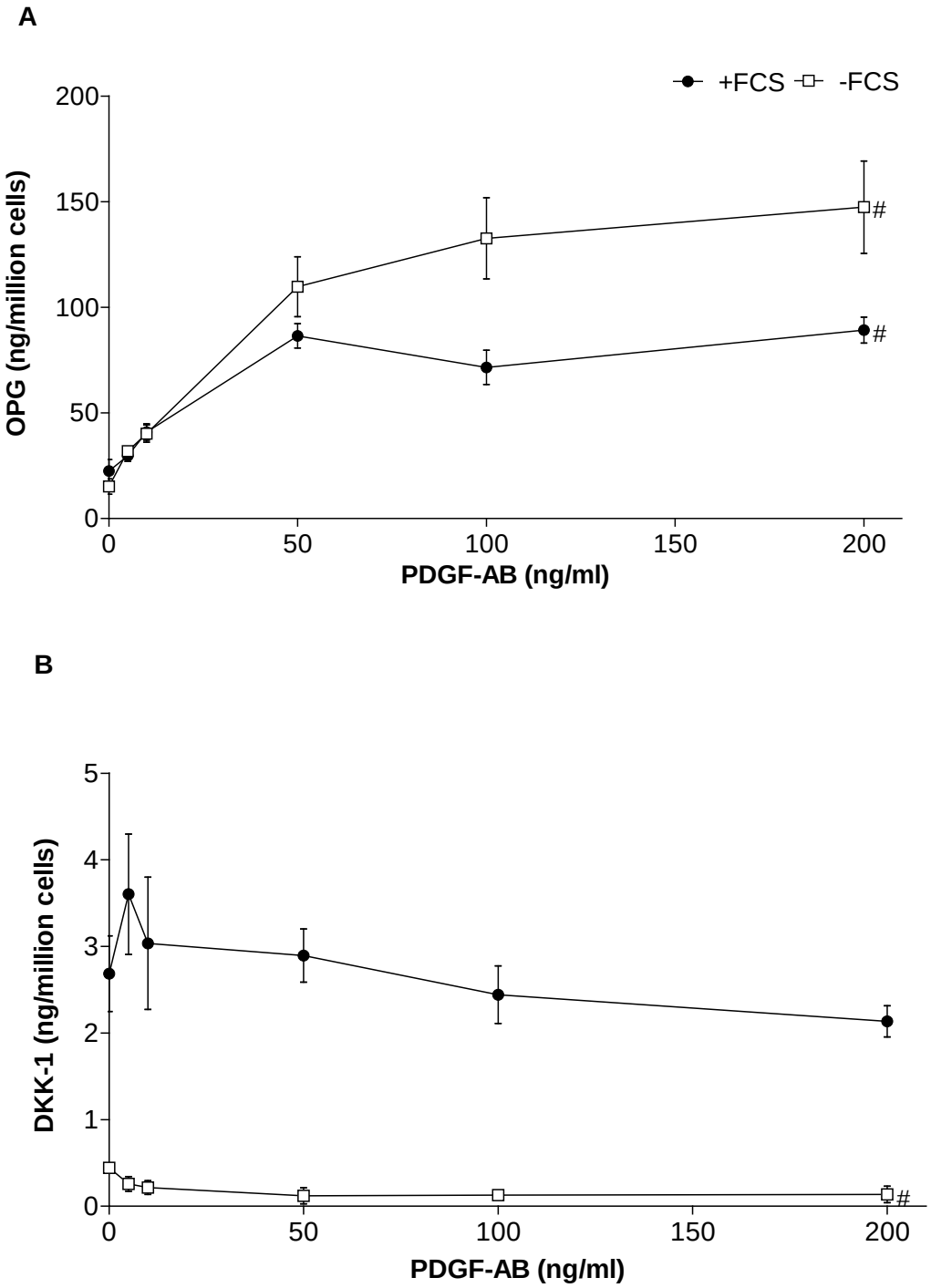

Fig 4.3 The effect of PDGF-AB on OPG and DKK-1 production in Saos-2 cells. Saos-2 cells were stimulated with 0-200ng/ml PDGF-AB for 24 hours with and without FCS. Culture medium was assayed for A) OPG and B) DKK-1. Data shown are mean ± SD, n=4. #P<0.001 versus 0ng/ml PDGF-AB (one-way ANOVA using Dunnett's multiple comparison test).

The time course of PDGF-AB on OPG production by MG63 and Saos-2 cells was determined. In control samples, production of OPG increased with time in both MG63 and Saos-2 ($P<0.001$, Fig 4.4). The earliest time to exhibit a significant response to PDGF-AB was 12 hours in MG63 cells and 6 hours in Saos-2 cells. At 24 hours, addition of 100ng/ml PDGF-AB had significantly increased the rate of OPG production above that of control at the same time point by 150% and 170% for MG63 cells and Saos-2 cells respectively ($P<0.001$, Fig 4.4).

MG63 and Saos-2 cells were stimulated with 100ng/ml of three isoforms of PDGF (-AA, -AB and -BB). Each isoform was found to significantly stimulate production of OPG in both MG63 and Saos-2 cells ($P<0.001$, Fig 4.5). Another PDGF family member is vascular endothelial growth factor (VEGF). However, VEGF at 100ng/ml in complete media for 24 hours did not significantly affect OPG production in MG63 or Saos-2 cells (Fig 4.5).

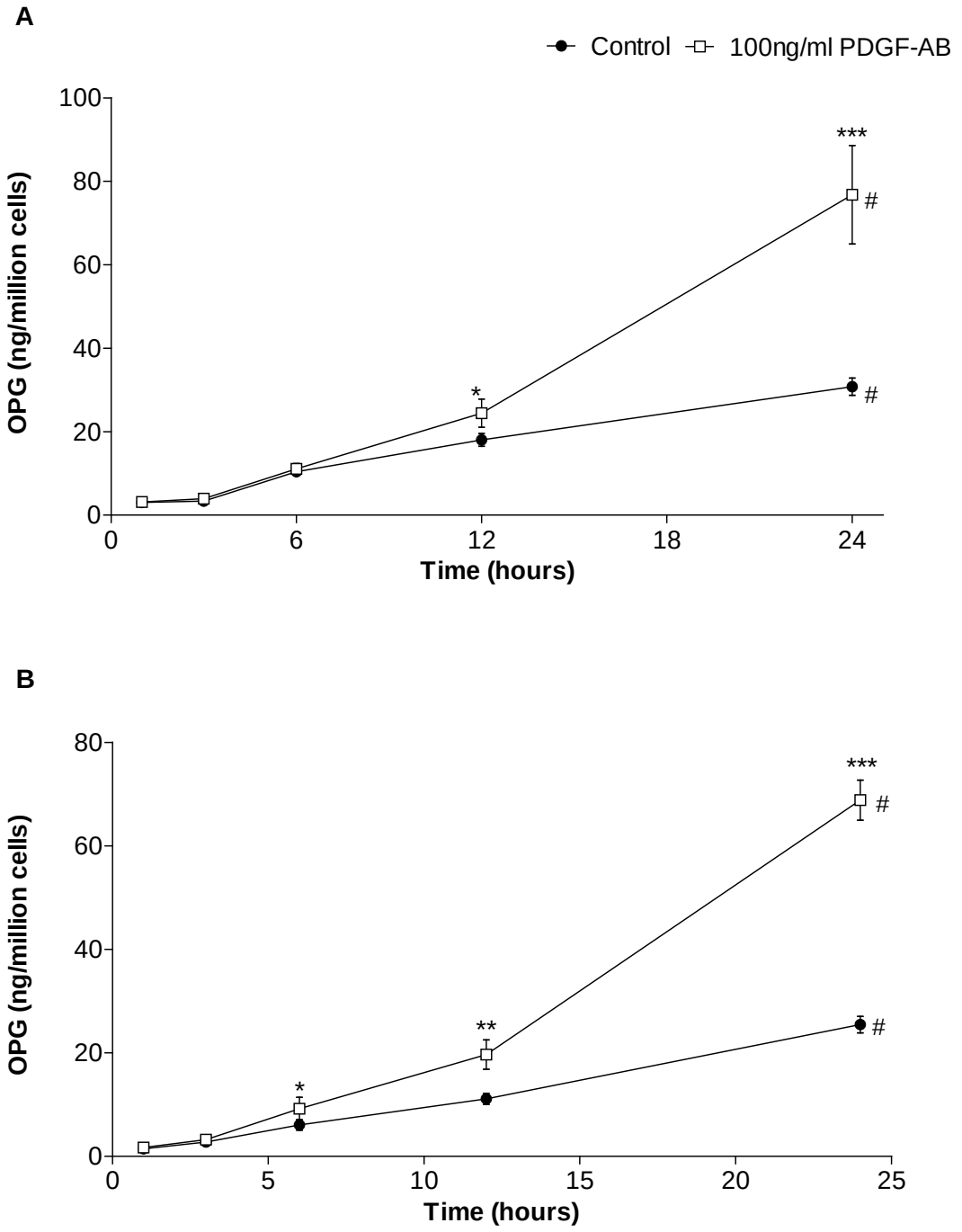

Fig 4.4 PDGF-AB-induced OPG production in osteoblastic cells is time dependent. A) MG63 cells and B) Saos-2 cells were stimulated with 100ng/ml PDGF-AB for 3, 6, 12 and 24 hours. Culture medium was assayed for OPG at each time point. Data shown are mean ± SD, n=4. #P<0.001 versus control (one-way ANOVA using Dunnett's multiple comparison test), *P<0.05, **P<0.01, ***P<0.001 versus same time point control (unpaired t-test).

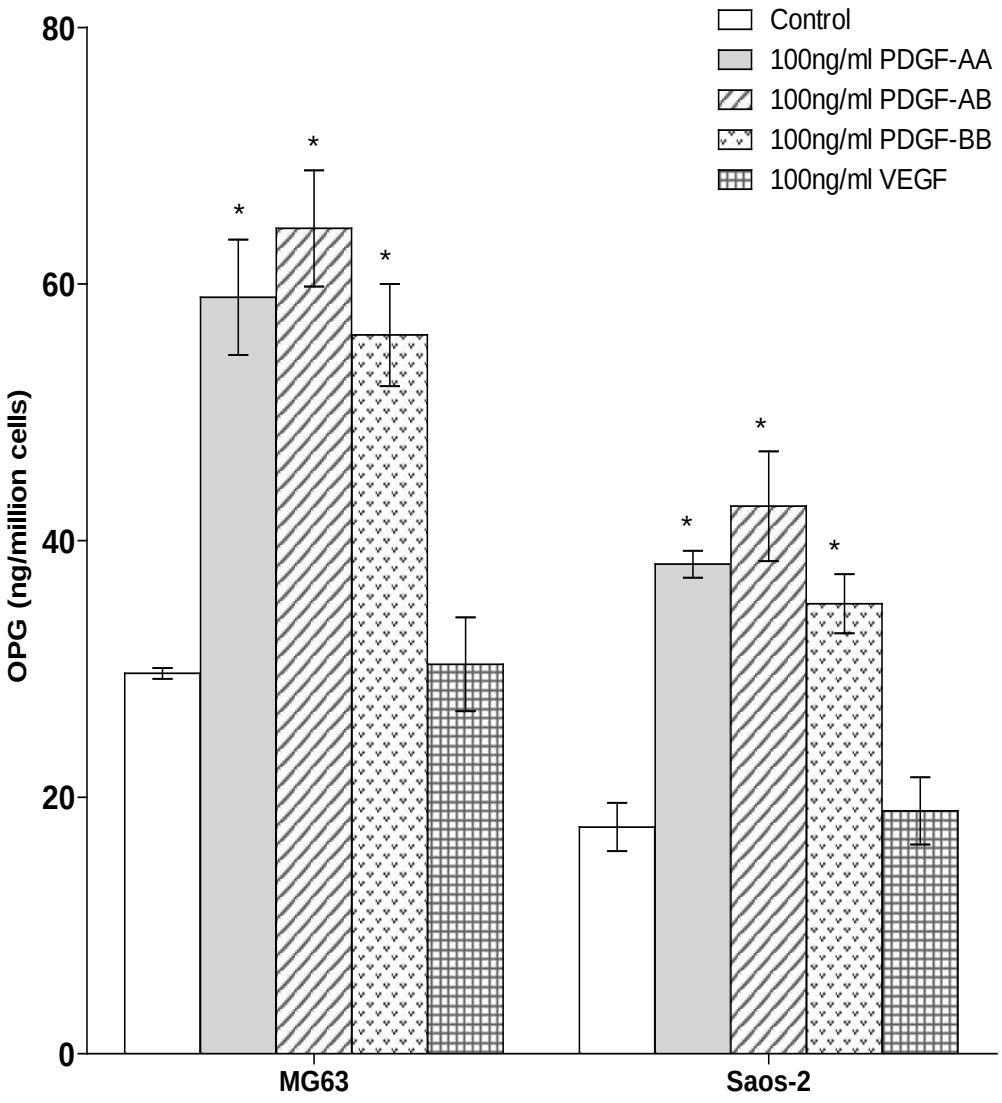

Fig 4.5 All PDGF isoforms stimulate OPG production in osteoblastic cells. MG63 and Saos-2 cells were stimulated with 100ng/ml PDGF-AA, -AB, -BB or VEGF for 24 hours. Culture medium was assayed for OPG. Data shown are mean ± SD, n=4. *P<0.001 versus own cell type control (one-way ANOVA using Dunnett's multiple comparison test).

As MG63 and Saos-2 osteosarcoma cells lines are both transformed, the effect of PDGF-AB was examined for its ability to stimulate OPG in non-transformed cells. MC3T3-E1 (a normal mouse pre-osteoblastic cell line), human mesenchymal stem cells (hMSC), human foetal osteoblasts (hFOB, an immortalised but non-transformed cell line) and 149BR (a normal human skin fibroblast) were stimulated with 100ng/ml PDGF-AB for 24 hours. PDGF-AB significantly stimulated OPG production in both MC3T3-E1 cells and hMSCs by 113% ($P<0.001$) and 45% ($P<0.01$) respectively (Fig 4.6). Although both hFOB and 149BR cells produced detectable amounts of OPG, their production of OPG was not stimulated by PDGF-AB.

To confirm that the OPG production observed with the addition of PDGF-AB is not the result of cross-reactivity with another receptor, MG63 cells were exposed to 0-30µM AG1296, a specific PDGFR inhibitor, either with or without 100ng/ml PDGF-AB in complete medium for 24 hours. AG1296 exhibited the ability to inhibit both constitutive and PDGF-AB stimulated OPG production in a dose dependent manner ($P<0.001$, Fig 4.7A). At 30µM AG1296 significantly inhibited OPG production 30% below that of control in the absence of PDGF-AB ($P<0.001$). The lowest effective concentration of AG1296 to exhibit a significant response in cells stimulated with 100ng/ml PDGF-AB was 2.5µM. A maximal effect was observed at 5µM AG1296 where PDGF-AB failed to significantly stimulate OPG production above that of control. Saos-2 and MC3T3-E1 cells were also exposed to 5µM AG1296 either with or without 100ng/ml PDGF-AB. PDGF-AB stimulated OPG production was significantly inhibited by 5µM AG1296 in both cell types ($P<0.001$, Fig 4.7B)

To determine whether the effect on OPG secretion required an increase in *OPG* gene expression, MG63 and Saos-2 cells were incubated for 24 hours in complete medium with 100ng/ml PDGF-AB either with or without 5µM AG1296 before extracting total RNA. Quantitative RT-PCR for *OPG* and *RANKL* relative to *GAPDH* showed PDGF-AB significantly increased messenger RNA (mRNA) production for *OPG* in both MG63 and Saos-2 by 3.2 ($P<0.01$) and 4.4 ($P<0.001$) fold respectively (Fig 4.8A). The addition of AG1296 suppressed the expression of *OPG* mRNA to the same levels as controls. Neither PDGF-AB nor AG1296 affected mRNA for *RANKL* in either cell type (Fig 4.8B).

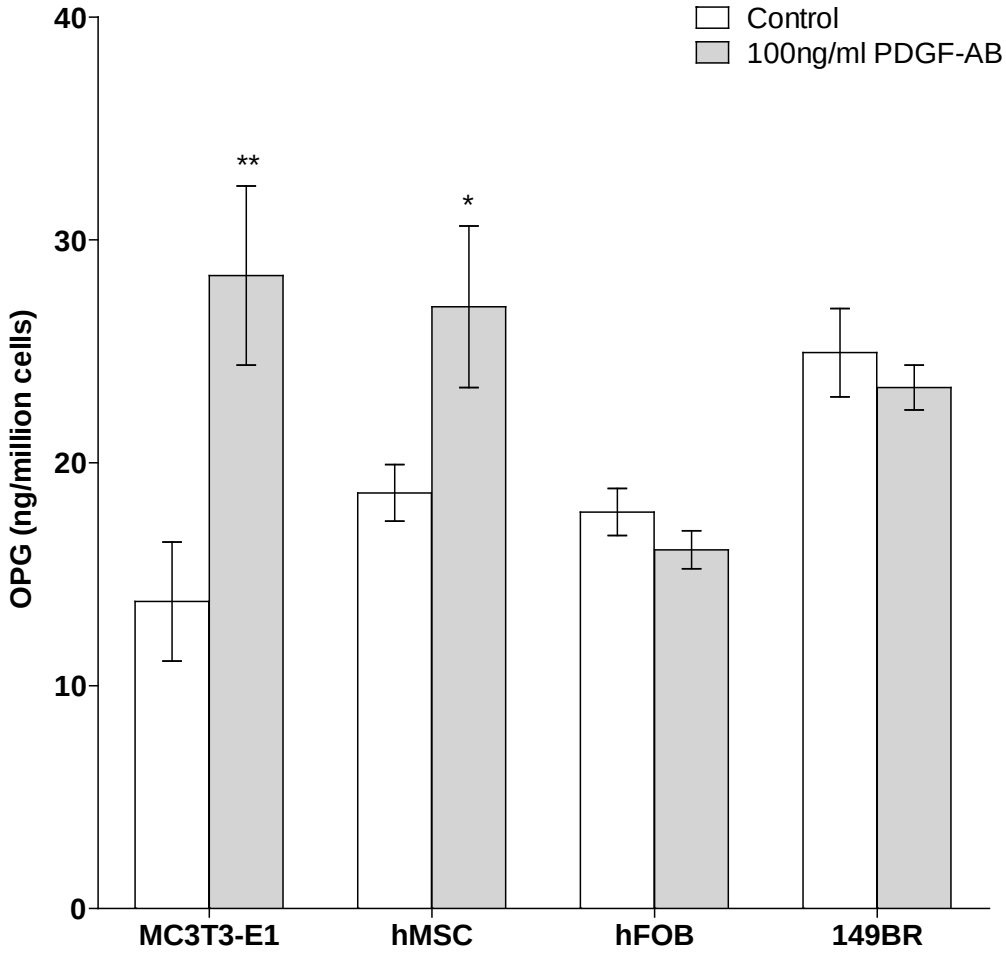

Fig 4.6 PDGF-AB stimulates OPG protein production in MC3T3-E1 and hMSC but not in hFOB or skin fibroblasts. MC3T3-E1, hMSC, hFOB and 149BR (skin fibroblasts) cells were stimulated with 100ng/ml PDGF-AB for 24 hours. Culture medium was assayed for OPG. Data shown are mean ± SD, n=4. *P<0.05, **P<0.01 versus own cell type control (unpaired t-test).

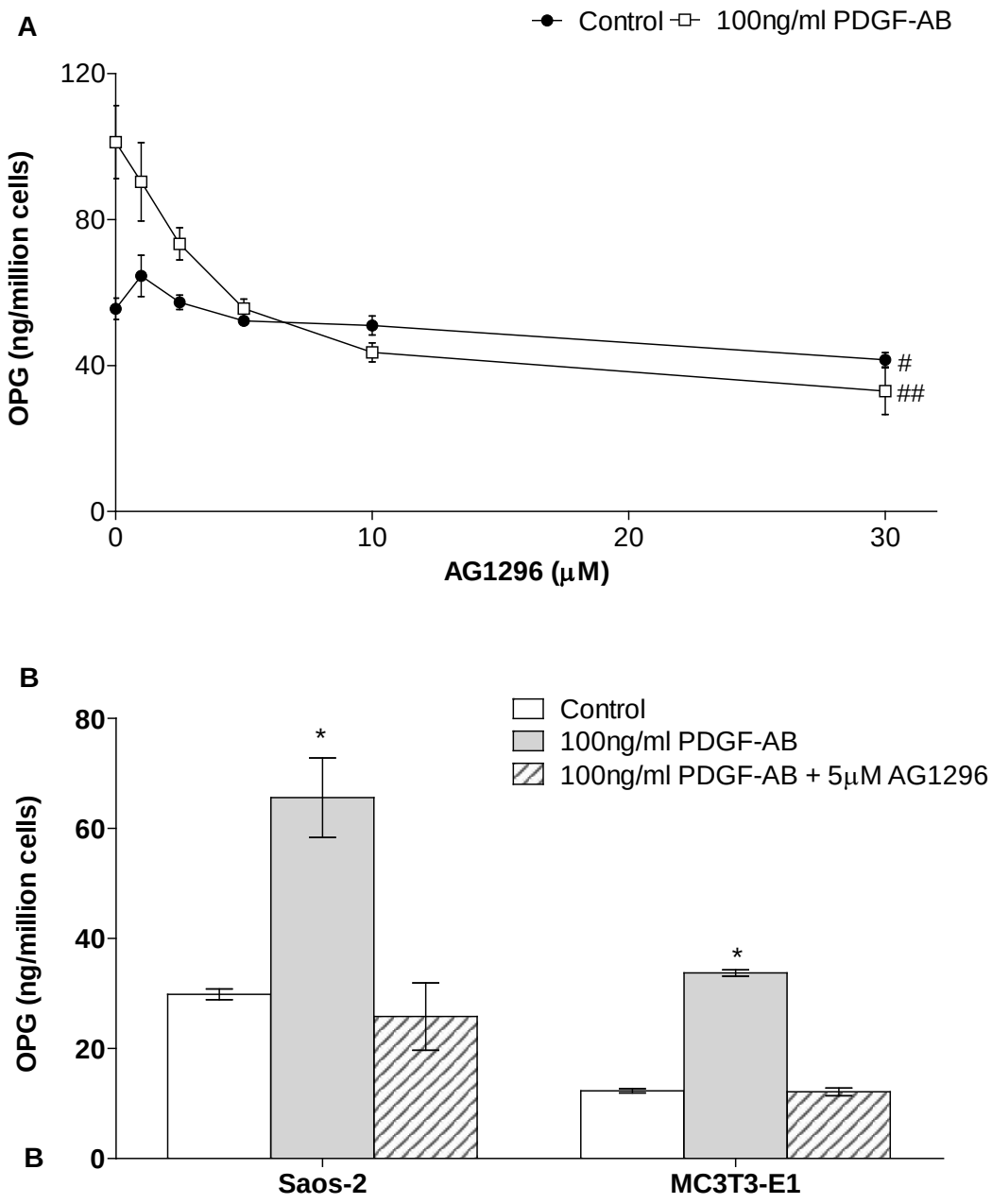

Fig 4.7 PDGF-AB-stimulated OPG production is inhibited by AG1296. A) MG63 cells were stimulated with 0-30μM AG1296 for 24 hours either with or without 100ng/ml PDGF-AB. Culture medium was assayed for OPG. Data shown are mean ± SD, n=4. #P<0.05, ##P<0.001 versus 0μM AG1296 (one-way ANOVA using Dunnett's multiple comparison test). B) Saos-2 and MC3T3-E1 cells were incubated with 100ng/ml PDGF-AB with or without 5μM AG1296 for 24 hours. Culture medium was assayed for OPG. Data shown are mean ± SD, n=4. *P<0.001 versus own cell type control (unpaired t-test).

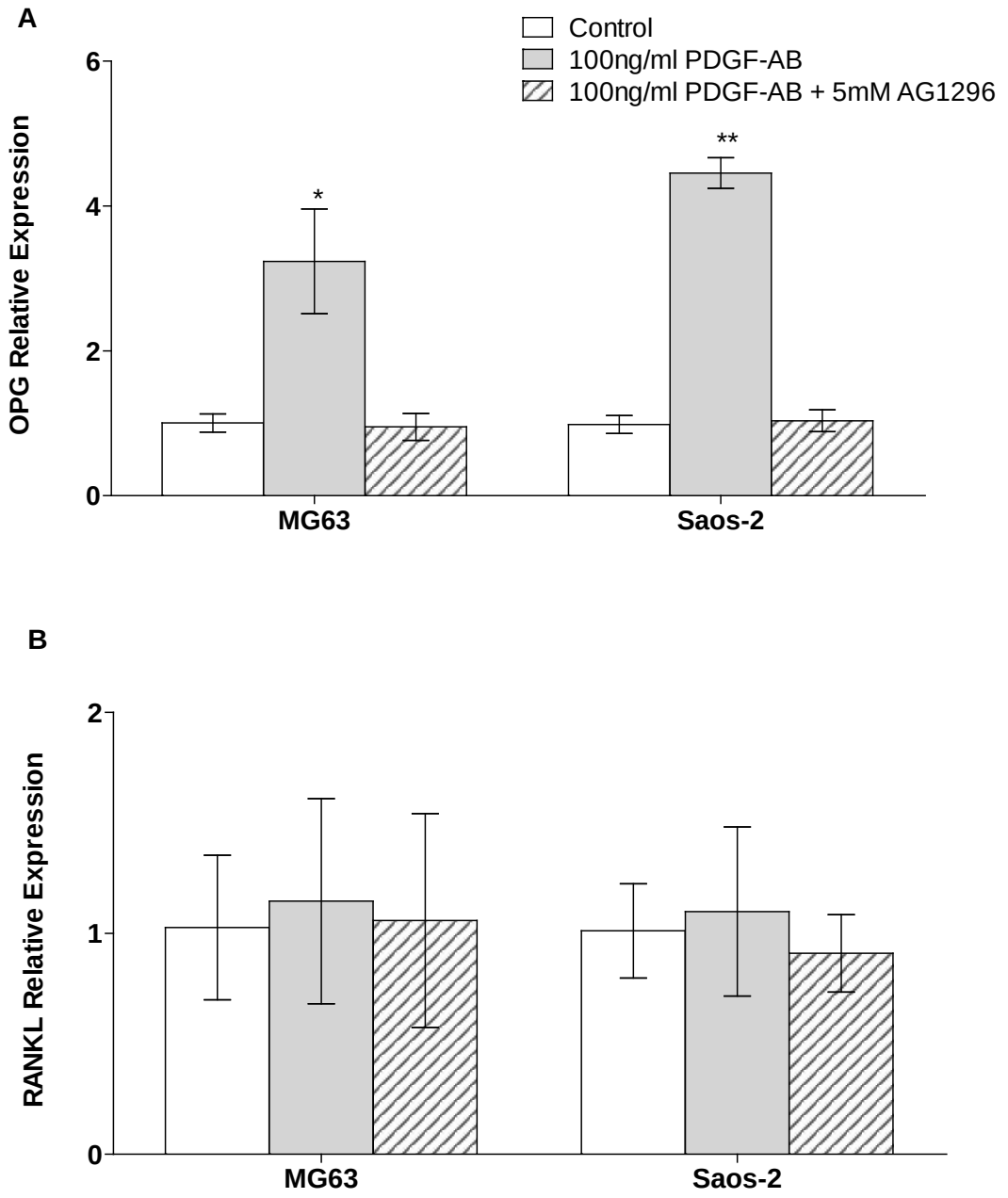

Fig 4.8 PDGF-AB-stimulated OPG production is accompanied by increased mRNA expression and is inhibited by AG1296. MG63 and Saos-2 cells were incubated with 100ng/ml PDGF-AB with or without 5µM AG1296 for 24 hours. RNA was extracted and expression of A) *OPG* and B) *RANKL* mRNA was assayed using RT-qPCR. Data shown are expressed relative to control (24 hours, no stimulation) normalised to *GAPDH* housekeeper expression (mean ± range, n=3). *P<0.01, **P<0.001 versus own cell type control (unpaired t-test).

To investigate the signalling pathway through which PDGF-AB was stimulating the increase in OPG production, MG63, Saos-2 and MC3T3-E1 cells were treated in complete medium for 24 hours with 10μM inhibitor for the following pathways: JNK (SP600125), P38MAPK (SB202190), ERK/MEK (U0126), PI3K (LY294002) and NF-kB (BAY 11-7082). U0126 significantly inhibited PDGF-AB-stimulated OPG production in both MG63 ($P<0.001$) and Saos-2 ($P<0.001$) but not MC3T3-E1 (Fig 4.9). LY294002 inhibited PDGF-AB-induced OPG production in MC3T3-E1 ($P<0.001$), partially inhibited PDGF-AB-induced OPG production in Saos-2 ($P<0.01$) and had no effect in MG63. SB202190 inhibited PDGF-AB-induced OPG production in MG63 ($P<0.001$) but not in Saos-2 or MC3T3-E1. SP600125 and BAY 11-7082 had no significant effect on PDGF-AB-stimulated OPG production in any of the three cell types investigated (Table 4.1A). At the concentration used, 10μM, SB202190 significantly inhibited OPG production in control MG63 cells as observed previously ($P<0.001$, Fig 3.3). There was no other significant effect of these inhibitors on OPG production in any of the three cells types investigated (Table 4.1B).

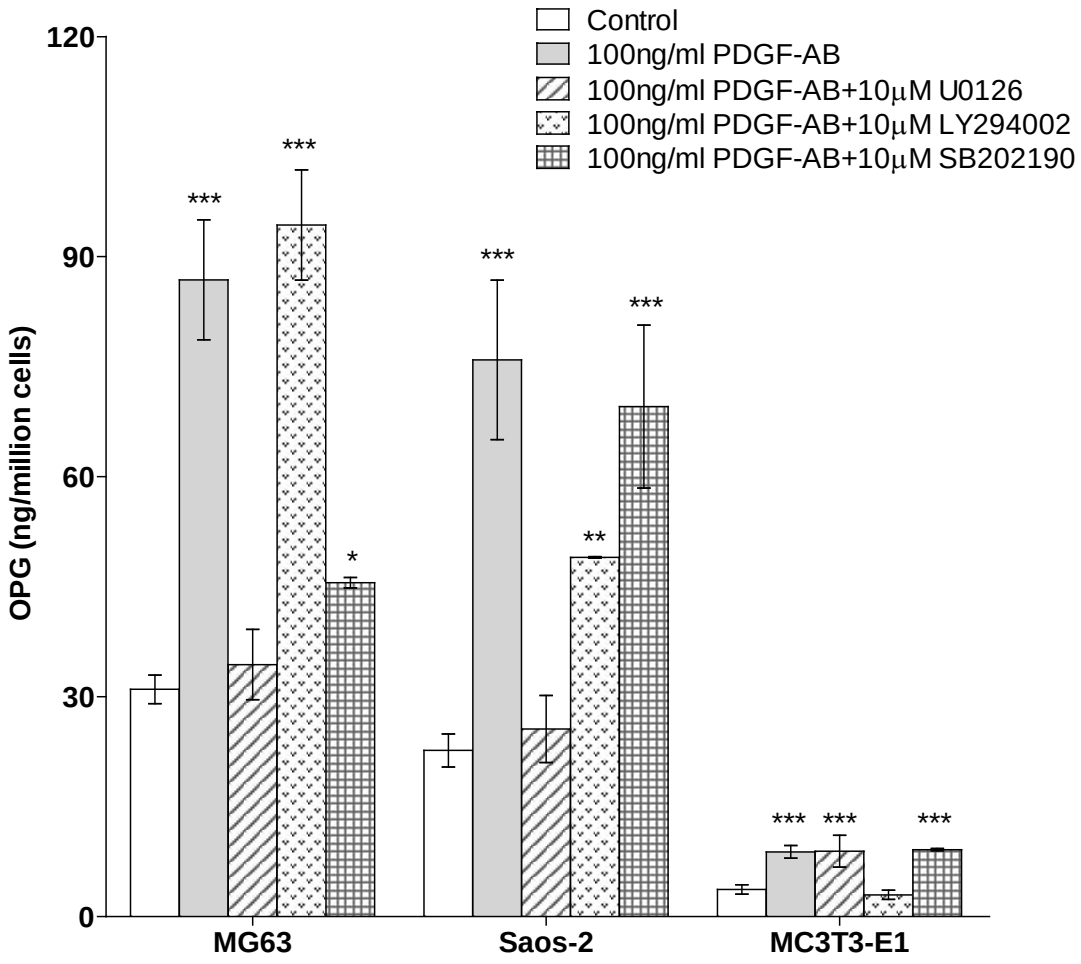

Fig 4.9 PDGF-AB signals through PI3K, ERK and p38. MG63, Saos-2 and MC3T3-E1 cells were incubated with 10μM U0126, LY294002 or SB202190 with 100ng/ml PDGF-AB for 24 hours. Culture medium was assayed for OPG. Data shown are expressed as mean ± SD, n=3. *P<0.05, **P<0.01, ***P<0.001 versus own cell type control (one-way ANOVA using Dunnett's multiple comparison test).

Table 4.1 Signalling inhibitors and OPG production. A) PDGF-AB induced OPG production is not mediated via JNK or NFkB. B) The effect of signalling inhibitors on OPG production under normal culture conditions over 24 hours. Data shown are OPG (ng/million cells) expressed as mean ± SD, n=3. *P<0.001 versus own cell type control (unpaired t-test).

A

	MG63	***Saos-2***	***MC3T3-E1***
100ng/ml PDGF-AB	86.8 ± 8.2	75.9 ± 10.8	8.8 ± 0.9
10μM SP600125	91.4 ± 4.2	91.1 ± 2.3	7.1 ± 1.4
10μM BAY 11-7082	81.8 ± 7.1	73.8 ± 5.7	9.9 ± 1.1

B

	MG63	***Saos-2***	***MC3T3-E1***
Control	30.9 ± 1.9	22.7 ± 2.3	3.7 ± 0.7
10μM SP600125	33.5 ± 4.6	23.0 ± 2.5	3.8 ± 0.1
10μM SB202190	19.2 ± 2.6*	25.3 ± 3.2	2.5 ± 0.3
10μM U0126	29.7 ± 0.7	23.1 ± 4.8	4.8 ± 0.7
10μM LY294002	36.3 ± 1.9	19.2 ± 0.8	2.9 ± 0.5
10μM BAY 11-7082	34.5 ± 3.7	20.9 ± 0.9	4.4 ± 0.7

To further investigate the signalling mechanisms responsible for PDGF-AB stimulated OPG production, MG63 and Saos-2 cells were incubated with 0-50nM staurosporine (STS), a wide specificity protein kinase inhibitor with or without 100ng/ml PDGF-AB for 24 hours in complete medium. STS significantly decreased cell number in both cell types both in the presence and absence of 100ng/ml PDGF-AB ($P<0.001$, Fig 4.10). Above 10nM STS there was a significant decrease in cell number in both cell types ($P<0.001$). Therefore, the maximum effective dose without inducing cell death deemed suitable for use in further experiments was 10nM.

STS significantly stimulated OPG production in both MG63 and Saos-2 cells ($P<0.001$, Fig 4.11). The lowest effective concentration of STS to exhibit a significant response was 1nM. At 10nM STS, OPG production was significantly increased by 87% and 526% in MG63 and Saos-2 cells respectively compared to controls ($P<0.001$). STS also significantly stimulated the production of OPG in the presence of 100ng/ml PDGF-AB ($P<0.001$, Fig 4.11). In MG63 cells, 10nM STS significantly increased PDGF-AB-induced OPG production 199% above that of PDGF-AB only control whereas in Saos-2 cells 10nM STS stimulated PDGF-AB-induced OPG production by 321% above PDGF-AB only control.

MG63 and Saos-2 cells were incubated with 0-10µM bisindolylmaleamide (BIM), a specific PKC inhibitor, with or without 100ng/ml PDGF-AB for 24 hours in complete medium. BIM inhibited constitutive production of OPG in MG63 cells ($P<0.01$, Fig 4.12A) but not Saos-2 cells. At 10µM BIM, constitutive OPG production in MG63 cells was inhibited by 35% compared to control. BIM significantly inhibited PDGF-AB induced OPG production in both MG63 and Saos-2 cells ($P<0.001$, Fig 4.12). The lowest effective concentration of BIM to exhibit a significant response in both cell types was 1µM. A maximal effect was observed at 10µM where PDGF-AB stimulated OPG production in MG63 cells was significantly inhibited by 70%, equivalent to control levels ($P<0.001$, Fig 4.12A). In Saos-2 cells, 10µM BIM significantly inhibited PDGF-AB-induced OPG production 41% compared to control ($P<0.001$, Fig 4.12B).

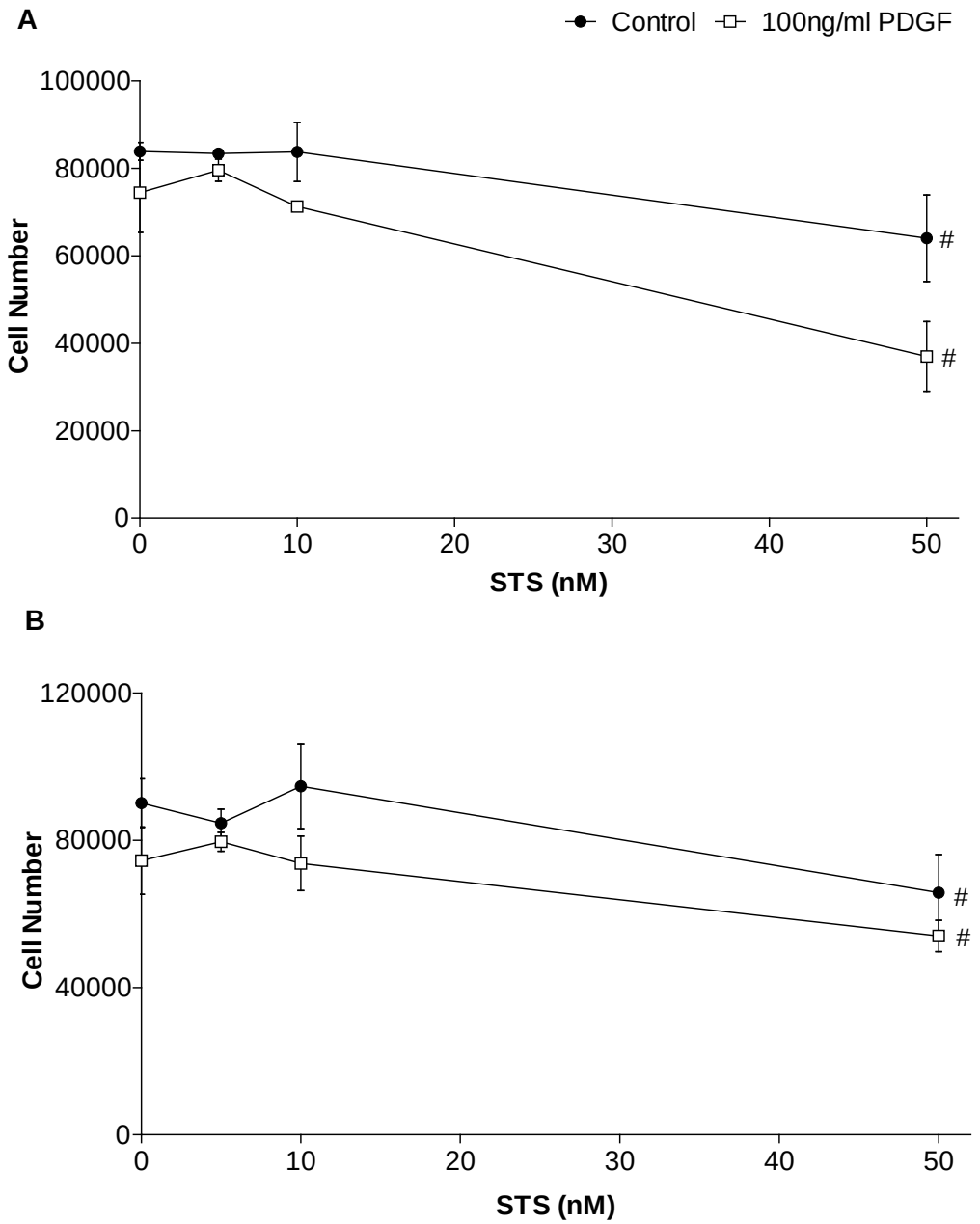

Fig 4.10 The effect of STS on cell number in osteoblastic cells. A) MG63 and B) Saos-2 cells were incubated with 0-50nM STS with or without 100ng/ml PDGF-AB for 24 hours. Culture medium was removed and cell number estimated. Data shown are expressed as mean ± SD, n=3. #P<0.001 versus 0nM STS (one-way ANOVA using Dunnett's multiple comparison test).

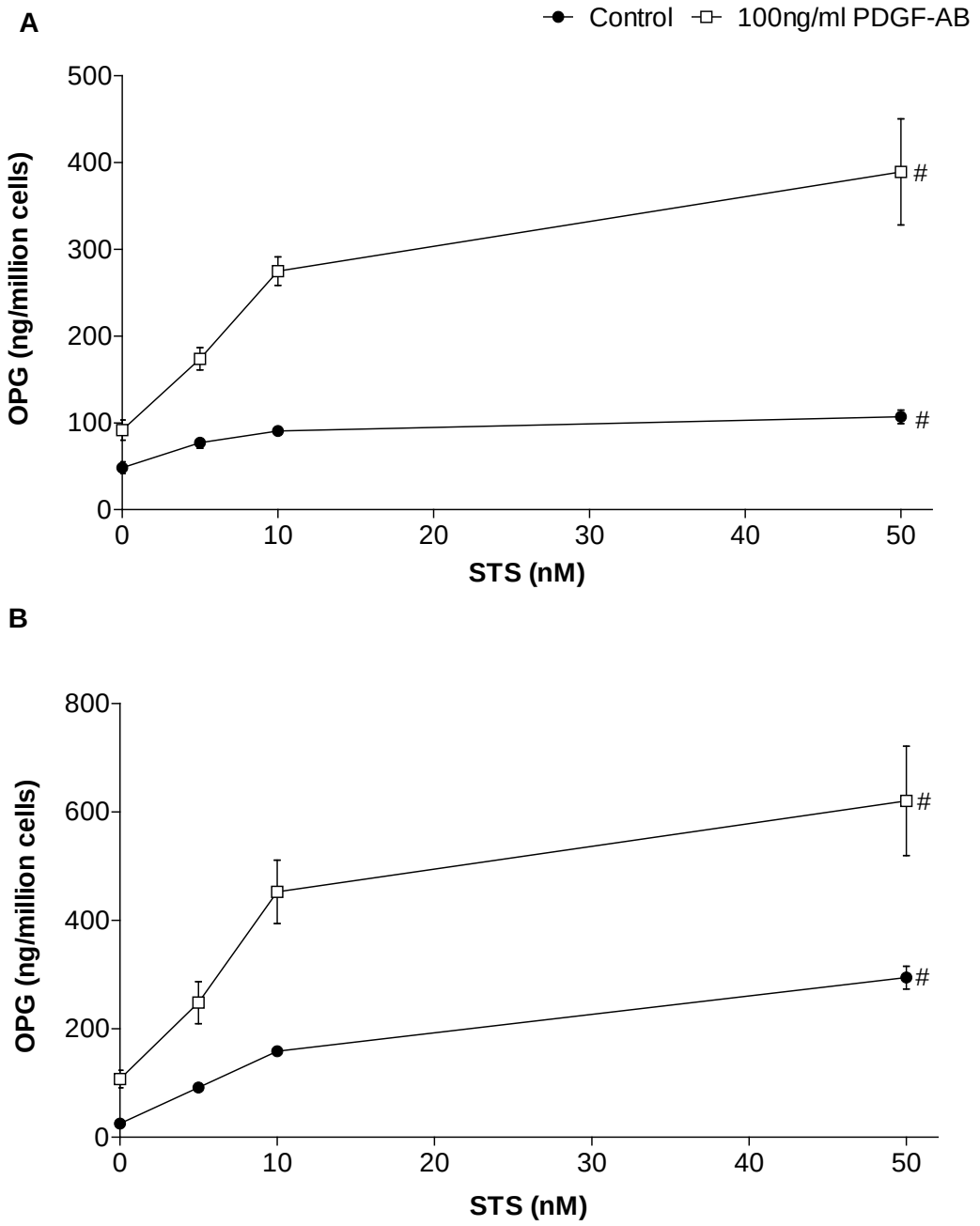

Fig 4.11 PDGF-AB induced OPG production is stimulated by STS. A) MG63 and B) Saos-2 cells were incubated with 0-50nM STS with or without 100ng/ml PDGF-AB for 24 hours. Culture medium was assayed for OPG. Data shown are expressed as mean ± SD, n=3. #P<0.001 versus 0nM STS (one-way ANOVA using Dunnett's multiple comparison test).

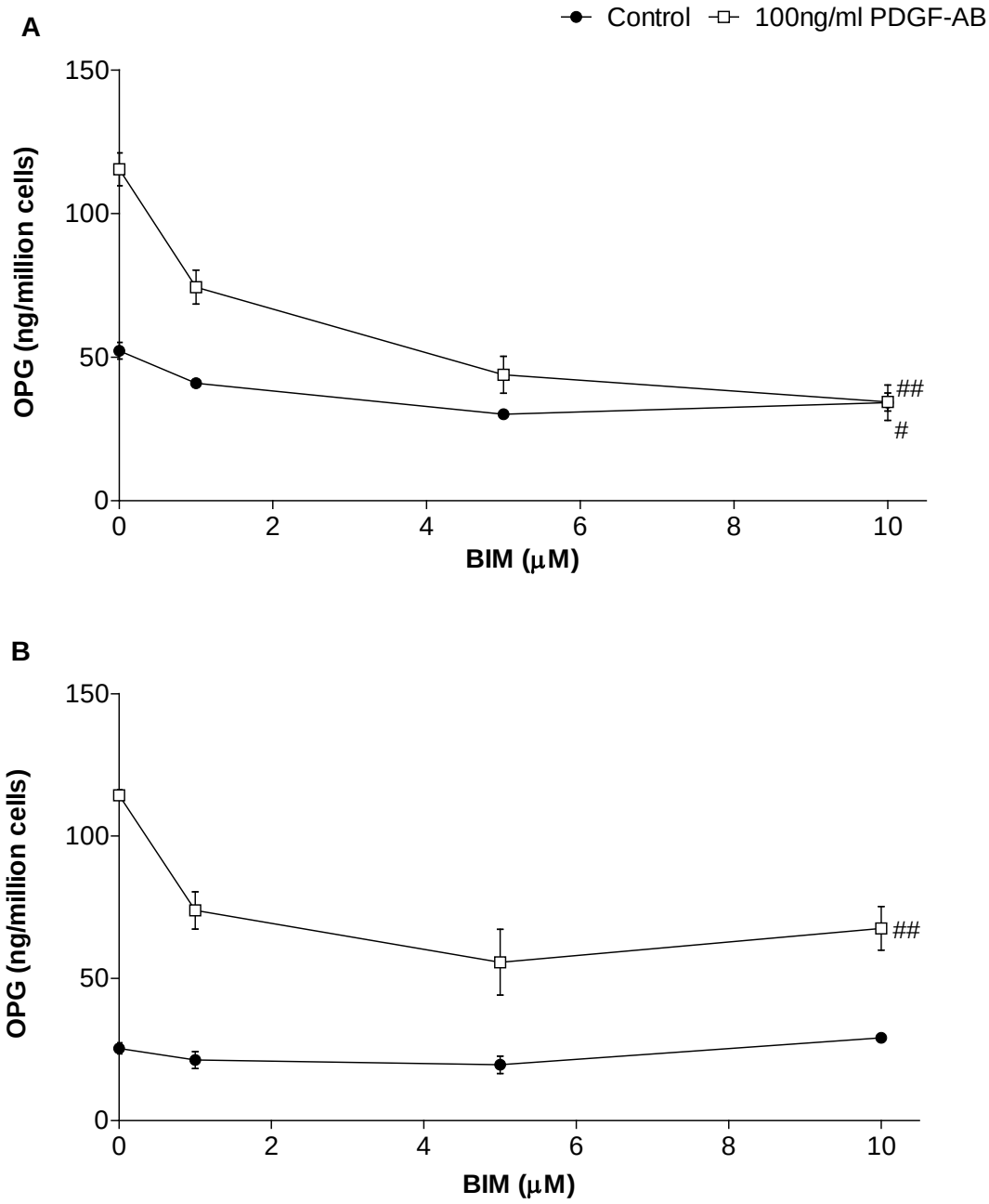

Fig 4.12 PDGF-AB induced OPG production is inhibited by BIM. A) MG63 and B) Saos-2 cells were incubated with 0-10μM BIM with or without 100ng/ml PDGF-AB for 24 hours. Culture medium was assayed for OPG. Data shown are expressed as mean ± SD, n=4. #P<0.01, ##P<0.001 versus 0μM BIM (one-way ANOVA using Dunnett's multiple comparison test).

4.2 The effect of PDGF on differentiated osteoblastic cells

hFOB's show a more differentiated phenotype than MG63, Saos-2, MC3T3-E1 and hMSC. As OPG production was not stimulated in response to PDGF-AB in the cells, it was hypothesised that an osteoblast may become less responsive to PDGF-AB-induced OPG production as it becomes more differentiated. Therefore, the effect of differentiation was investigated. MG63 cells do not mineralise in culture, so Saos-2 cells were cultured in either complete medium or osteogenic medium for a total of 21 days. Medium was refreshed every 3/4 days. On days 9, 16 and 21 cells were stimulated with 100ng/ml PDGF-AB for 24 hours. Culture medium was removed for OPG assay and cellular ALP activity measured. Representative cells were stained with Alizarin Red on day 21 to determine the extent of mineralisation.

In osteogenic media, cellular ALP activity was significantly increased above that of cells in cultured in complete medium ($P<0.05$, Fig 4.113A). ALP activity showed a peak on day 16 with significantly more activity in osteogenic medium than complete media ($P<0.001$). On day 21 there was no significant difference in ALP activity between the two culture conditions. Cells in both complete medium and osteogenic medium stained positive for mineralisation with Alizarin Red on day 21, although mineralised nodules were only observed in cells incubated in osteogenic media (Fig 4.13B).

On days 9 and 16, PDGF-AB induced OPG production in cells cultured in complete medium was significantly increased above that of the same time point and medium control by 76% and 43% respectively ($P<0.01$, Fig 4.14). PDGF-AB did not induce OPG production in Saos-2 cells after 21 days of culture in complete medium. PDGF-AB did not stimulate the production of OPG in Saos-2 cells cultured in osteogenic medium at any of the time points. Cells cultured in osteogenic medium produced significantly less OPG at each of the three time points than those cultured in complete medium.

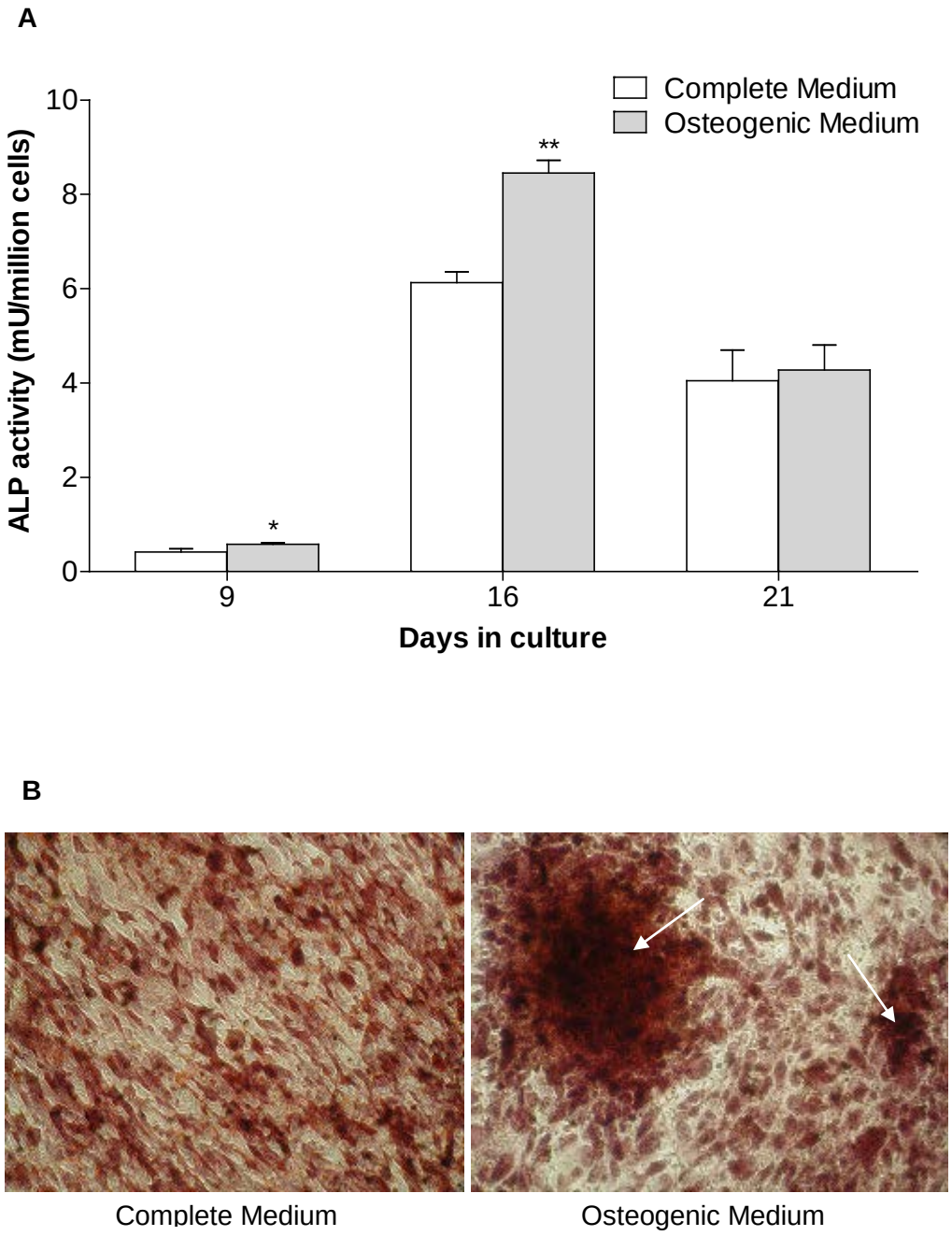

Fig 4.13 ALP expression in Saos-2 cells with differentiation. Saos-2 cells were incubated in either complete medium or osteogenic medium for 21 days. A) Cells were assayed for ALP activity on days 9, 16 and 21. Data shown are mean ± standard deviation, n=3. *P<0.05, **P<0.001, versus control (complete medium at same time point, unpaired t-test). B) Cells were stained for mineralised calcium deposits using Alizarin Red on day 21. White arrows indicate mineralised nodules.

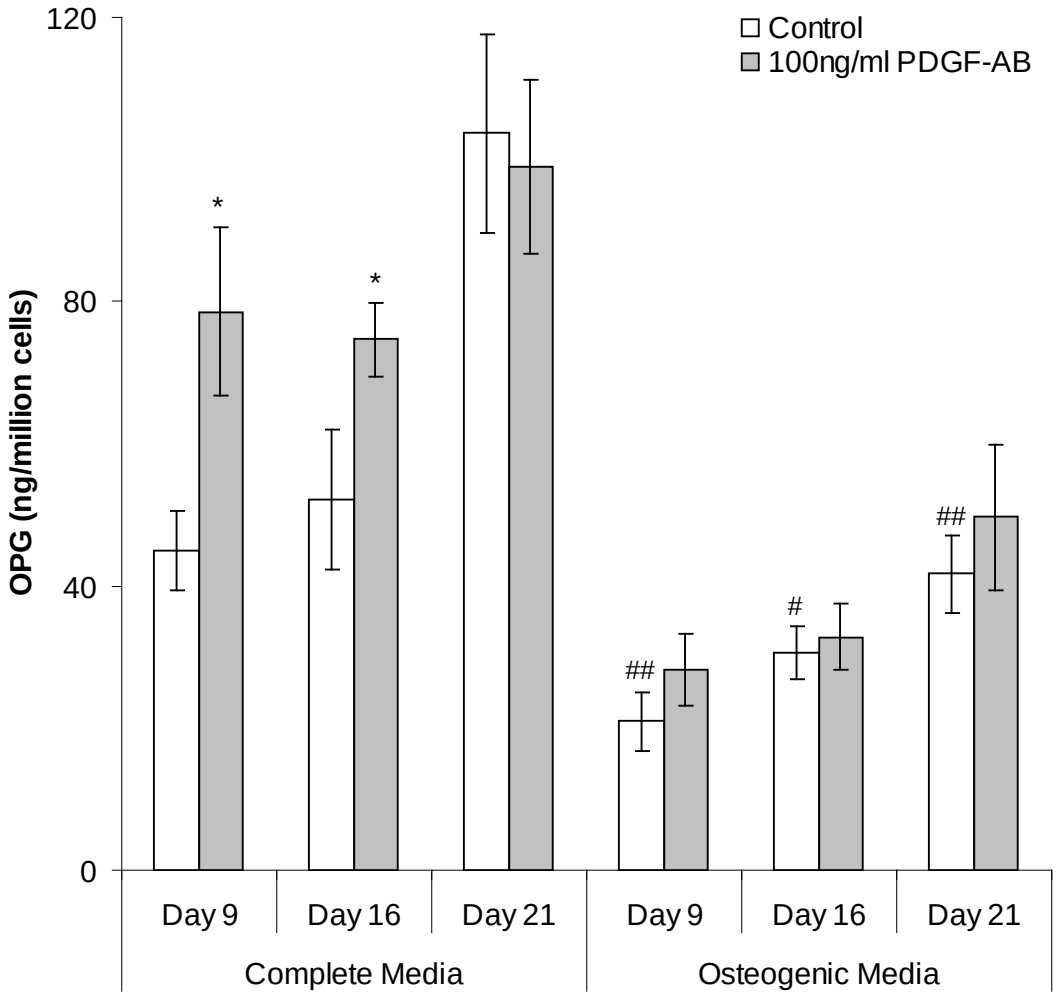

Fig 4.14 The ability for PDGF-AB to stimulate OPG production is lost with osteoblastic differentiation. Saos-2 cells were incubated in either complete medium or osteogenic medium for 21 days. On days 9, 16 and 21 medium was removed and cells were stimulated with 100ng/ml PDGF-AB in complete medium for 24 hours. Culture media was assayed for OPG. Data shown are mean ± standard deviation, n=4. *P<0.01, versus control of same time point and culture condition (unpaired t-test). #P<0.01, ##P<0.001 versus control of same time point of complete medium (unpaired t-test)

The expression of the PDGF receptors α and β (PDGFRα and PDGFRβ respectively) was analysed in Saos-2 cells during differentiation. Saos-2 cells were cultured in osteogenic medium for 1, 7, 14 and 21 days. Medium was refreshed every 3/4 days when required. On days 1, 7, 14 and 21 RNA was extracted and quantitative RT-PCR for both *PDGFRα* and *PDGFRβ* performed. In Saos-2 cells *PDGFRα* expression steadily rose significantly during the course of differentiation, reaching 4-fold above control (day 1) after 21 days ($P<0.01$, Fig 4.15). The expression of *PDGFRβ* was significantly up-regulated during differentiation, peaking at 15-fold higher than control (day 1) by day14 ($P<0.001$). By day 21, expression of *PDGFRβ* remained 7.5-fold above control ($P<0.001$).

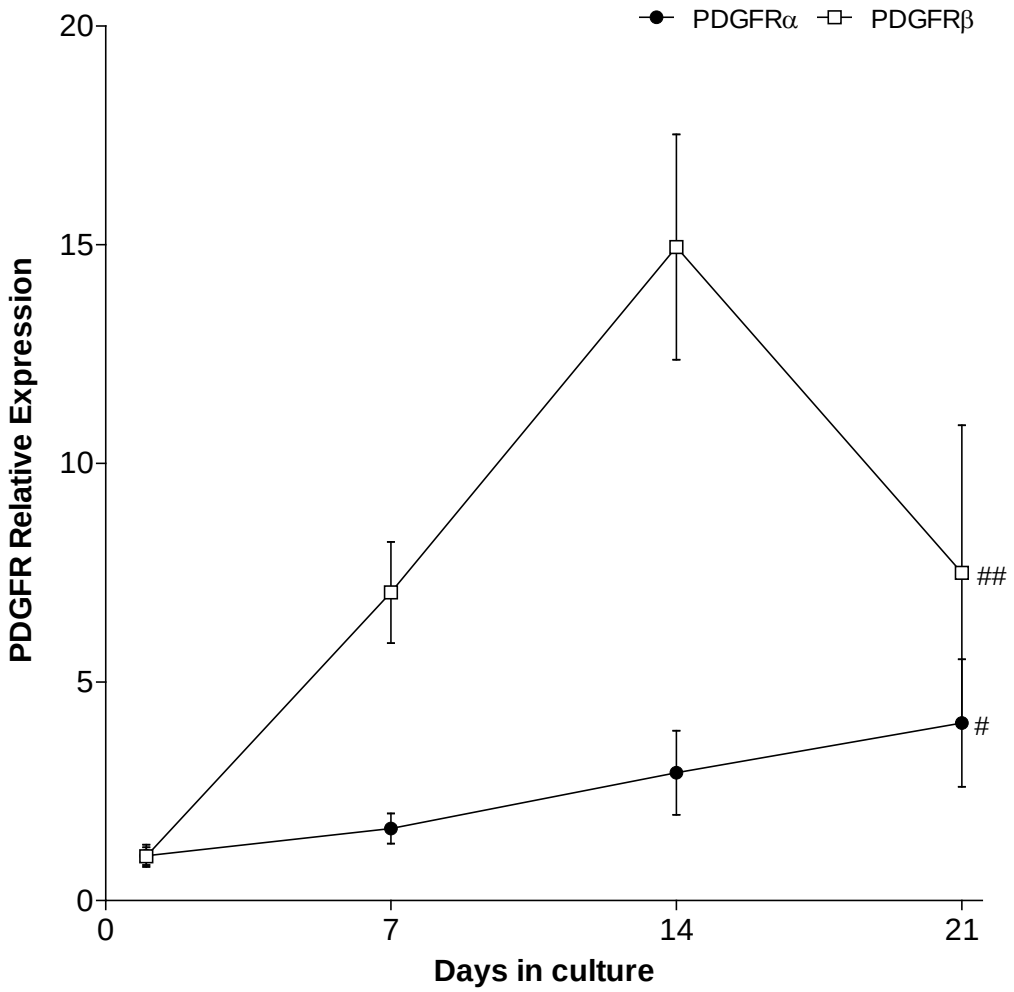

Fig 4.15 The expression of the PDGF receptors α and β during differentiation. Saos-2 cells were cultured in osteogenic media for 1, 7, 14 and 21 days. RNA was extracted at each time point and mRNA for *PDGFRα* and *PDGFRβ* assayed using RT-qPCR. Data shown are expressed relative to control (day 1) normalised to *GAPDH* housekeeper expression (mean ± range, n=3). #P<0.01, ##P<0.001 versus day 1 (one-way ANOVA using Dunnett's multiple comparison test).