CHAPTER 3

The Effect of FCS on Osteoblastic Cells

3.1 The effect of FCS on OPG production

Whilst studying the regulation of OPG and DKK-1 production in osteoblastic cells, an unexpected dependency upon FCS for the production of OPG and DKK-1 was observed. MG63 cells were cultured under standard conditions for 24 hours either in complete medium containing 10% FCS (control) or FCS-free medium. After 24 hours the culture medium was removed and assayed for OPG. Culturing cells in the absence of FCS significantly inhibited the production of OPG from MG63 cells by 65% compared to control ($P<0.001$, Fig 3.1A).

MG63 cells were cultured in complete medium for either 24 hours or 48 hours with or without addition of further FCS at the 24 hour time point. There was no significant difference in OPG production between 24 and 48 hours (Fig 3.1B) indicating that OPG production ceases after 24 hours. Addition of extra FCS at 24 hours significantly stimulated OPG production by 30% ($P<0.001$) and 27% ($P<0.01$) above the 24 and 48 hour controls respectively. Addition of PBS at 24 hours had no effect on the production of OPG (Fig 3.1B).

Other medium constituents were analysed for their ability to stimulate OPG production. MG63 cells were cultured for 24 hours in either full medium containing extra amino acids, vitamins or glucose, or full medium lacking nucleotides. Altering these media constituents had no significant effect on OPG production in MG63 cells (Fig 3.2).

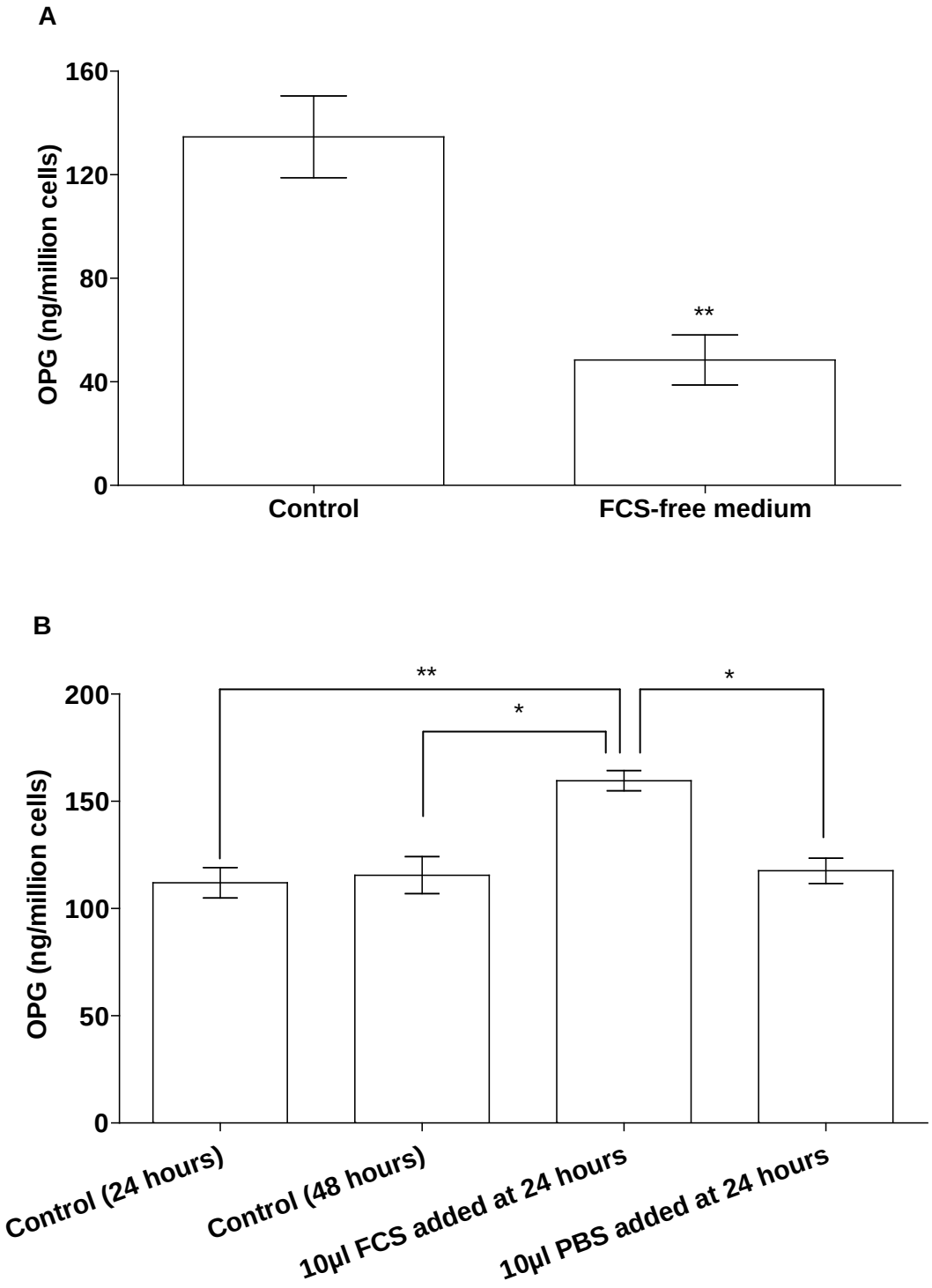

Fig 3.1 The effect of FCS on OPG production in osteoblastic cells. A) MG63 cells were cultured for 24 hours with or without FCS. B) MG63 cells were cultured for either 24 hours or 48 hours with or without addition of further FCS at the 24 hour time point. Culture medium was assayed for OPG. Data are presented as mean ± SD, n=4. *P<0.01 and **P<0.001 versus control (unpaired t-test).

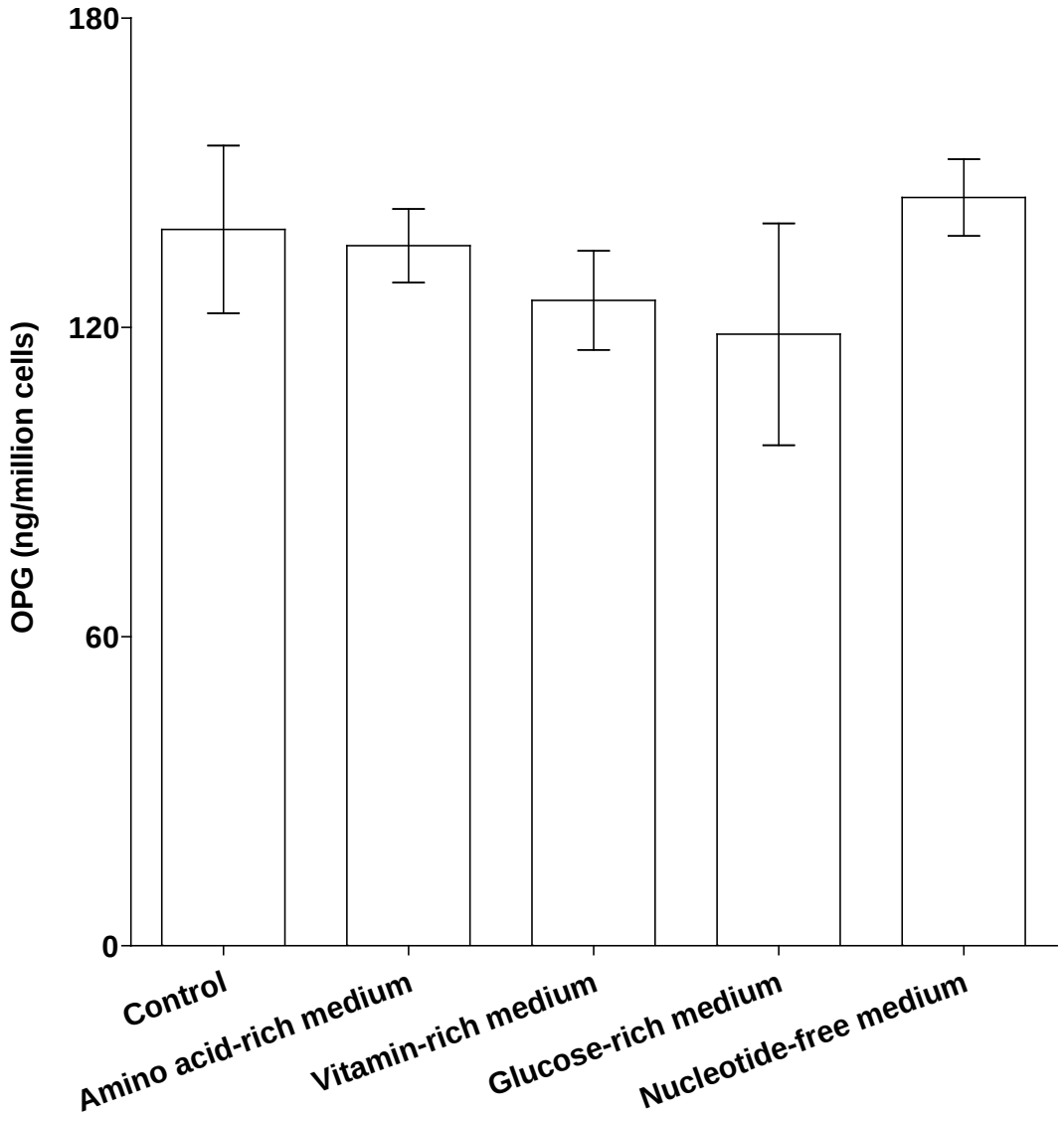

Fig 3.2 The effect of various media constituents on OPG production in osteoblastic cells. MG63 cells were cultured for 24 hours in either complete medium containing extra amino acids, vitamins or glucose, or medium lacking nucleotides. Culture medium was assayed for OPG. Data are presented as mean ± SD, n=4.

The effect of pathway inhibitors on the production of OPG was investigated. MG63 cells were cultured for 24 hours in complete medium with 0-25µM various pathway signalling inhibitors for PI3K (LY294002), ERK (U0126), P38MAPK (SB202190), NF-κB (BAY11-7082) and JNK (SP600125). Assay of the culture medium for OPG showed U0126 and SB202190 significantly inhibited FCS-induced / constitutive OPG production in a dose-dependent manner ($P<0.001$, Fig 3.3). The lowest effective concentration of U0126 and SB202190 to exhibit a significant response was 10µM and 5µM respectively. A maximal response was seen at 50µM, where U0126 and SB202190 significantly inhibited OPG production in MG63 cells by 50% and 66% respectively compared to control ($P<0.001$, Fig 3.3). SP600125 significantly increased production of OPG in a dose-dependent manner ($P<0.001$). The lowest effective concentration of SP600125 to exhibit a significant response was 25µM and a maximal response was seen at 50µM where OPG production in MG63 cells was 3-fold above that of control. LY294002 and BAY11-7082 did not significantly affect OPG production in these cells. At 50µM, OPG production in the presence of LY294002 and BAY11-7082 was 87.76ng/ml ± 13.87ng/ml and 106.65ng/ml ± 6.36ng/ml respectively.

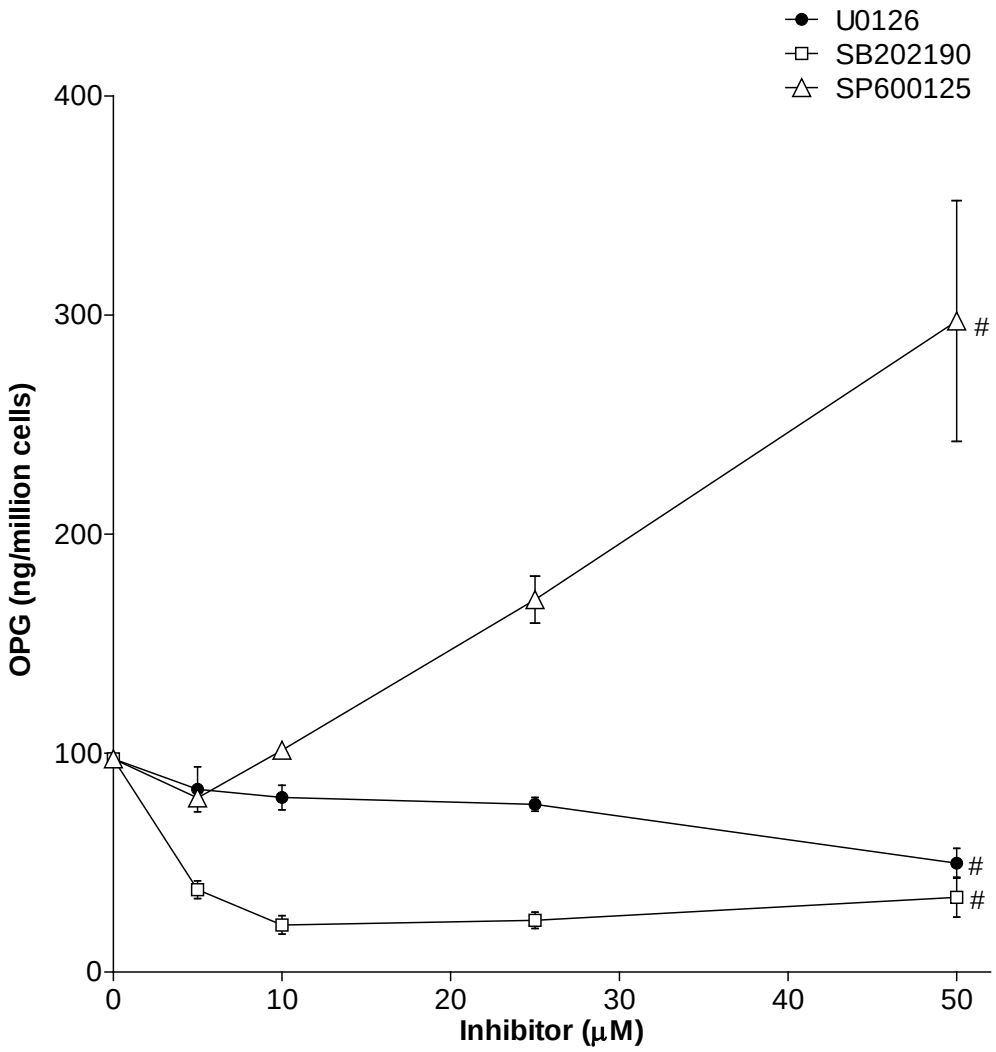

Fig 3.3 The effect of pathway inhibitors on OPG production in osteoblastic cells. MG63 cells were cultured for 24 hours with 0-50μM inhibitors for ERK (U0126), P38MAPK (SB202190) and JNK (SP600125). Culture medium was assayed for OPG. Data are presented as mean ± SD, n=4. #P<0.01 and ##P<0.001 versus control (0μM inhibitor, one-way ANOVA using Dunnett's multiple comparison test).

3.2 The effect of FCS on DKK-1 production and Wnt expression

When MG63 cells are cultured under standard conditions for 24 hours in complete medium they produce on average 10-30ng/million cells DKK-1. However, when this medium was substituted for FCS free medium, DKK-1 production was significantly reduced by 88% compared to control ($P<0.001$, Fig 3.4A).

As DKK-1 is a potent soluble antagonist of canonical Wnt signalling, mRNA expression of secreted Wnt ligands was examined in response to FCS starvation. MG63 cells were cultured for 24 hours in FCS free medium. RNA was extracted and reverse transcribed. Expression of *Wnt1, Wnt2a, Wnt3a, Wnt4, Wnt5a, Wnt7b, Wnt10a and Wnt11* and *DKK-1* was analysed by RT-PCR using GAPDH as a housekeeper. FCS starvation significantly up-regulated *Wnt4* and *Wnt11* by 18- and 14-fold respectively compared to control ($P<0.001$), blocked the expression of *Wnt7b* and significantly down-regulated the expression of *DKK-1* by 81% compared to control ($P<0.01$, Fig 3.4B). Expression of *Wnt3a, Wnt5a and Wnt10a* was not affected by FCS starvation. Neither *Wnt1* nor *Wnt2a* were detected in either control or FCS-starved cells.

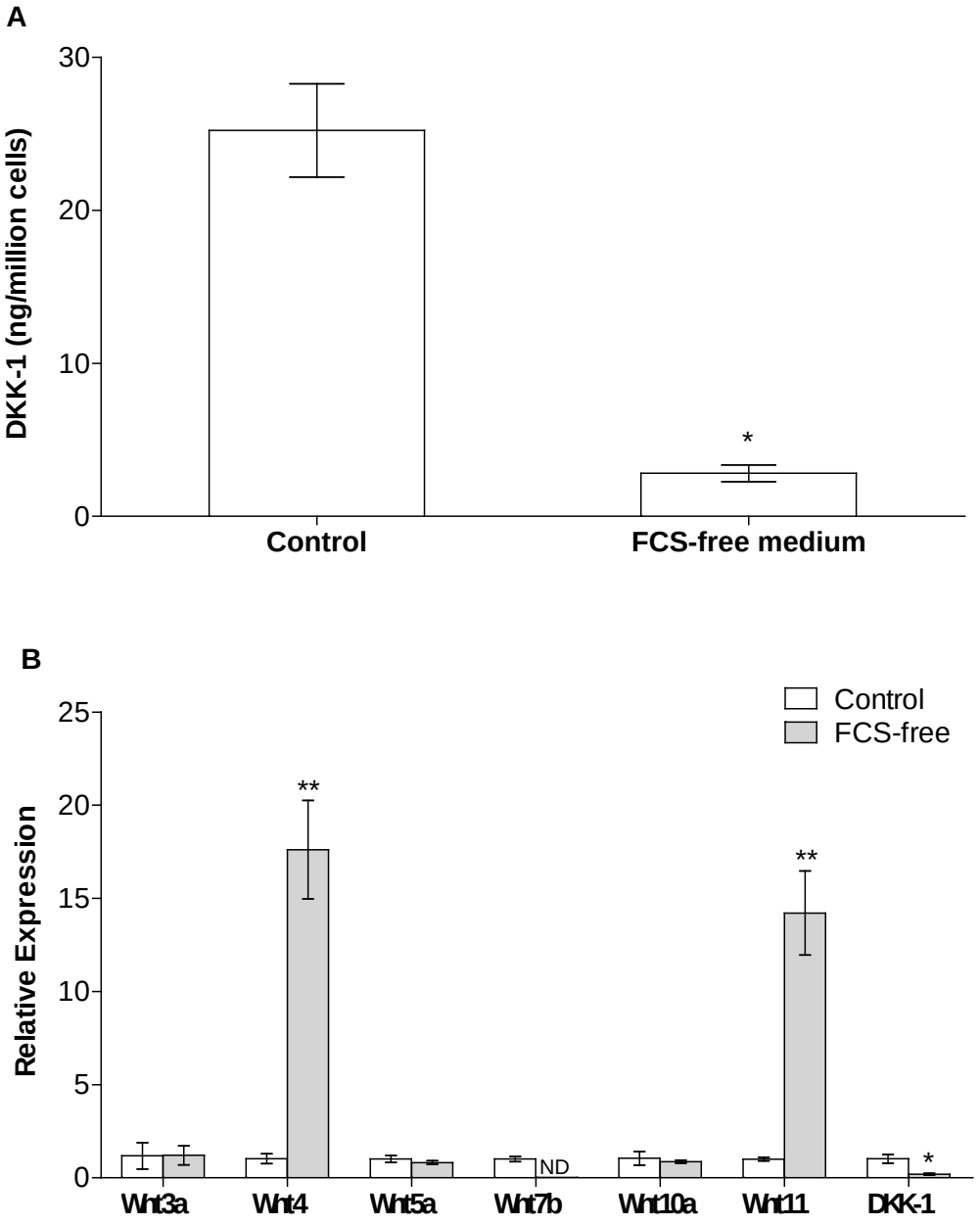

Fig 3.4 The effect of FCS starvation on DKK-1 production and Wnt expression in osteoblastic cells. MG63 cells were cultured for 24 hours with or without FCS. A) Culture medium was assayed for DKK-1. Data are presented as mean ± SD, n=4. *P<0.001 versus control (unpaired t-test). B) RNA was extracted and mRNA analysed using RT-PCR. Data shown are expressed relative to control (24 hours culture with FCS) normalised to *GAPDH* housekeeper expression (mean ± range, n=3). ND = not detected. *P<0.01 and **P<0.001 versus own mRNA control (unpaired t-test).

MG63 cells were cultured for 24 hours with 0-50μM various pathway signalling inhibitors for PI3K (LY294002), ERK (U0126), P38MAPK (SB202190), NF-κB (BAY11-7082) and JNK (SP600125). Analysis of the culture medium for DKK-1 revealed LY294002, U0126 and SB202190 inhibited DKK-1 production in a dose-dependent manner ($P<0.001$ Fig 3.5) while SP600125 stimulated DKK-1 production ($P<0.001$). The lowest effective concentration to exhibit a significant response was 5μM for LY294002, U0126 and SB202190 and 10μM for SP600125. A maximal response was seen at 10μM for SB202190 where DKK-1 production in MG63 cells was inhibited by 72% compared to control ($P<0.01$, Fig 3.5). For U0126 and LY294002, maximal responses were observed at 50μM inhibiting DKK-1 production by 47% ($P<0.01$) and 82% ($P<0.001$) respectively compared to control (Fig 3.5). The maximal response observed for SP600125 was 10μM where DKK-1 production was stimulated 71% compared to control ($P<0.001$, Fig 3.5). BAY11-7082 had no effect on FCS-induced DKK-1 production. At 50μM, DKK-1 production in the presence of BAY11-7082 was 11.87ng/ml ± 2.87ng/ml.

To investigate the effect these inhibitors on Wnt expression, MG63 cells were then cultured with 25μM of each inhibitor in complete medium for 24 hours. Expression of *Wnt3a, Wnt4, Wnt5a, Wnt7b, Wnt10a and Wnt11* and *DKK-1* was analysed in response to the inhibitors. LY294002 significantly up-regulated the expression of *Wnt4* ($P<0.01$) and *Wnt11* ($P<0.01$) compared to control whilst significantly down-regulating the expression of *Wnt5a* ($P<0.05$) and *DKK-1* ($P<0.001$) compared to control whilst significantly (Fig 3.6A). SB202190 significantly up-regulated the expression of *Wnt4* ($P<0.05$), *Wnt7b* ($P<0.001$) and *Wnt11* ($P<0.01$) compared to control whilst significantly down-regulating the expression of *Wnt5a* ($P<0.05$) and *DKK-1* ($P<0.001$) compared to control (Fig 3.6A). SP600125 significantly up-regulated the expression of *Wnt4* ($P<0.05$) and *Wnt7b* ($P<0.001$) compared to control (Fig 3.6B). U0126 significantly up-regulated the expression of *Wnt3a* ($P<0.05$), *Wnt7b* ($P<0.05$) and *Wnt11* ($P<0.05$) compared to control whilst significantly down-regulating the expression of *Wnt4* ($P<0.01$) and *DKK-1* ($P<0.001$) compared to control (Fig 3.6B). There was no change in expression of *Wnt10a* in response to any pathway inhibitor.

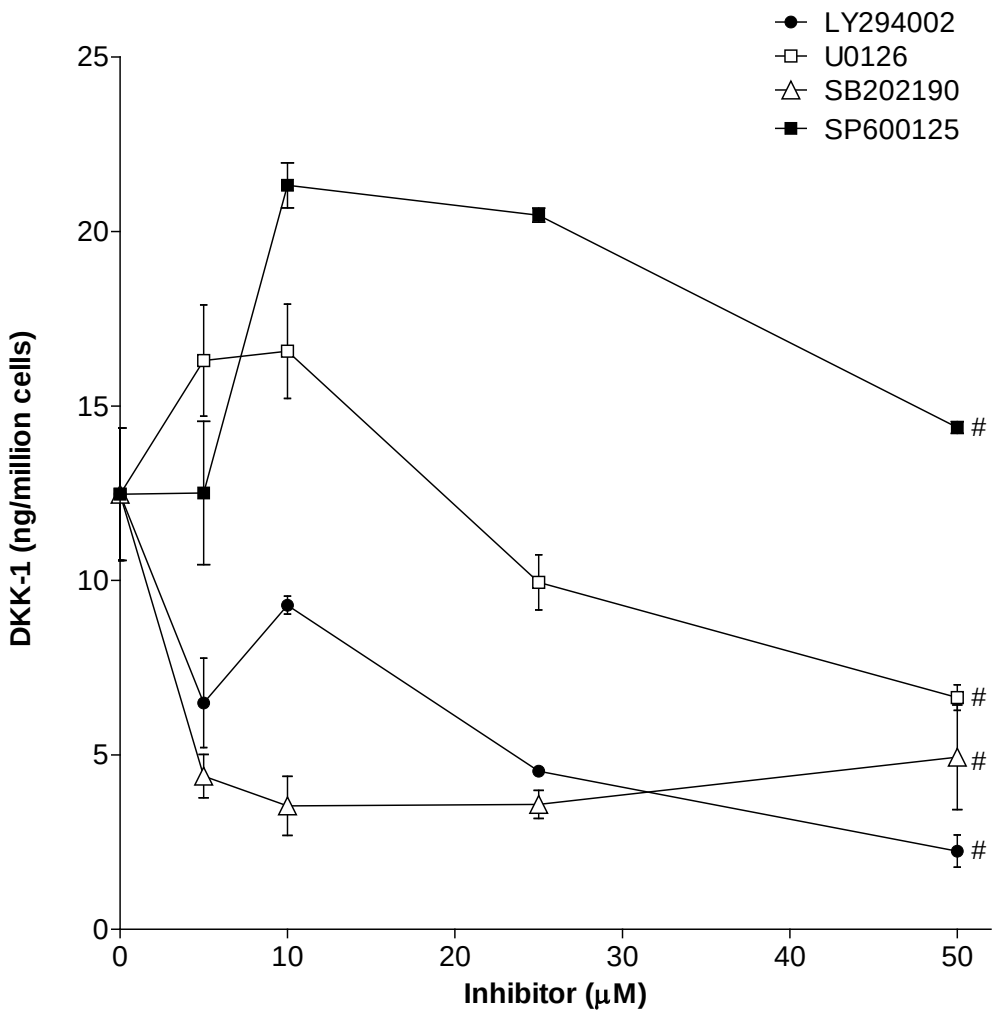

Fig 3.5 FCS-induced DKK-1 production and signals through Pi3K, ERK and P38MAPK. MG63 cells were incubated with 0-50μM of inhibitors for Pi3K (LY294002), ERK (U0126) and P38MAPK (SB202190) in complete medium for 24 hours. Culture medium was assayed for DKK-1. Data are presented as mean ± SD, n=3. #P<0.001 versus control (0μM inhibitor, one-way ANOVA using Dunnett's multiple comparison test).

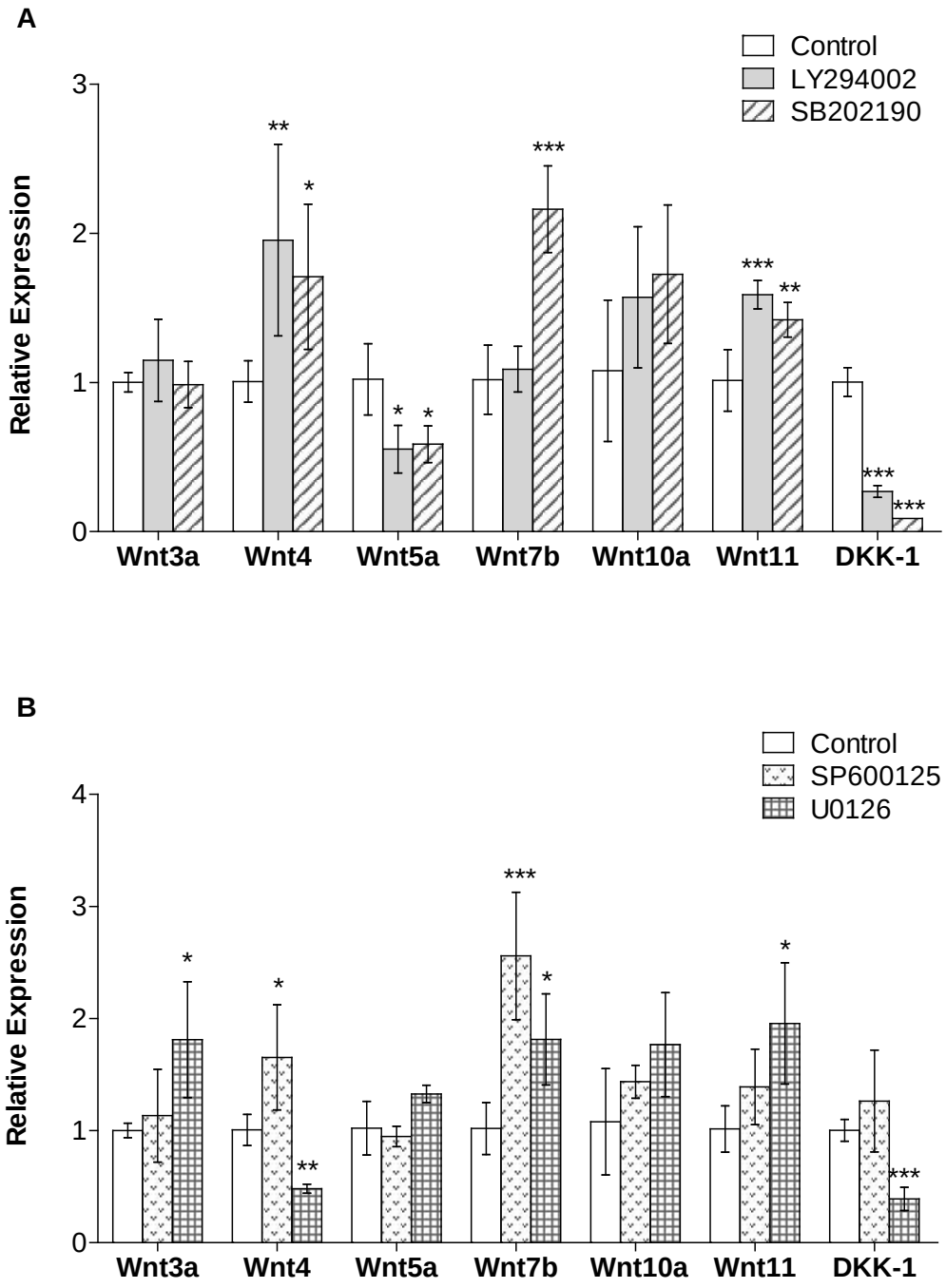

Fig 3.6 The effect of pathway inhibitors for PI3K, P38MAPK, JNK and ERK/MEK on Wnt expression in osteoblastic cells. MG63 cells were stimulated for 24 hours in complete medium in the presence of 25µM inhibitor. RNA was extracted and mRNA analysed using RT-PCR. Data shown are expressed relative to control (24 hours culture with FCS) normalised to *GAPDH* housekeeper expression (mean ± range, n=3). *P<0.05, **P<0.01 and ***P<0.001 versus own mRNA control (unpaired t-test).

To establish the time course of expression of Wnts and DKK-1 during FCS starvation, MG63 cells were incubated for 24 hours in complete medium, then serum starved for 0.5, 1, 3, 6, 9, 12 and 24 hours. For each time point, culture medium was removed and assayed for DKK-1, RNA was extracted, reverse transcribed and the expression of *Wnt4, Wnt7b, Wnt11* and *DKK-1* was analysed. For the purpose of this experiment, time zero (control) refers to the end of the 24 hour culture period in complete medium and the beginning of serum starvation.

DKK-1 production was significantly increased in a time-dependent bi-phasic manner both in the presence and absence of FCS ($P<0.001$, Fig 3.7A). In the presence of FCS, the earliest time for serum starvation to exhibit a significant response was 0.5 hours where DKK-1 production per hour was 8-fold above control ($P<0.001$). At 3 hours, DKK-1 production per hour in the presence of FCS had reached a maximum 10-fold above control ($P<0.001$). Between 3 hours and 24 hours the rate of DKK-1 production per hour significantly decreased in a time-dependent manner. At 12 hours, the rate of DKK-1 production in the presence of FCS was not significantly different to control.

In the absence of FCS however, the earliest time for serum starvation to exhibit a significant response was 3 hours where the rate of DKK-1 production per hour was 3 fold above control ($P<0.05$, Fig 3.7A). Between 3 hours and 24 hours the rate of DKK-1 production per hour significantly decreased in a time-dependent manner. At 24 hours, the rate of DKK-1 production in the absence of FCS had been significantly reduced by 99% compared to control ($P<0.001$).

The expression of *DKK-1* in the presence of FCS was also significantly increased in a time-dependent biphasic manner ($P<0.001$, Fig 3.7B). The earliest time for serum starvation to exhibit a significant response was 0.5 hours where the expression of *DKK-1* was 6.5-fold above control ($P<0.001$). Between 3 hours and 24 hours the expression of *DKK-1* significantly decreased in a time-dependent manner. At 12 hours, the expression of *DKK-1* production in the presence of FCS was not significantly different to control.

In the absence of FCS, the expression of *DKK-1* was significantly inhibited in a time-dependent manner ($P<0.001$, Fig 3.7B). The earliest time for serum starvation to exhibit a significant response was 0.5 hours. A maximal effect was observed at 24 hours where the expression of *DKK-1* was significantly inhibited 94% compared to control ($P<0.001$).

The expression of *Wnt4* and *Wnt11* was significantly up-regulated in response to serum starvation in a time-dependent manner (P<0.001, Fig 3.8). The earliest time for serum starvation to exhibit a significant response was 9 hours and 6 hours for *Wnt4* and *Wnt11* respectively. A maximal effect was observed after 24 hours serum starvation, where the expression of *Wnt4* and *Wnt11* was up-regulated 25-fold and 7-fold respectively compared to control (P<0.001).

The expression of *Wnt7b* was significantly down-regulated in response to serum starvation in a time-dependent manner (P<0.001, Fig 3.8). The earliest time for serum starvation to exhibit a significant effect was 3 hours. A maximal effect was observed after 24 hours serum starvation where the expression of *Wnt7b* was inhibited by 73% compared to control (P<0.001).

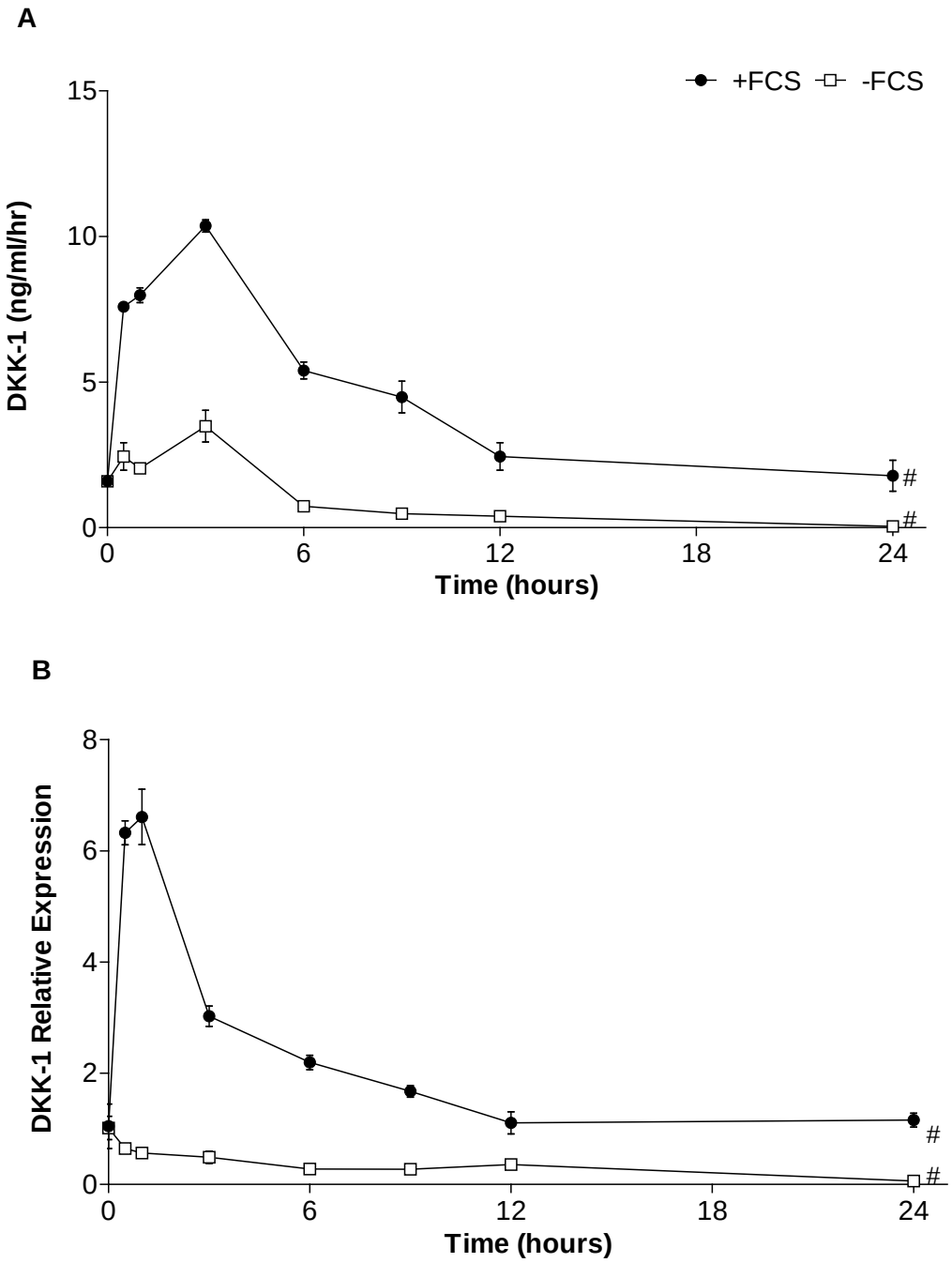

Fig 3.7 The effect of serum starvation on DKK-1 with time. MG63 cells were cultured for 0.5, 1, 3, 6, 9, 12 and 24 hours either with or without FCS following a 24 hour period in the presence of FCS (time zero). A) Culture medium was assayed for DKK-1. Data expressed as mean DKK-1 production per hour ± SD, n=4. B) RNA was extracted and mRNA assayed using RT-PCR. Data shown are expressed relative to control normalised to *GAPDH* housekeeper expression (mean ± range, n=3). #P<0.001 versus control (time zero, one-way ANOVA using Dunnett's multiple comparison test).

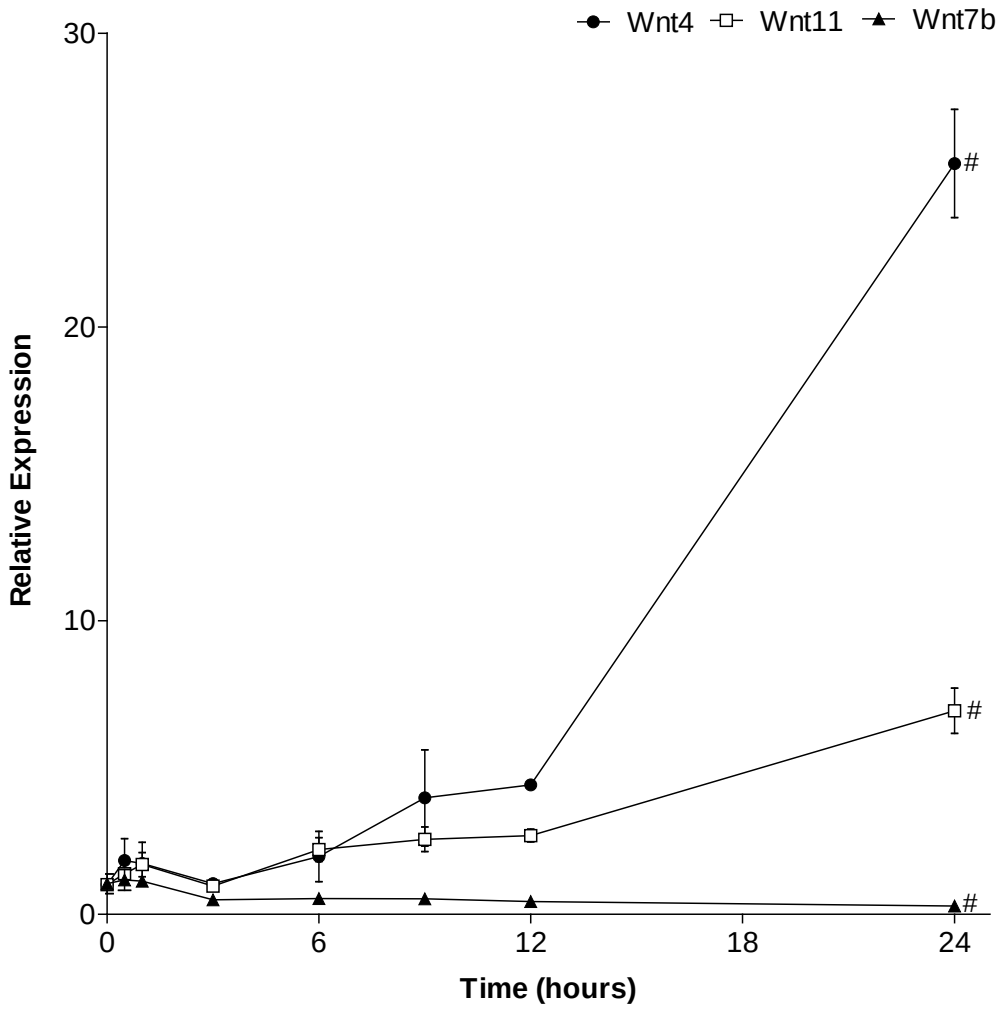

Fig 3.8 The effect of serum starvation on the expression of *Wnt4, Wnt7b* and *Wnt11* with time. MG63 cells were FCS starved for 0.5, 1, 3, 6, 9, 12 and 24 hours. RNA was extracted and mRNA assayed using RT-PCR. Data shown are expressed relative to control (24 hours culture with FCS) normalised to *GAPDH* housekeeper expression (mean ± range, n=3). #P<0.001 versus control (one-way ANOVA using Dunnett's multiple comparison test).

MG63 cells were then cultured in FCS-free medium for 24 hours. Medium was replaced with complete medium for a further 1, 3, 6 and 9 hours. RNA was extracted at each time point and reverse transcribed. Expression of *Wnt4*, *Wnt7b* and *Wnt11* and *DKK-1* was analysed as previously described. Replacement of FCS-free medium with complete medium caused the expression of both *Wnt4* and *Wnt11* to be significantly down-regulated in a time-dependent manner, exhibiting a significant response at 1 hour ($P<0.001$) and 3 hours ($P<0.01$) respectively compared to expression levels at the end of the 24 hour FCS-starvation period, termed time zero from here on (Fig 3.9A). The expression of both *Wnt4* and *Wnt11* had reached control levels at 6 hours. Replacement of FCS-free medium with complete medium also significantly up-regulated the expression of *Wnt7b* and *DKK-1* in a time-dependent manner ($P<0.01$ and $P<0.001$ respectively, Fig 3.9A). The time taken for the expression of *Wnt7b* and *DKK-1* to exhibit a significant response was 3 hours and 1 hour respectively compared to time zero. Expression of *DKK-1* was significantly up-regulated earlier than *Wnt7b* in response to replacement of complete medium, reaching a peak (13-fold expression compared to control) at 6 hours.

DKK-1 protein was also assayed in the culture medium. As already established, FCS starvation inhibits DKK-1 production. Replacement of FCS-free medium after 24 hours with complete medium caused a significant time-dependent increase in DKK-1 protein ($P<0.001$). The time taken for the production of DKK-1 to exhibit a significant response was 6 hours compared to time zero, returning to 70% of control levels at 9 hours (Fig 3.9B).

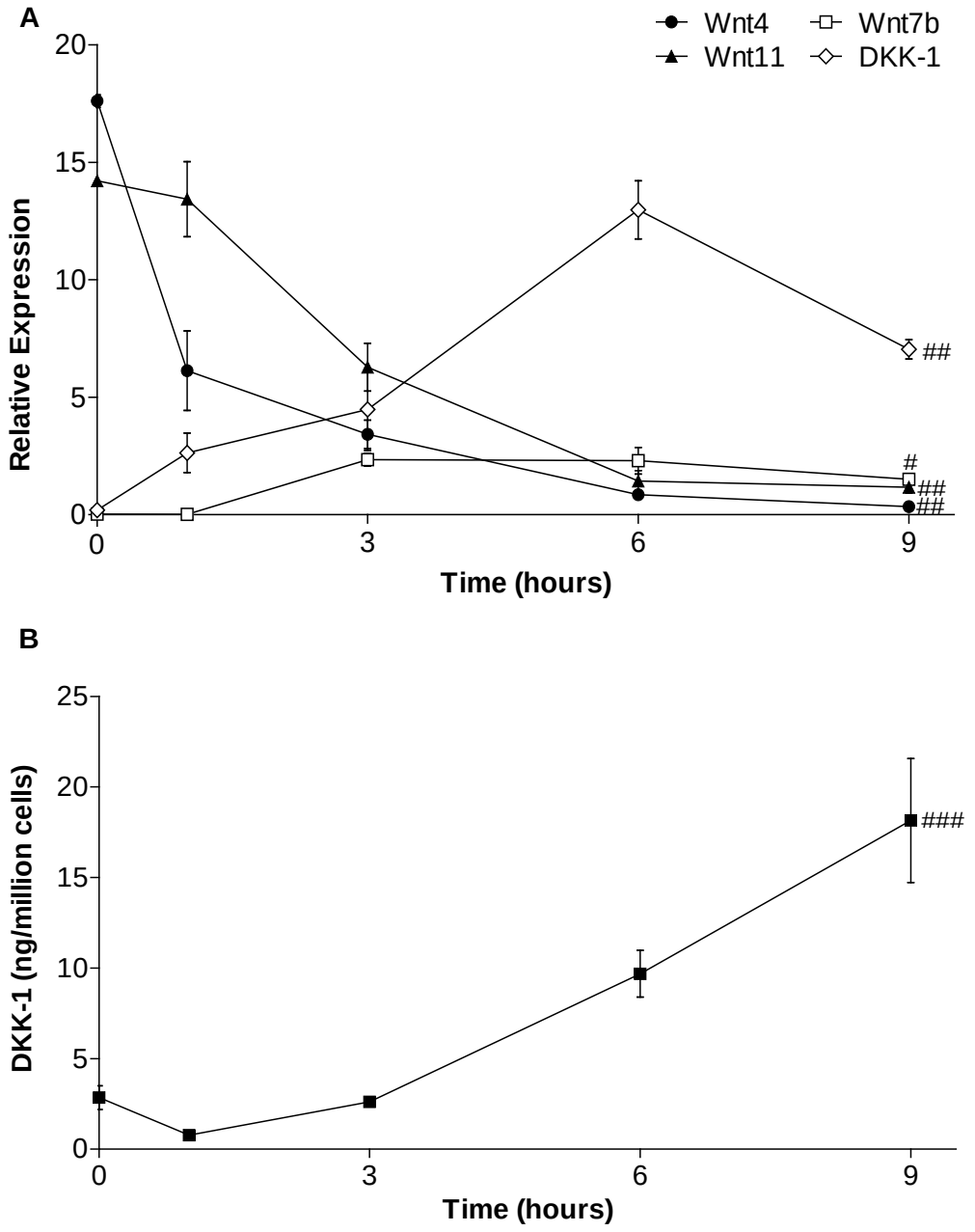

Fig 3.9 The effect of re-addition of FCS. MG63 cells were FCS starved for 24 hours before medium was replaced with full medium for a further 1, 3, 6 and 9 hours. A) RNA was extracted and mRNA assayed using RT-PCR. Time zero (control) refers to the end of the 24 hour FCS starvation period. Data shown are expressed relative to control (24 hours culture with FCS) normalised to *GAPDH* housekeeper expression (mean ± range, n=3). #P<0.01 and ##P<0.001 versus control (one-way ANOVA using Dunnett's multiple comparison test). B) Culture medium was assayed for accumulated DKK-1. Data are presented as mean ± SD, n=4. ###P<0.01 versus control (one-way ANOVA using Dunnett's multiple comparison test).

3.3 Identifying possible candidate effectors responsible for the FCS-induced OPG and DKK-1

The previous results have suggested that there is something in FCS responsible for causing the production of OPG and DKK-1. FCS contains many different hormones, cytokines and growth factors, any of which could be responsible for stimulating OPG and/or DKK-1 production directly or indirectly. Hormones cytokines and growth factors known for their effect on bone were investigated for their ability to stimulate OPG and DKK-1 production.

3.3.1 The effect of BMP-2 and BMP-7 on OPG and DKK-1 production

MG63 cells were stimulated for 24 hours in complete medium with 0-200ng/ml bone morphogenetic protein-2 (BMP-2) with or without 200ng/ml noggin, a BMP inhibitor. Culture medium was removed and assayed for OPG and DKK-1. BMP-2 significantly increased OPG and DKK-1 in a dose-dependent manner ($P<0.001$, Fig 3.10). The lowest effective concentration of BMP-2 to exhibit a significant response was 50ng/ml for both OPG and DKK-1 production. A maximal effect was seen at 200ng/ml BMP-2 where OPG and DKK-1 production was significantly increased in MG63 cells by 108% and 409% respectively compared to control ($P<0.001$). Noggin significantly attenuated BMP-2 induced OPG and DKK-1 production although to different extents ($P<0.01$ and $P<0.001$ respectively, Fig 3.10). At 200ng/ml BMP-2 in the presence of 200ng/ml noggin, OPG production had been increased by only 37% compared to control whereas DKK-1 production had been increased by 281% compared to control. Noggin had no effect on either OPG or DKK-1 production in the absence of BMP-2.

The effect of BMP-2 in Saos-2 cells was investigated. Saos-2 cells were stimulated for 24 hours in complete medium with 100ng/ml BMP-2. OPG production was significantly increased 36% above control ($P<0.05$, Fig 3.11A). BMP-2 had no effect on DKK-1 production in Saos-2 cells (Fig 3.11B).

BMP-7 was also investigated for its ability to stimulate both OPG and DKK-1 production. MG63 and Saos-2 cells were stimulated for 24 hours in complete medium with or without 100ng/ml BMP-7. BMP-7 had no effect on OPG production in either MG63 or Saos-2 cells (Fig 3.12A). DKK-1 production was significantly increased by 166% and 108% above control in MG63 and Saos-2 cells respectively ($P<0.001$ and $P<0.01$ respectively, Fig 3.12B).

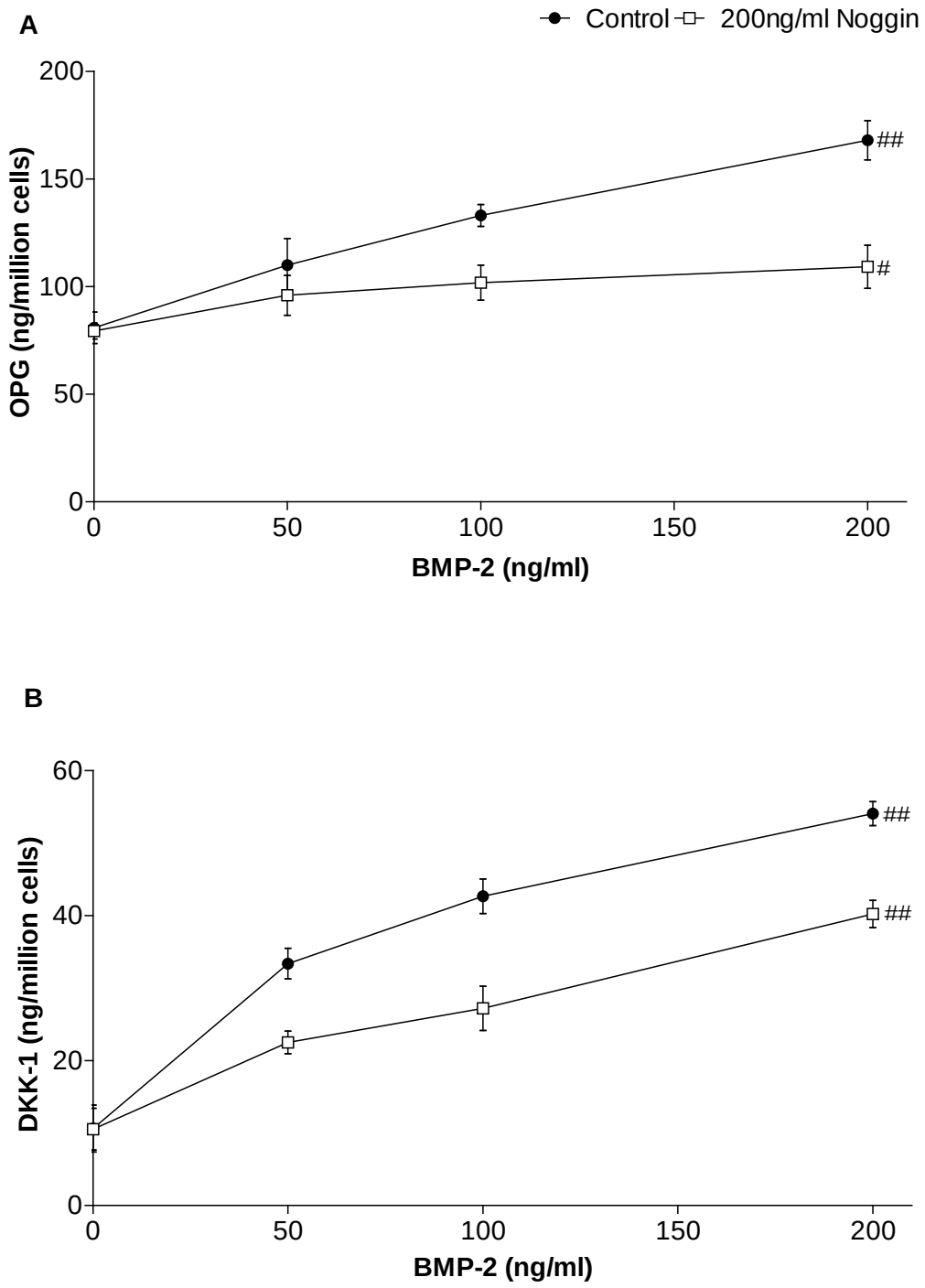

Fig 3.10 BMP-2 stimulates both OPG and DKK-1 production in MG63 cells. MG63 cells were cultured for 24 hours with various concentrations of BMP-2 with or without 200ng/ml noggin. Culture medium was removed and assayed for A) OPG and B) DKK-1. Data are presented as mean ± SD, n=4. #P<0.01 and ##P<0.001 versus control (0ng/ml BMP-2, one-way ANOVA using Dunnett's multiple comparison test).

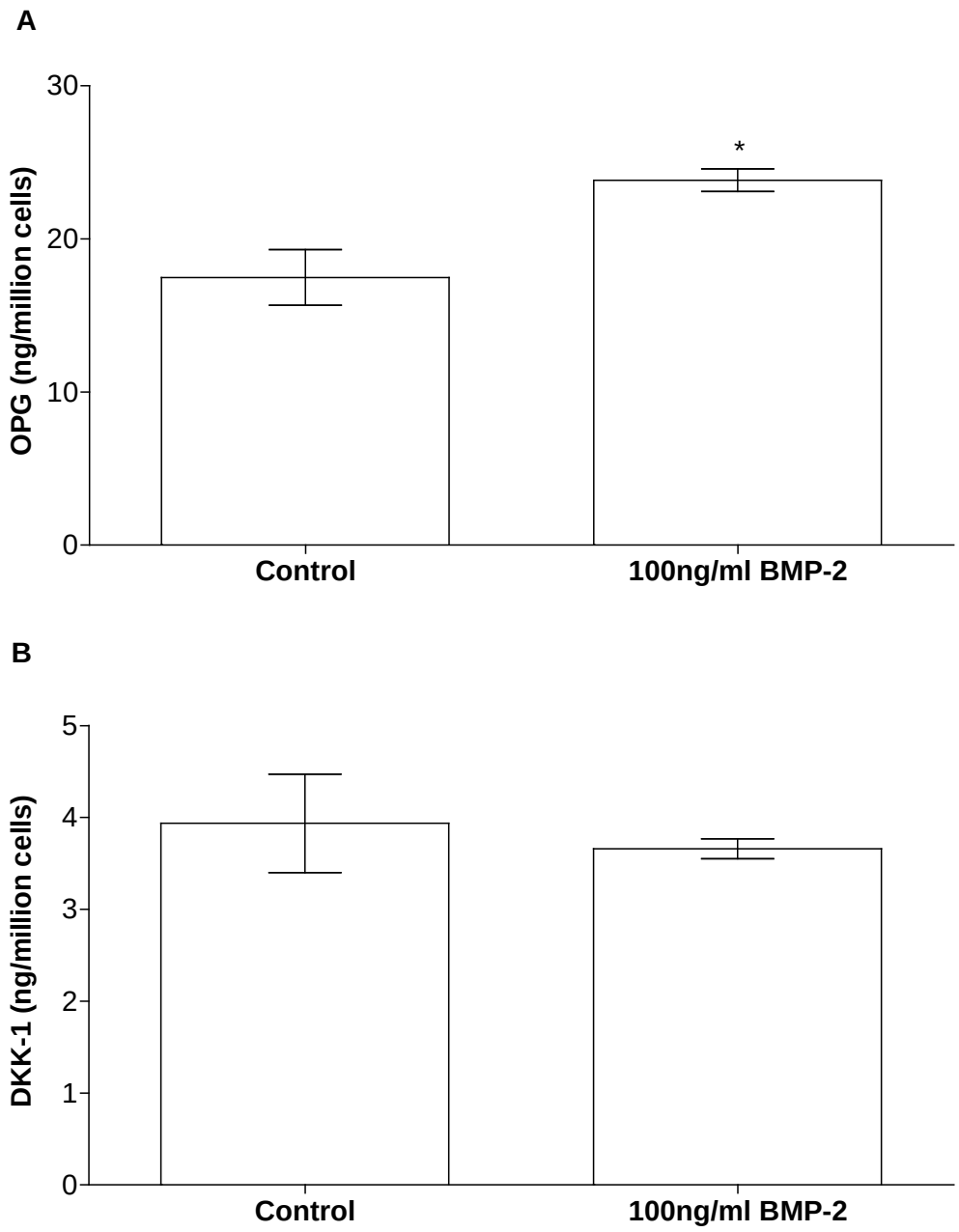

Fig 3.11 The effect of BMP-2 on OPG and DKK-1 production in Saos-2 cells. Saos-2 cells were stimulated for 24 hours with or without 100ng/ml BMP-2. Culture medium was removed and assayed for A) OPG and B) DKK-1. Data are presented as mean ± SD, n=4. *P<0.05 versus control (unpaired t-test).

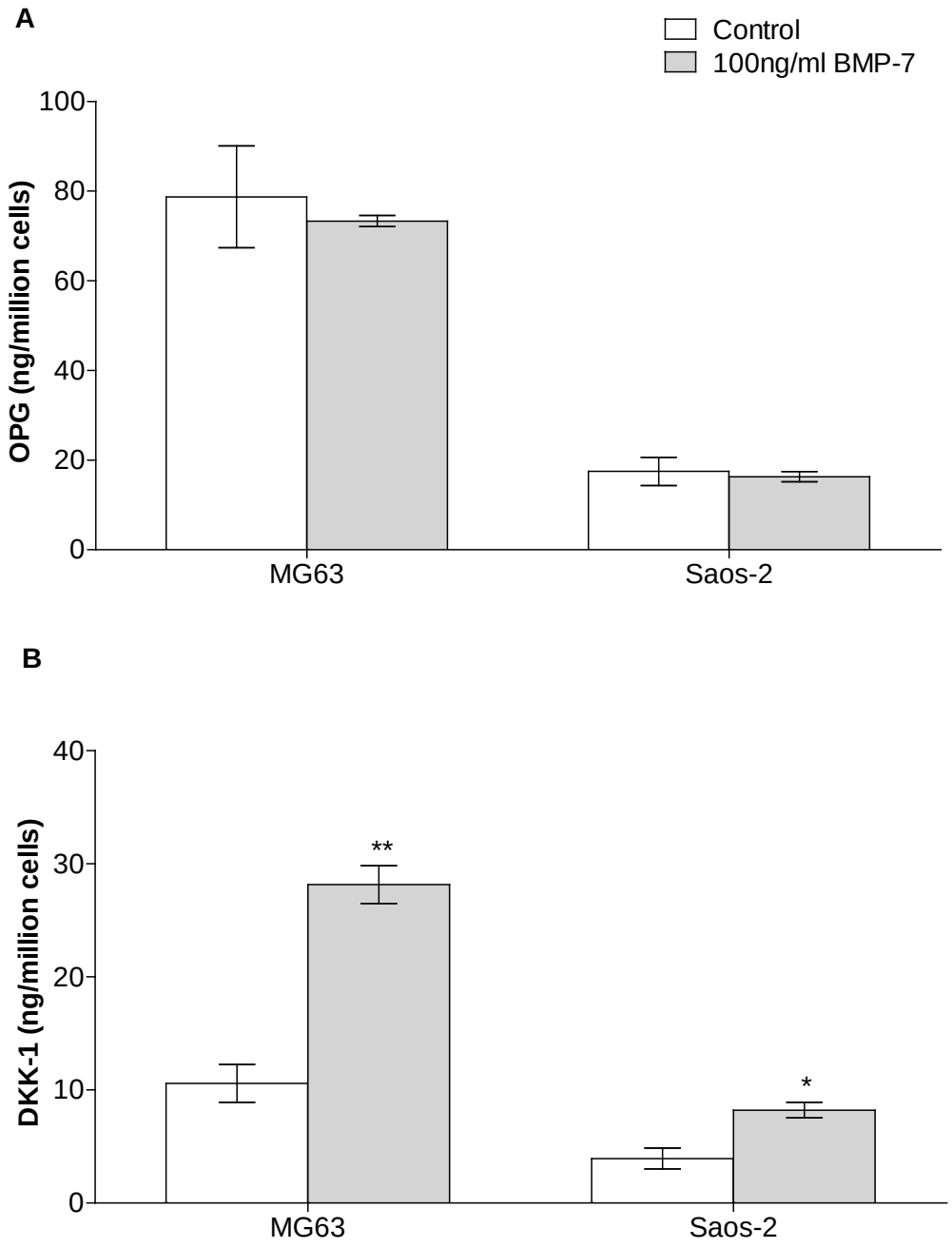

Fig 3.12 The effect of BMP-7 on OPG and DKK-1 production in osteoblastic cells. MG63 and Saos-2 cells were stimulated for 24 hours with or without 100ng/ml BMP-7. Culture medium was removed and assayed for A) OPG and B) DKK-1. Data are presented as mean ± SD, n=4. *P<0.01 and **P<0.001 versus same cell type control (unpaired t-test).

3.3.2 The effect of TGFβ on OPG and DKK-1 production

The effect of transforming growth factor-beta (TGFβ) on OPG and DKK-1 production was investigated. MG63 and Saos-2 cells were stimulated for 24 hours with 0-10ng/ml TGFβ either with or without FCS. Culture medium was assayed for OPG and DKK-1 after the 24 hour period. TGFβ significantly stimulated OPG production in MG63 cells in a dose-dependent manner both in the presence and absence of FCS ($P<0.001$, Fig 3.13A). The lowest effective concentration to exhibit a maximal effect was 5ng/ml in both culture conditions. In the presence of FCS, a maximal effect was observed at 5ng/ml where OPG production was increased by 160% compared to control ($P<0.001$). In the absence of FCS, a maximal effect was observed at 10ng/ml where OPG production was increased by 129% compared to control ($P<0.001$).

DKK-1 production in MG63 cells was significantly increased in a biphasic manner in the presence of FCS ($P<0.001$, Fig 3.13B). The lowest effective concentration to exhibit a significant response was 1ng/ml. A maximal effect was observed at 2.5ng/ml where DKK-1 production was increased 65% compared to control ($P<0.001$, Fig 3.13B). At 10ng/ml TGFβ in the presence of FCS, DKK-1 production in MG63 cells was significantly increased 29% compared to control ($P<0.01$). In the absence of FCS, TGFβ did not significantly affect DKK-1 production in MG63 cells.

In Saos-2 cells, there was no significant effect of TGFβ on the production of OPG either in the presence or absence of FCS (Fig 3.14A). DKK-1 production in Saos-2 cells in response to TGFβ was similar to that of MG63 cells. In the presence of FCS, DKK-1 production was significantly increased in a biphasic manner ($P<0.001$, Fig 3.14B). The lowest effective concentration to exhibit a significant response was 1ng/ml. A maximal effect was observed at 2.5ng/ml where DKK-1 production was increased 54% compared to control ($P<0.001$). At 10ng/ml TGFβ in the presence of FCS, DKK-1 production in Saos-2 cells was not significantly different from control. In the absence of FCS, TGFβ significantly stimulated DKK-1 production in Saos-2 cells in a dose-dependent manner ($P<0.001$, Fig 3.14B). The maximal and only concentration to exhibit a significant response was observed at 10ng/ml, where DKK-1 production was stimulated 119% compared to control ($P<0.001$).

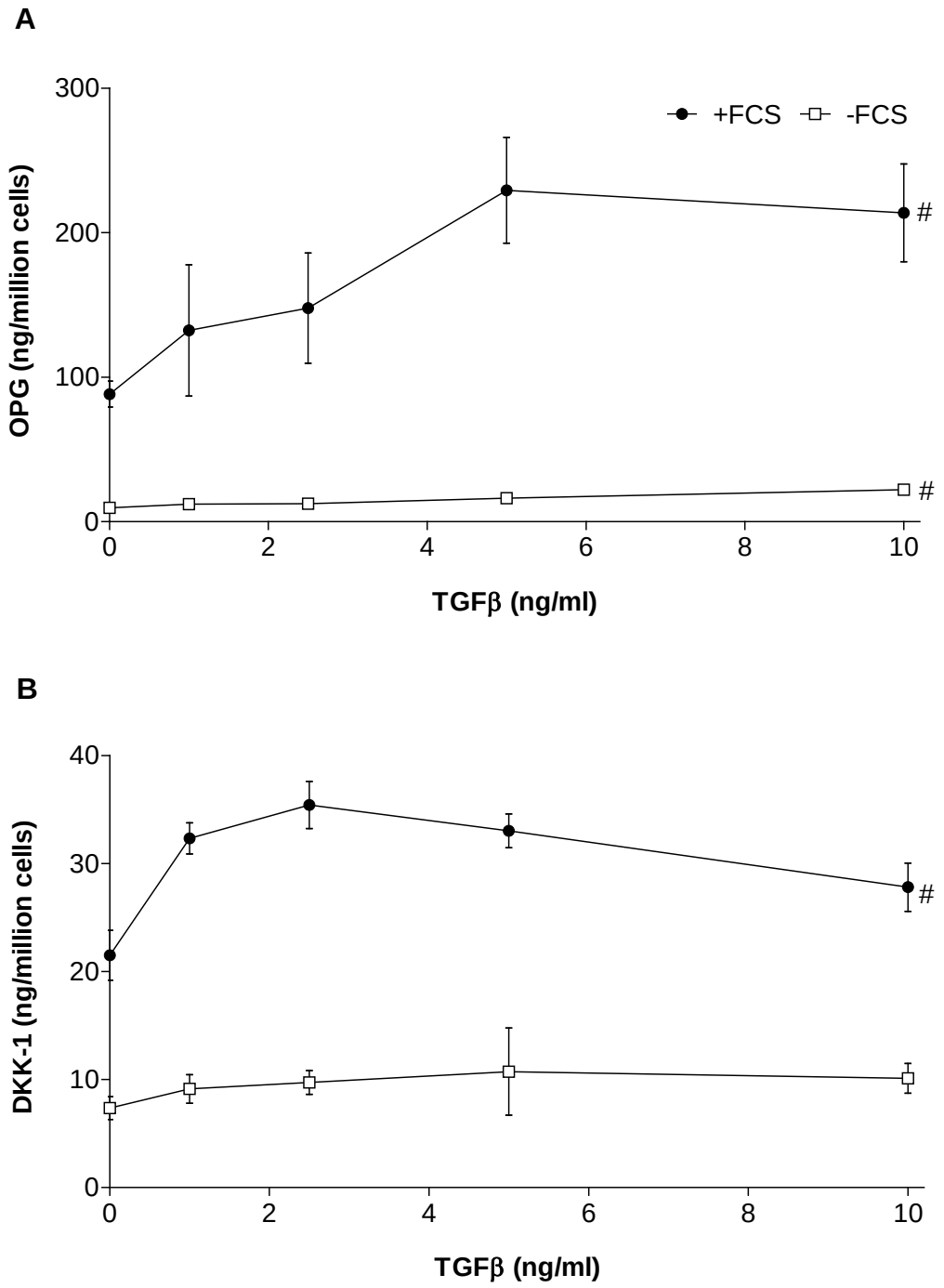

Fig 3.13 The effect of TGFβ on OPG and DKK-1 production in MG63 cells. MG63 cells were stimulated for 24 hours with 0-10ng/ml TGFβ with or without FCS. Culture medium was removed and assayed for A) OPG and B) DKK-1. Data are presented as mean ± SD, n=4. #P<0.001 versus control (0ng/ml TGFβ, one-way ANOVA using Dunnett's multiple comparison test).

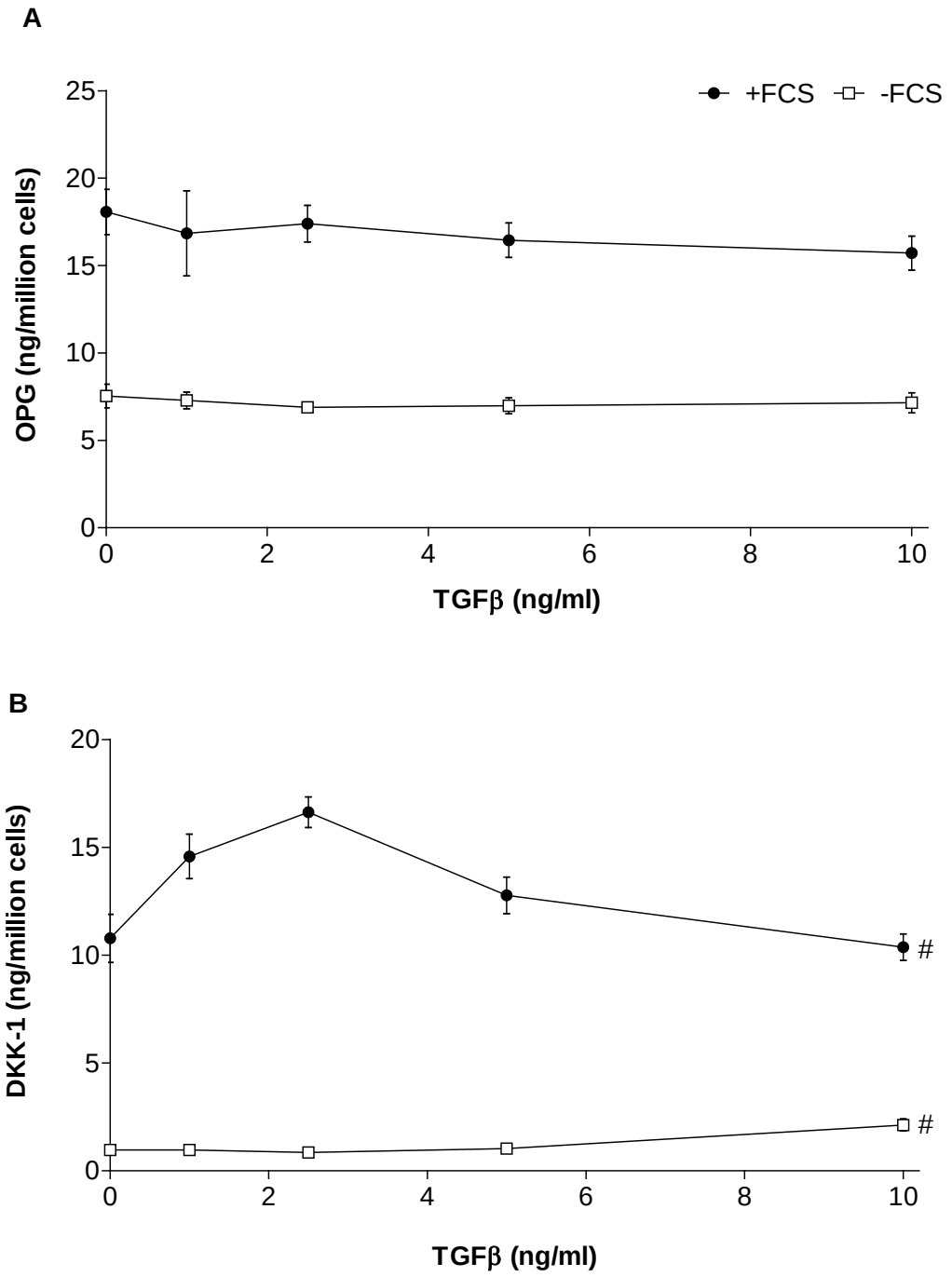

Fig 3.14 The effect of TGFβ on OPG and DKK-1 production in Saos-2 cells. Saos-2 cells were stimulated for 24 hours with 0-10ng/ml TGFβ with or without FCS. Culture medium was removed and assayed for A) OPG and B) DKK-1. Data are presented as mean ± SD, n=4. #P<0.001 versus control (0ng/ml TGFβ, one-way ANOVA using Dunnett's multiple comparison test).

3.3.3 The effect of Activin A on OPG and DKK-1 production

The effect of Activin A on OPG and DKK-1 production was investigated. MG63 and Saos-2 cells were stimulated for 24 hours with 0-25ng/ml Activin A either with or without FCS. Culture medium was assayed for OPG and DKK-1 after 24 hours. In MG63 cells, Activin A did not have any significant effect on the production of OPG either in the presence or absence of FCS (Fig 3.15A). Activin A significantly increased DKK-1 production in the absence of FCS in a dose-dependent manner ($P<0.001$). The lowest and only concentration to exhibit a significant response was 25ng/ml, where DKK-1 production was stimulated by 150% compared to control ($P<0.001$, Fig 3.15B). There was no significant effect of Activin A on DKK-1 production in the presence of FCS.

In Saos-2 cells, Activin A did not have any significant effect on the production of OPG either in the presence or absence of FCS (Fig 3.16A). Activin A significantly inhibited DKK-1 production in the presence of FCS in a biphasic manner ($P<0.05$). The lowest and only concentration to exhibit a significant response was 5ng/ml, where DKK-1 production was inhibited by 15% compared to control ($P<0.05$, Fig 3.16B). At 25ng/ml, DKK-1 production was not significantly different to control levels. There was no significant effect of Activin A on DKK-1 production in the absence of FCS.

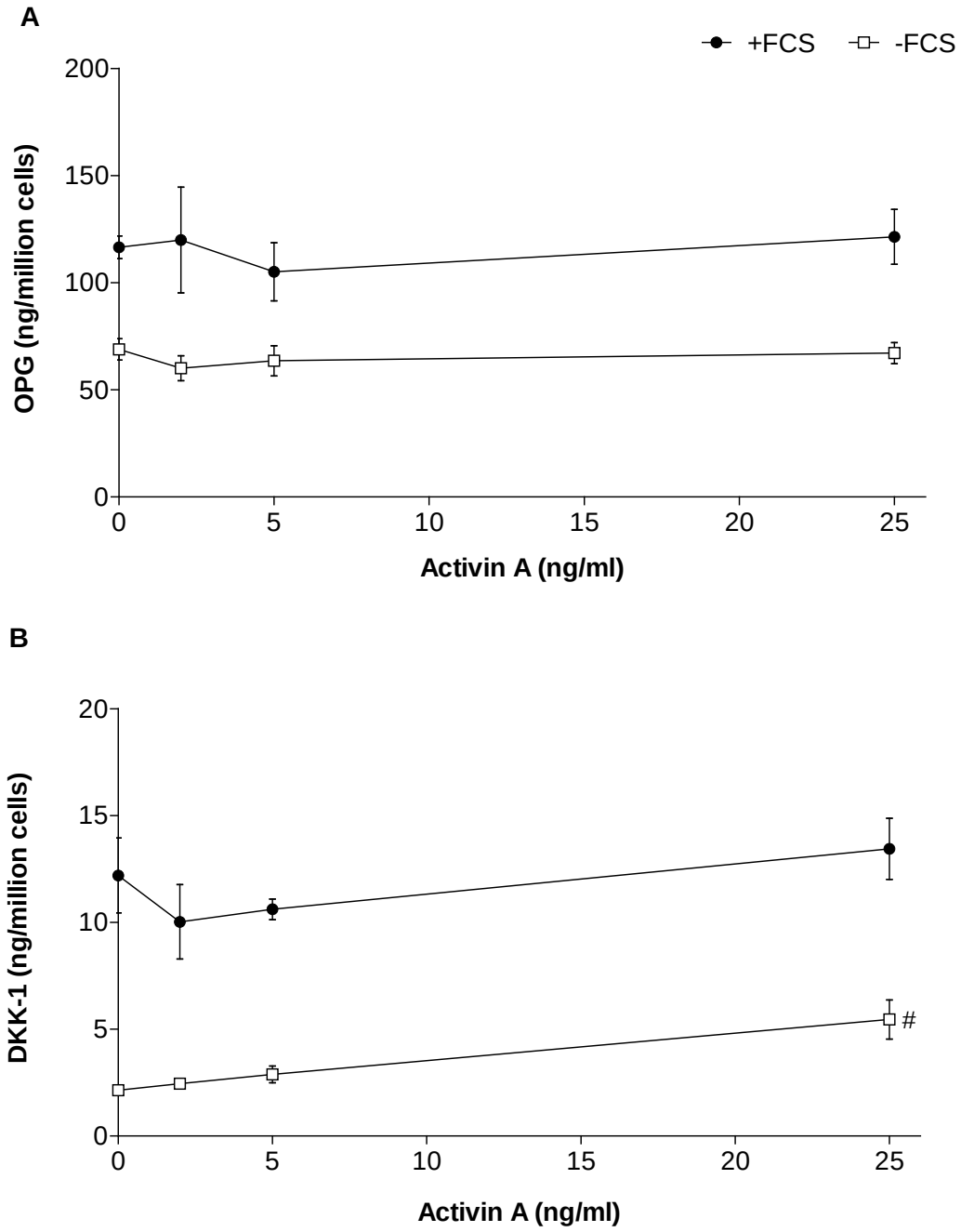

Fig 3.15 The effect of Activin A on OPG and DKK-1 production in MG63 cells. MG63 cells were stimulated for 24 hours with 0-25ng/ml Activin A with or without FCS. Culture medium was removed and assayed for A) OPG and B) DKK-1. Data are presented as mean ± SD, n=4. #P<0.001 versus control (0ng/ml Activin A, one-way ANOVA using Dunnett's multiple comparison test).

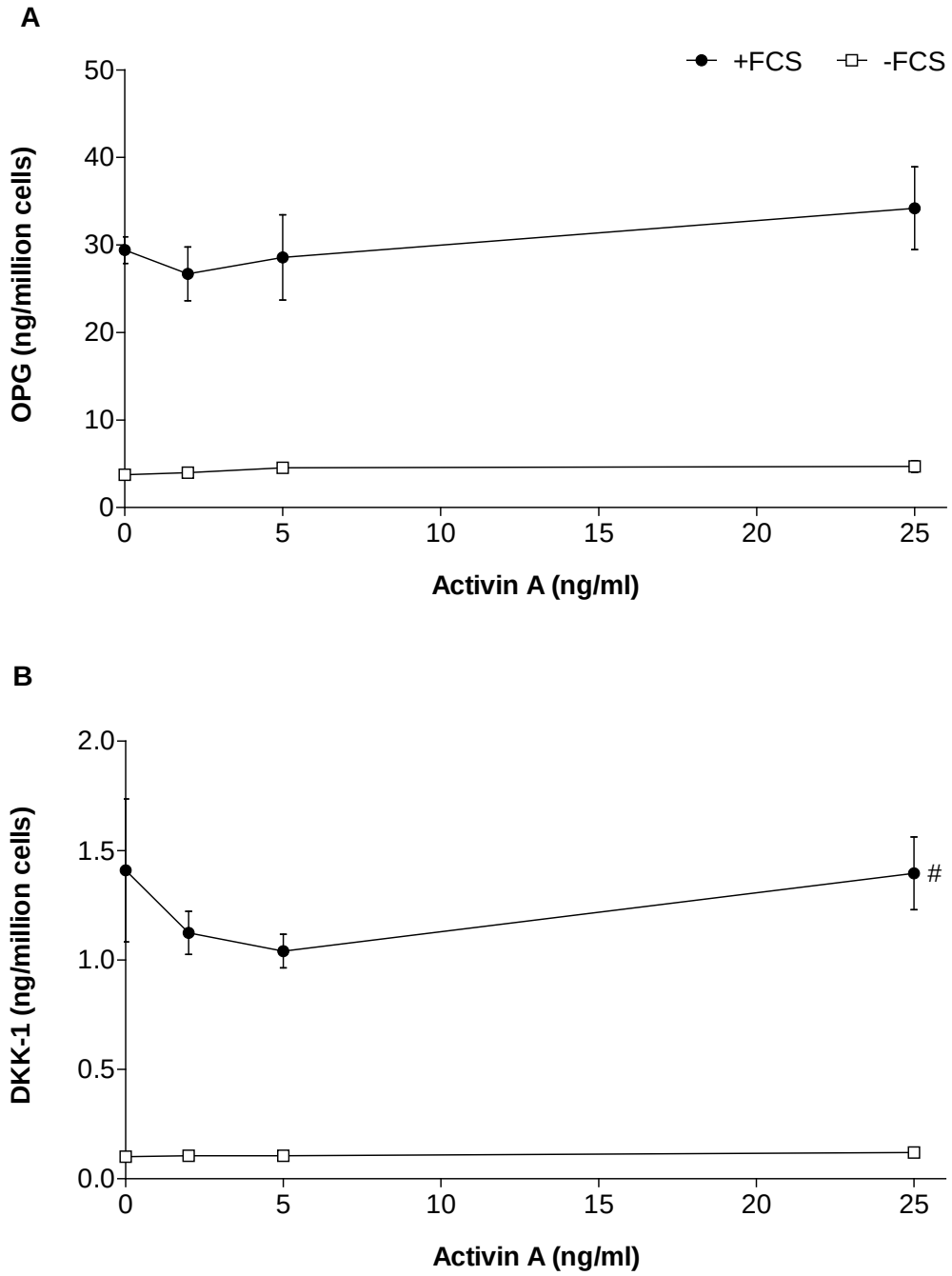

Fig 3.16 The effect of Activin A on OPG and DKK-1 production in Saos-2 cells. Saos-2 cells were stimulated for 24 hours with 0-25ng/ml Activin A with or without FCS. Culture medium was removed and assayed for A) OPG and B) DKK-1. Data are presented as mean ± SD, n=4. #P<0.05 versus control (0ng/ml Activin A, one-way ANOVA using Dunnett's multiple comparison test).

3.3.4 The effect of TNFα on OPG and DKK-1 production

The effect of tumour necrosis factor-alpha (TNFα) on OPG and DKK-1 production was investigated. MG63 and Saos-2 cells were stimulated for 24 hours with 0-10ng/ml TNFα either with or without FCS. Culture medium was assayed for OPG and DKK-1 after 24 hours. In MG63 cells, TNFα did not have any significant effect on the production of OPG in the presence of FCS. In the absence of FCS, only 10ng/ml TNFα had a significant effect on OPG production, where OPG production was stimulated by 380% compared to control ($P<0.05$, Fig 3.17A). There was not however, a significant dose response in the absence of FCS.

TNFα significantly stimulated DKK-1 production in MG63 cells in a dose-dependent manner both in the presence and absence of FCS ($P<0.01$ and $P<0.001$ respectively, Fig 3.17B). The lowest effective concentration of TNFα to exhibit a significant response was 10ng/ml in the presence of FCS and 5ng/ml in the absence of FCS. A maximal effect was seen at 10ng/ml TNFα where DKK-1 production was significantly increased in MG63 cells by 37% ($P<0.01$) and 480% ($P<0.001$) compared to control in the presence and absence of FCS respectively.

In Saos-2 cells, OPG production was significantly stimulated in response to TNFα in the presence of FCS in a biphasic manner ($P<0.05$, Fig 3.18A). The only concentration of TNFα to exhibit a significant response was at 2.5ng/ml, with OPG production returning to control levels by 10ng/ml. At 2.5ng/ml TNFα OPG production was stimulated by 60% compared to control. There was no effect of TNFα on OPG production in Saos-2 cells in the absence of FCS.

TNFα significantly stimulated DKK-1 production in Saos-2 cells in a dose-dependent manner both in the presence and absence of FCS ($P<0.01$ and $P<0.001$ respectively, Fig 3.18B). The lowest effective concentration of TNFα to exhibit a significant response was 10ng/ml in the presence of FCS and 5ng/ml in the absence of FCS. In the presence of FCS, 10ng/ml TNFα stimulated DKK-1 production was significantly increased in Saos-2 cells by 51% ($P<0.01$). DKK-1 was not detected in the culture medium of unstimulated Saos-2 cells in the absence of FCS, although in response to 10ng/ml TNFα DKK-1 production was re-instated at very low levels (Fig 3.18B).

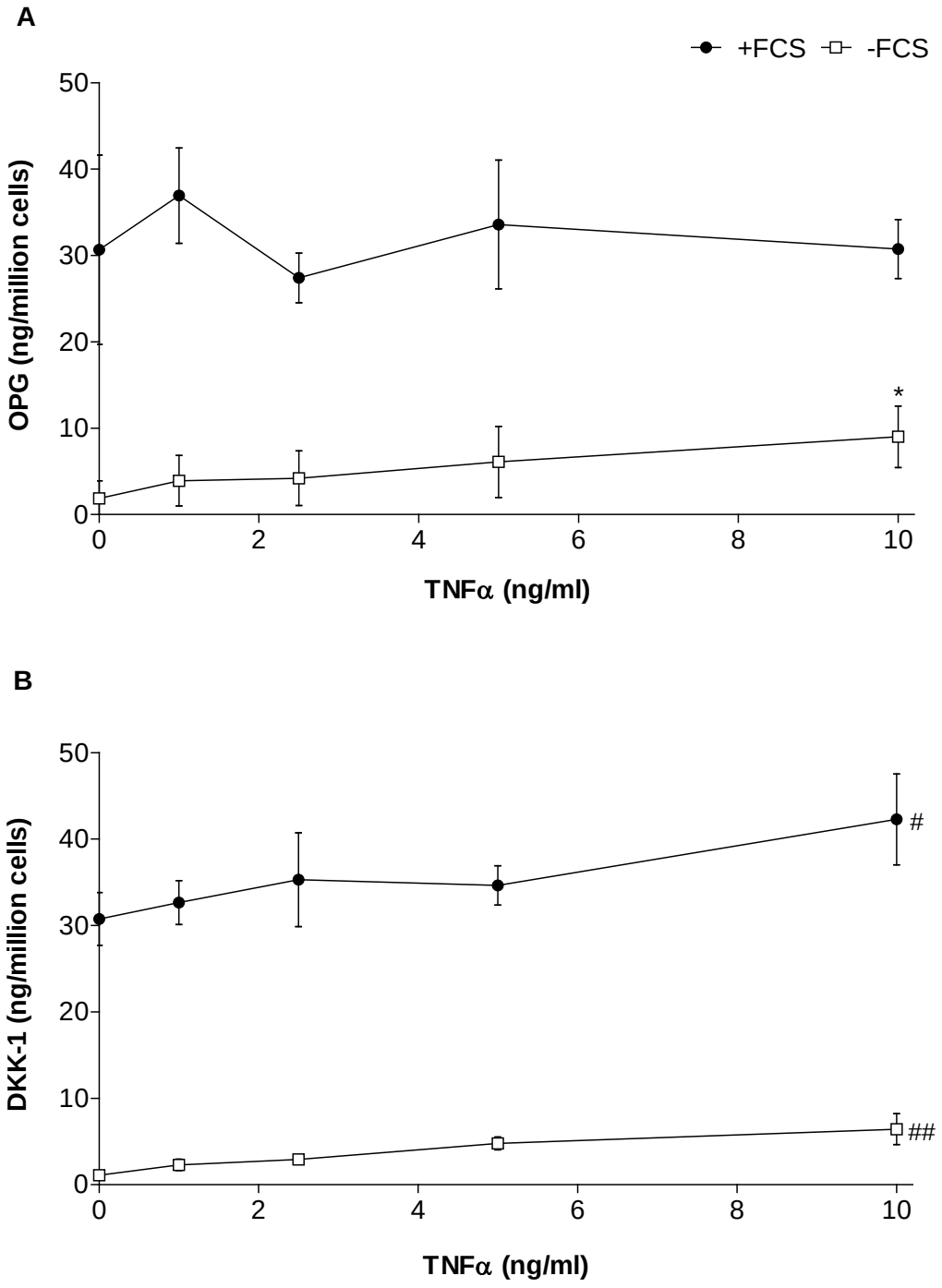

Fig 3.17 The effect of TNFα on OPG and DKK-1 production in MG63 cells. MG63 cells were stimulated for 24 hours with 0-10ng/ml TNFα with or without FCS. Culture medium was removed and assayed for A) OPG and B) DKK-1. Data are presented as mean ± SD, n=4. *P<0.05 versus control (0ng/ml TNFα, unpaired t-test). #P<0.01 and ##P<0.001 versus control (one-way ANOVA using Dunnett's multiple comparison test).

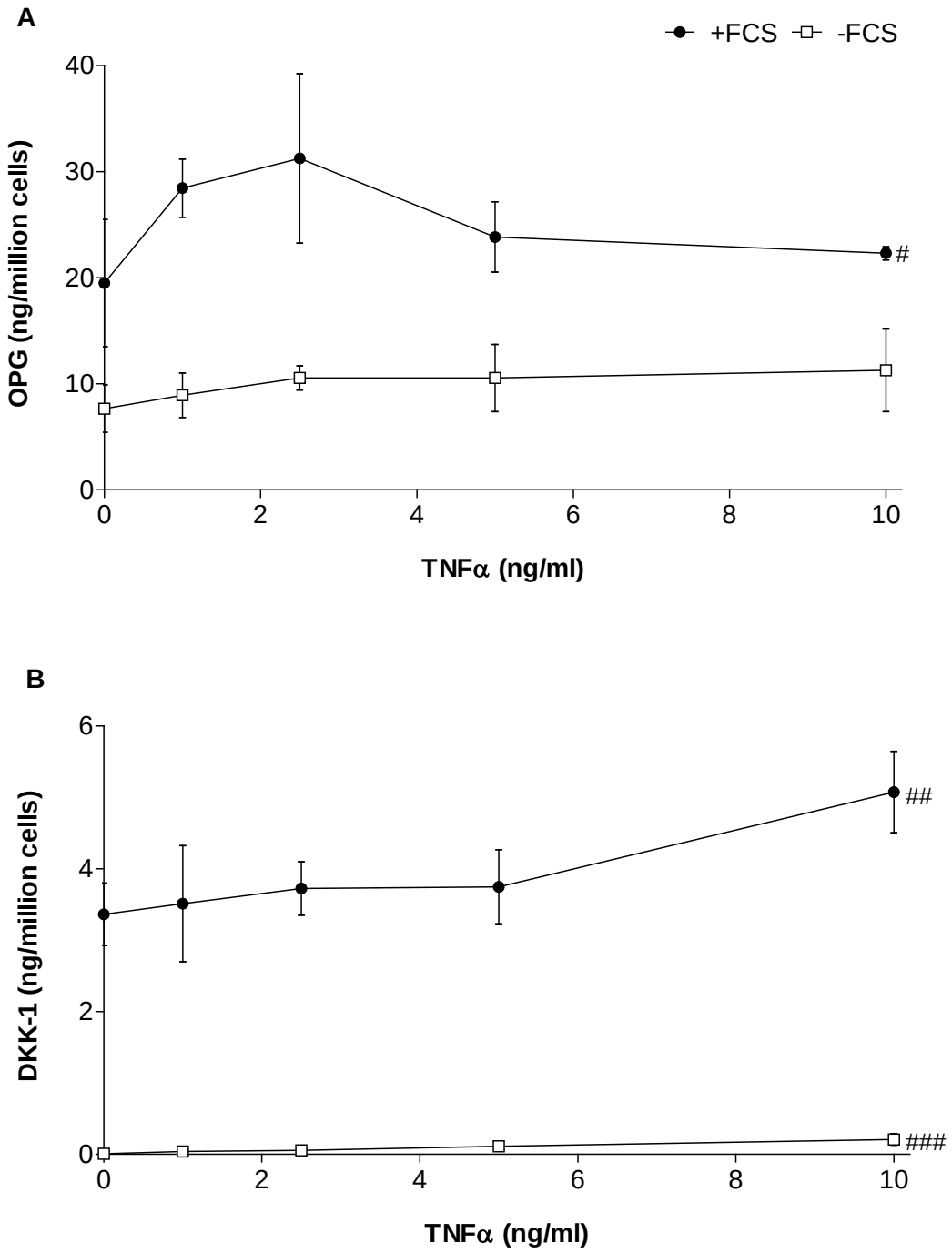

Fig 3.18 The effect of TNFα on OPG and DKK-1 production in Saos-2 cells. Saos-2 cells were stimulated for 24 hours with 0-10ng/ml TNFα with or without FCS. Culture medium was removed and assayed for A) OPG and B) DKK-1. Data are presented as mean ± SD, n=4. #P<0.05, ##P<0.01 and ###P<0.001 versus control (0ng/ml TNFα, one-way ANOVA using Dunnett's multiple comparison test).

3.3.5 The effect of insulin on OPG and DKK-1 production

The effect of insulin on OPG and DKK-1 production was investigated. MG63 and Saos-2 cells were stimulated for 24 hours with 0-100U/ml insulin either with or without FCS. Culture medium was assayed for OPG and DKK-1 after the 24 hour period. In MG63 cells, insulin significantly inhibited OPG production both in the presence and absence of FCS in a dose-dependent manner ($P<0.001$, Fig 3.19A). The lowest effective concentration of insulin to exhibit a significant response was 5U/ml in both culture conditions. A maximal effect was also observed at 5U/ml, where OPG production was inhibited 30% and 44% compared to control in the presence and absence of FCS respectively ($P<0.001$).

DKK-1 production in MG63 cells was significantly inhibited in a dose-dependent manner both in the presence and absence of FCS ($P<0.001$, Fig 3.19B). The lowest effective concentration of insulin to exhibit a significant response was 5U/ml in both culture conditions. A maximal effect was also observed at 5U/ml, where DKK-1 production was inhibited 29% and 69% compared to control in the presence and absence of FCS respectively (Fig $P<0.001$).

In Saos-2 cells, insulin also significantly inhibited OPG production both in the presence and absence of FCS in a dose-dependent manner ($P<0.01$, Fig 3.20A). The lowest effective concentration of insulin to exhibit a significant response was 5U/ml in both culture conditions. A maximal effect was also observed at 5U/ml, where OPG production was inhibited 17% ($P<0.01$) and 24% ($P<0.05$) compared to control in the presence and absence of FCS respectively.

DKK-1 production in Saos-2 cells was significantly inhibited in a dose-dependent manner while in the presence of FCS ($P<0.01$, Fig 3.20B). The lowest effective concentration of insulin to exhibit a significant response was 5U/ml. A maximal effect was also observed at 5U/ml, where DKK-1 production was inhibited 28% compared to control ($P<0.05$). Insulin did not have a significant effect on DKK-1 production in Saos-2 cells in the absence of FCS.

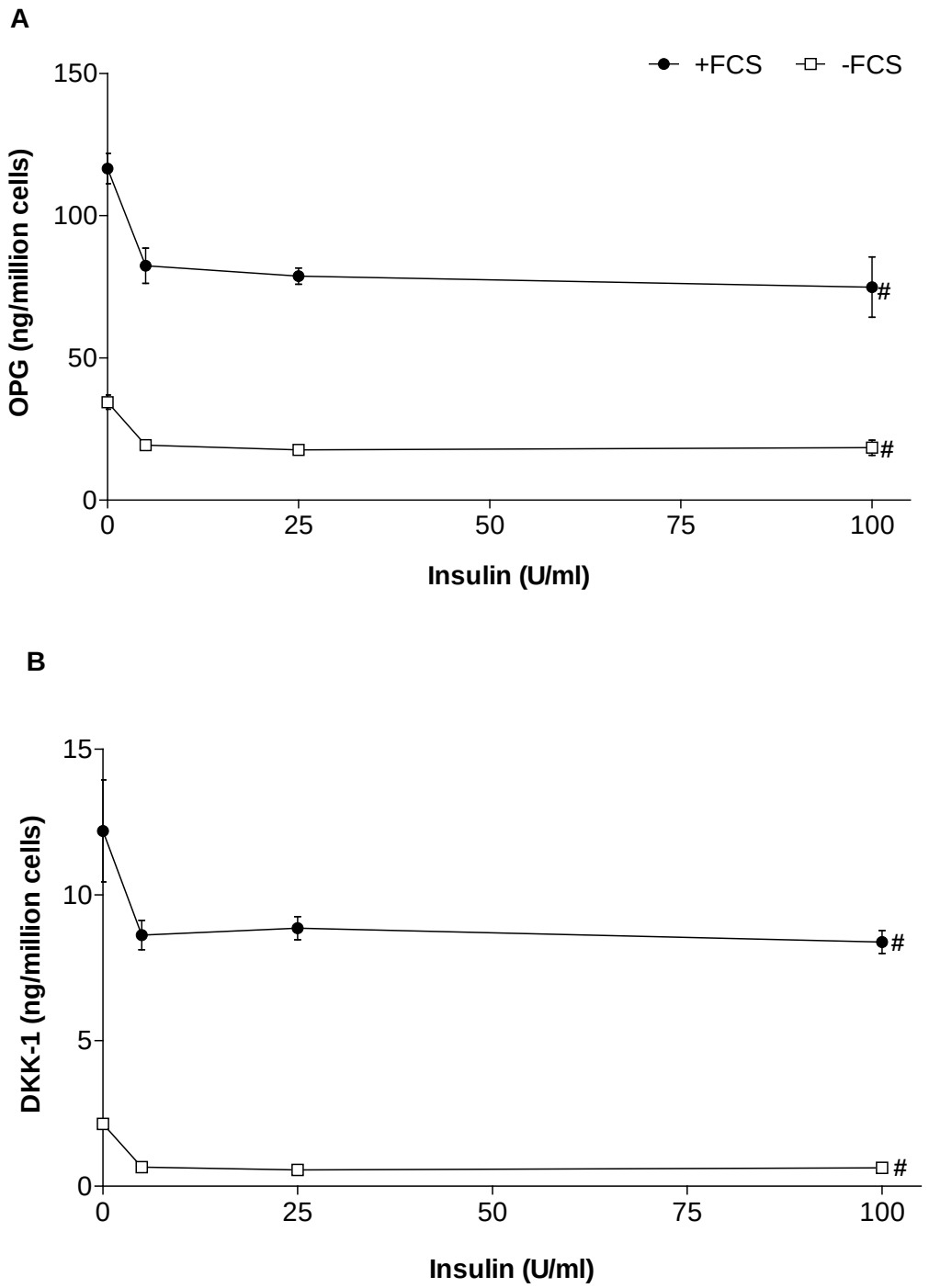

Fig 3.19 The effect of insulin on OPG and DKK-1 production in MG63 cells. MG63 cells were stimulated for 24 hours with 0-100U/ml insulin with or without FCS. Culture medium was removed and assayed for A) OPG and B) DKK-1. Data are presented as mean ± SD, n=4. #P<0.001 versus control (one-way ANOVA using Dunnett's multiple comparison test).

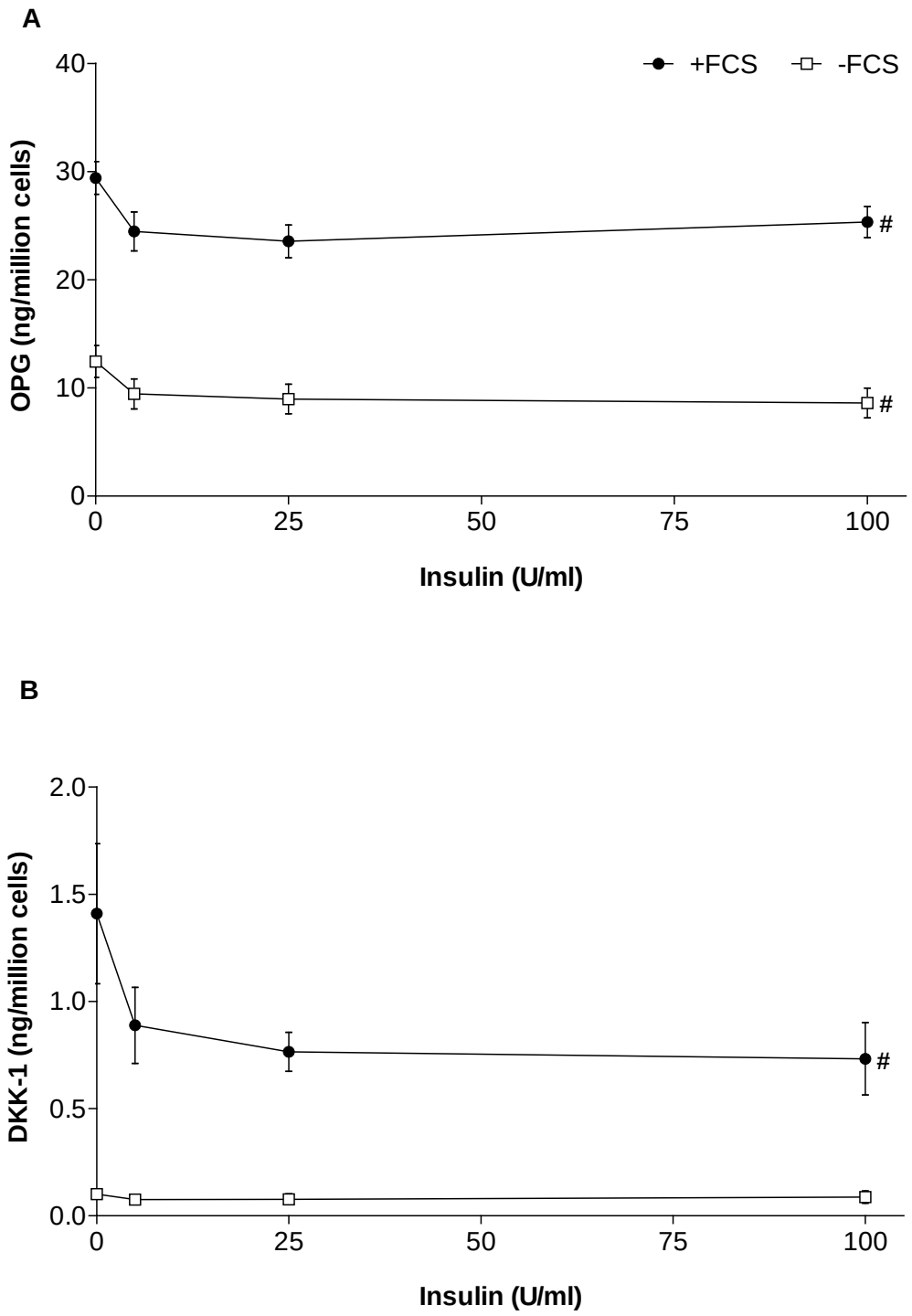

Fig 3.20 The effect of insulin on OPG and DKK-1 production in Saos-2 cells. Saos-2 cells were stimulated for 24 hours with 0-100U/ml insulin with or without FCS. Culture medium was removed and assayed for A) OPG and B) DKK-1. Data are presented as mean ± SD, n=4. #P<0.01 versus control (one-way ANOVA using Dunnett's multiple comparison test).

3.3.6 The effect of IGFII on OPG and DKK-1 production

The effect of insulin-like growth factor II (IGFII) on OPG and DKK-1 production was investigated. MG63 and Saos-2 cells were stimulated for 24 hours with 0-200ng/ml IGFII either with or without FCS. Culture medium was assayed for OPG and DKK-1 after the 24 hour period. In MG63 cells, IGFII only exhibited a significant effect on OPG production in the presence of FCS at 200ng/ml, where OPG production was stimulated by 22% compared to control ($P<0.001$, Fig 3.21A). IGFII did not significantly affect the production of OPG in MG63 cells in the absence of FCS.

DKK-1 production in MG63 cells was significantly stimulated in a biphasic manner in response to IGFII while in the presence of FCS ($P<0.001$, Fig 3.21B). The lowest effective concentration of IGFII to exhibit a significant response was 25ng/ml. A maximal effect was observed at 50ng/ml IGFII where DKK-1 production had been increased by 61% ($P<0.001$) compared to control, but had returned to control levels at 100ng/ml. In the absence of FCS, IGFII inhibited DKK-1 production in MG63 cells in a dose-dependent manner ($P<0.01$, Fig 3.21B). The lowest effective concentration of IGFII to exhibit a significant response was 25ng/ml. A maximal effect was observed at 200ng/ml where DKK-1 production was inhibited by 30% compared to control ($P<0.01$).

In Saos-2 cells, OPG production was significantly increased in response to TGFβ in both the presence and absence of FCS in a dose-dependent manner ($P<0.05$, Fig 3.22A). The lowest effective concentration to exhibit a significant response was 25ng/ml in both culture conditions ($P<0.05$). A maximum effect was observed at 25ng/ml where OPG production was significantly increased by 17% in both culture conditions compared to control ($P<0.05$).

In Saos-2 cells, IGFII inhibited DKK-1 production in a dose-dependent manner both with and without FCS ($P<0.001$, Fig 3.22B). The lowest effective concentration to exhibit a significant response was 50ng/ml both in the presence and absence of FCS. In the presence of FCS, a maximal response was observed at 200ng/ml, where DKK-1 production was inhibited by 53% compared to control ($P<0.001$). In the absence of FCS however, DKK-1 production was completely blocked at 50ng/ml (Fig 3.22B).

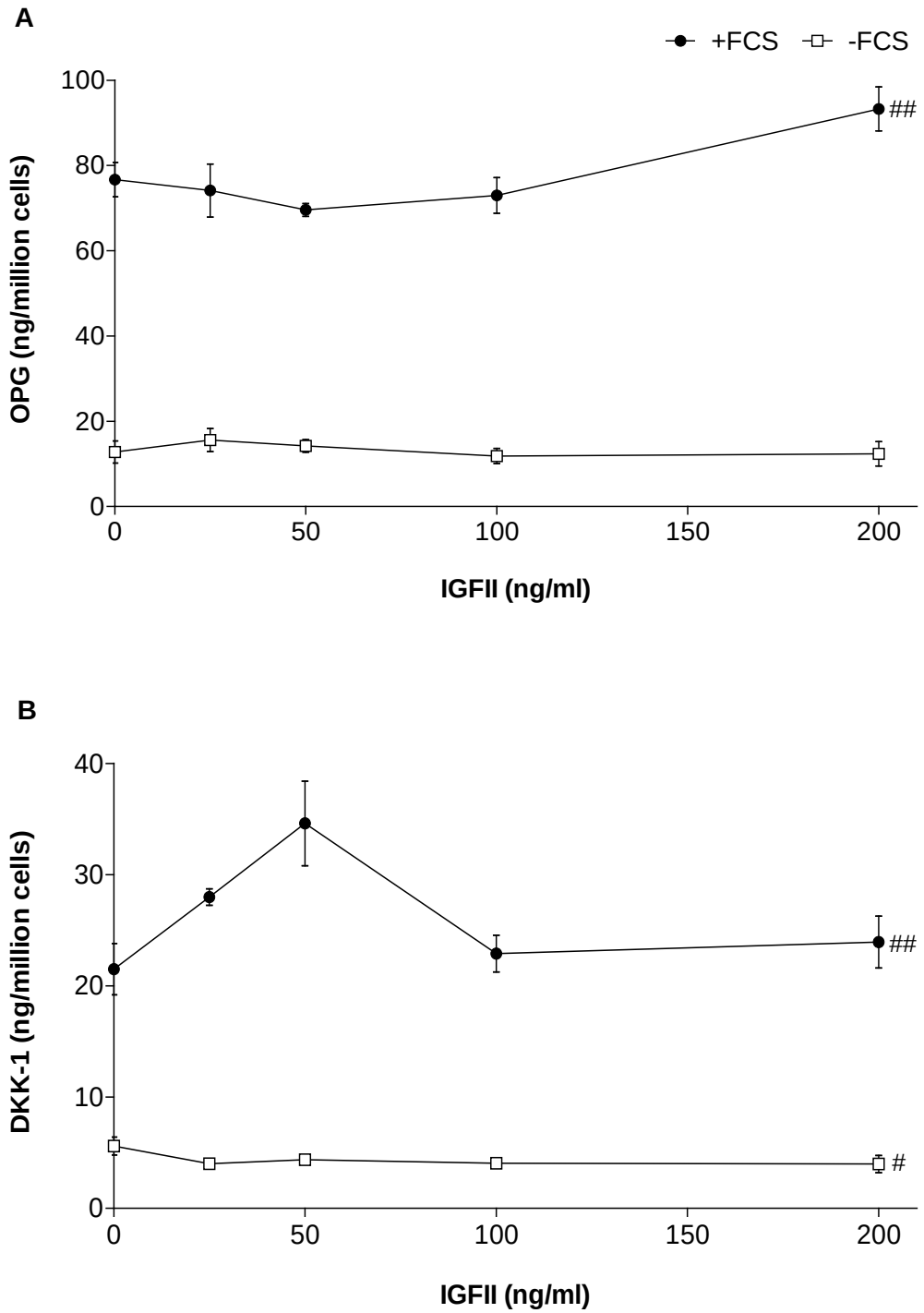

Fig 3.21 The effect of IGFII on OPG and DKK-1 production in MG63 cells. MG63 cells were stimulated for 24 hours with 0-200ng/ml IGFII with or without FCS. Culture medium was removed and assayed for A) OPG and B) DKK-1. Data are presented as mean ± SD, n=4. #P<0.01 and ##P<0.001 versus control (0ng/ml IGFII, one-way ANOVA using Dunnett's multiple comparison test).

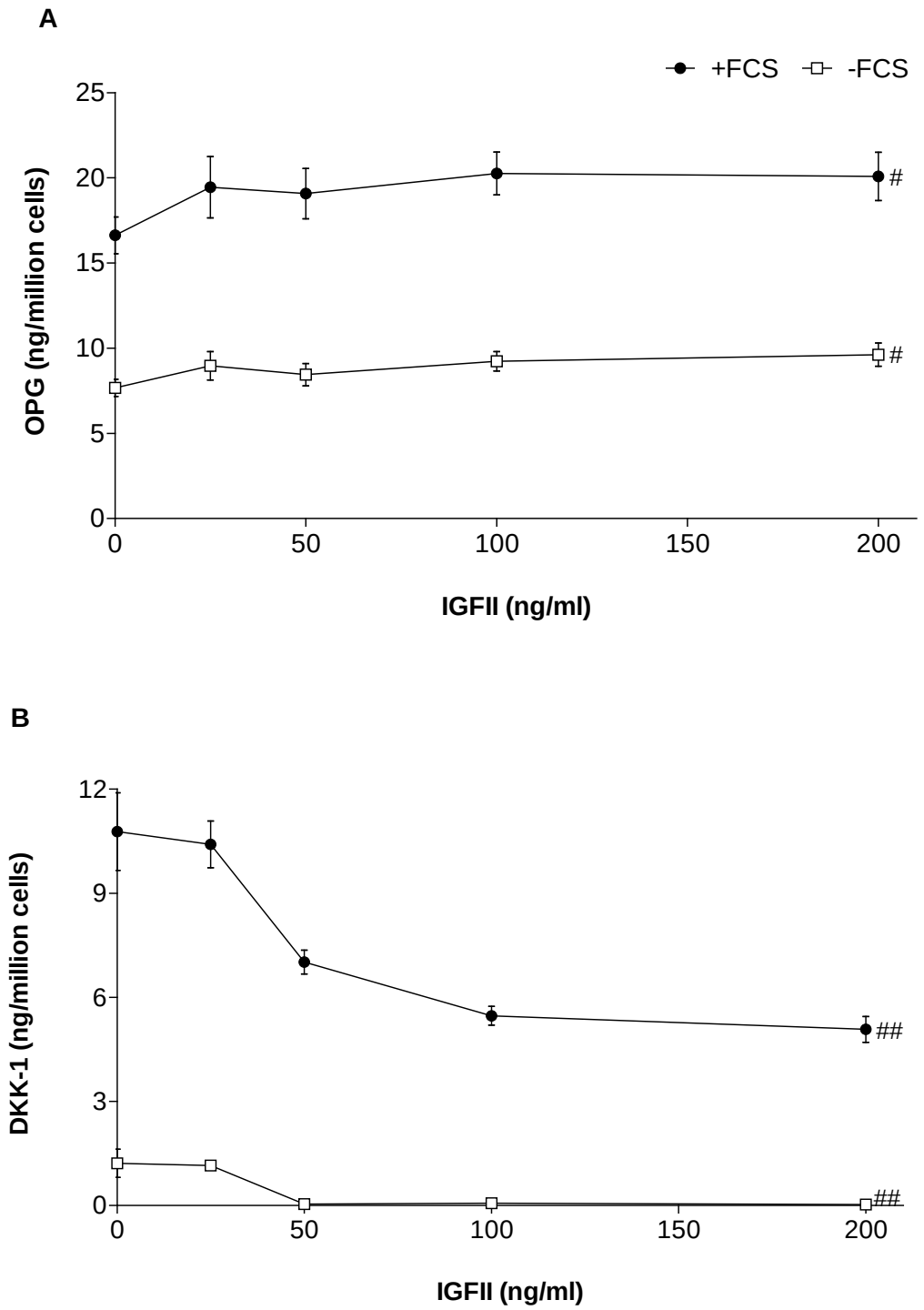

Fig 3.22 The effect of IGFII on OPG and DKK-1 production in Saos-2 cells. Saos-2 cells were stimulated for 24 hours with 0-200ng/ml IGFII with or without FCS. Culture medium was removed and assayed for A) OPG and B) DKK-1. Data are presented as mean ± SD, n=4. #P<0.05 and ##P<0.001 versus control (0ng/ml IGFII, one-way ANOVA using Dunnett's multiple comparison test).

3.3.7 The effect of IL-6 on OPG and DKK-1 production

The effect of interleukin-6 (IL-6) on OPG and DKK-1 production was investigated. MG63 and Saos-2 cells were stimulated for 24 hours with 0-10ng/ml IL-6 either with or without FCS. Culture medium was assayed for OPG and DKK-1 after the 24 hour period. In the presence of FCS, OPG production in MG63 cells was significantly inhibited in a dose-dependent manner ($P<0.001$, Fig 3.23A). The lowest concentration of IL-6 to exhibit a significant response was 0.5ng/ml. A maximal effect was observed at 2ng/ml where OPG production was inhibited 19% compared to control ($P<0.001$). There was no significant effect on OPG production in MG63 cells in response to IL-6 in the absence of FCS.

DKK-1 production in MG63 cells was significantly inhibited by IL-6 in the absence of FCS in a dose-dependent manner ($P<0.01$, Fig 3.23B). The lowest concentration of IL-6 to exhibit a significant response was 0.5ng/ml. A maximal effect observed at 10ng/ml where DKK-1 production was inhibited 25% compared to control ($P<0.01$). There was no significant effect on DKK-1 production in MG63 cells in response to IL-6 in the presence of FCS.

In Saos-2 cells, IL-6 did not have a significant effect on OPG production in either with or without FCS (Fig 3.24A). DKK-1 production in Saos-2 cells was significantly inhibited in response to IL-6 while in the presence of FCS ($P<0.01$). The lowest concentration of IL-6 to exhibit a significant response was 0.5ng/ml. A maximal effect was also observed at 0.5ng/ml where DKK-1 production was inhibited 30% compared to control (Fig 3.24B). There was no significant effect on DKK-1 production in Saos-2 cells in response to IL-6 in the absence of FCS.

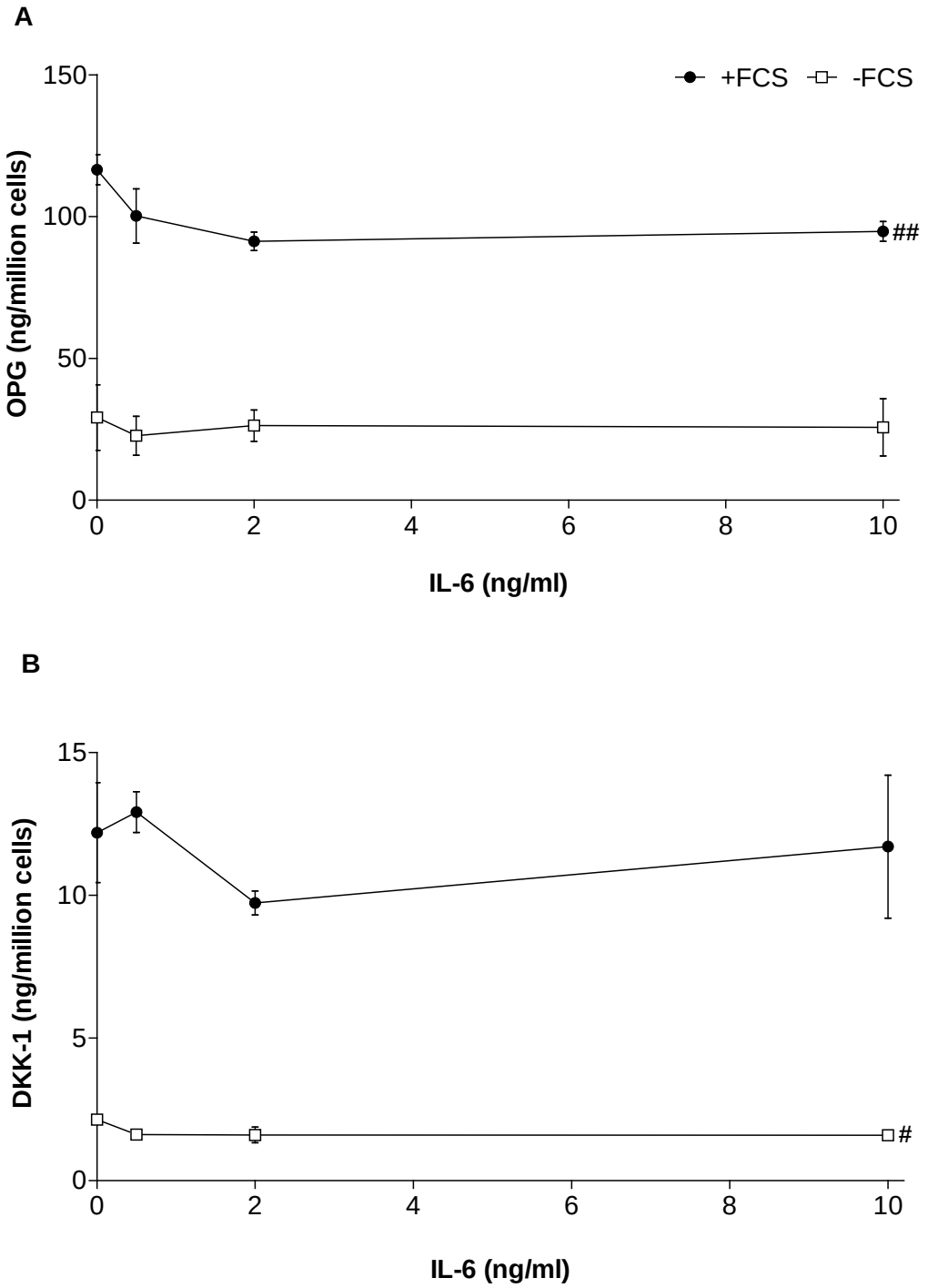

Fig 3.23 The effect of IL-6 on OPG and DKK-1 production in MG63 cells. MG63 cells were stimulated for 24 hours with 0-10ng/ml IL-6 with or without FCS. Culture medium was removed and assayed for A) OPG and B) DKK-1. Data are presented as mean ± SD, n=4. #P<0.01 and ##P<0.001 versus control (0ng/ml IL-6, one-way ANOVA using Dunnett's multiple comparison test).

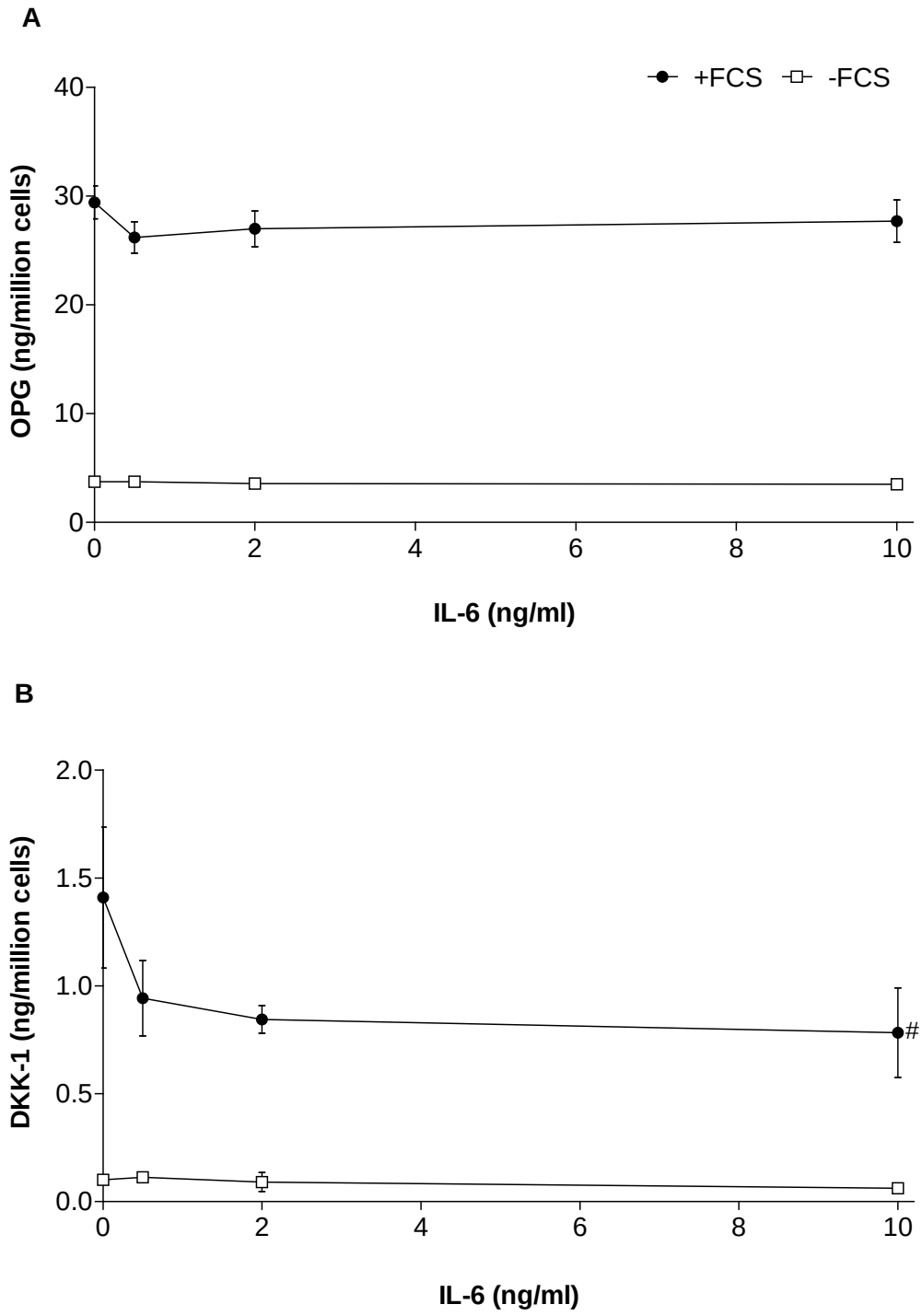

Fig 3.24 The effect of IL-6 on OPG and DKK-1 production in Saos-2 cells. Saos-2 cells were stimulated for 24 hours with 0-10ng/ml IL-6 with or without FCS. Culture medium was removed and assayed for A) OPG and B) DKK-1. Data are presented as mean ± SD, n=4. #P<0.01 and ##P<0.001 versus control (0ng/ml IL-6, one-way ANOVA using Dunnett's multiple comparison test).

3.3.8 The effect of FGF-1 on OPG and DKK-1 production

The effect of fibroblast growth factor-1 (FGF-1) on OPG and DKK-1 production was investigated. MG63 cells were stimulated for 24 hours with 0-50ng/ml FGF-1 either with or without FCS. Culture medium was assayed for OPG and DKK-1 after the 24 hour period. In the presence of FCS, OPG production in MG63 cells was significantly inhibited in a dose-dependent manner ($P<0.001$, Fig 3.25A). The lowest concentration of FGF-1 to exhibit a significant response was 10ng/ml. A maximal effect was observed at 50ng/ml where OPG production was inhibited 67% compared to control ($P<0.001$). There was no significant effect on OPG production in MG63 cells in response to FGF-1 in the absence of FCS.

DKK-1 production in MG63 cells was also significantly inhibited by FGF-1 in a dose-dependent manner ($P<0.01$, Fig 3.25B). The lowest concentration of FGF-1 to exhibit a significant response was 20ng/ml with a maximal effect observed at 50ng/ml where DKK-1 production was inhibited 38% compared to control ($P<0.01$). There was no significant effect on DKK-1 production in MG63 cells in response to FGF-1 in the absence of FCS.

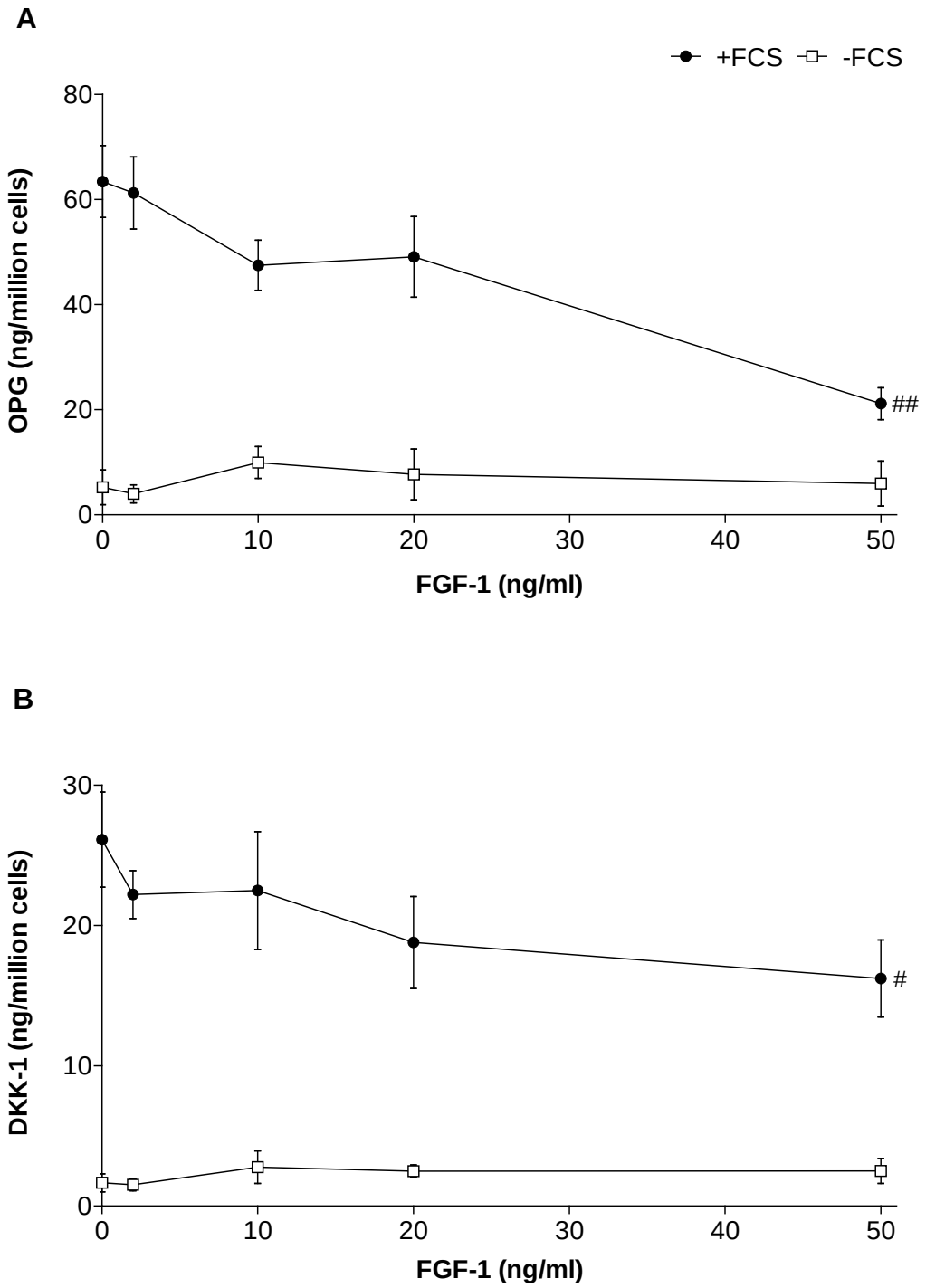

Fig 3.25 The effect of FGF-1 on OPG and DKK-1 production in osteoblastic cells. MG63 cells were stimulated for 24 hours with 0-50ng/ml FGF-1 with or without FCS. Culture medium was removed and assayed for A) OPG and B) DKK-1. Data are presented as mean ± SD, n=4. #P<0.01 and ##P<0.001 versus control (0ng/ml FGF-1, one-way ANOVA using Dunnett's multiple comparison test).

3.3.9 The effect of serotonin, SCF and HGF on OPG and DKK-1 production

The effect of serotonin, stem cell factor (SCF) and hepatocyte growth factor (HGF) on OPG and DKK-1 production was investigated. MG63 cells were stimulated for 24 hours with 0-50µM serotonin, 0-100ng/ml SCF or 0-50ng/ml HGF either with or without FCS. Culture medium was assayed for OPG and DKK-1 after the 24 hour period. Serotonin did not have any significant effects on either OPG production (Fig 3.26A) or DKK-1 production (Fig 3.26B) either in the presence or absence of FCS.

SCF in the presence of FCS significantly increased OPG production in MG63 cells ($P<0.01$, Fig 3.27A). The lowest effective concentration to exhibit a significant response was 5ng/ml. A maximal effect was also observed at 5ng/ml, where OPG production was significantly increased 25% compared to control ($P<0.01$). There was no significant effect of SCF on OPG production in the absence of FCS. SCF did not significantly affect the production of DKK-1 in MG63 cells either in the presence or absence of FCS (Fig 3.27B).

In the absence of FCS, HGF significantly inhibited the production of OPG in MG63 cells in a dose-dependent manner ($P<0.05$, Fig 3.28A). The lowest effective concentration to exhibit a significant response was 5ng/ml. A maximal effect was also observed at 5ng/ml, where OPG production was significantly inhibited 11% compared to control ($P<0.05$). There was no significant effect of HGF on OPG production in the presence of FCS. HGF did not significantly affect the production of DKK-1 in MG63 cells either in the presence or absence of FCS (Fig 3.28B).

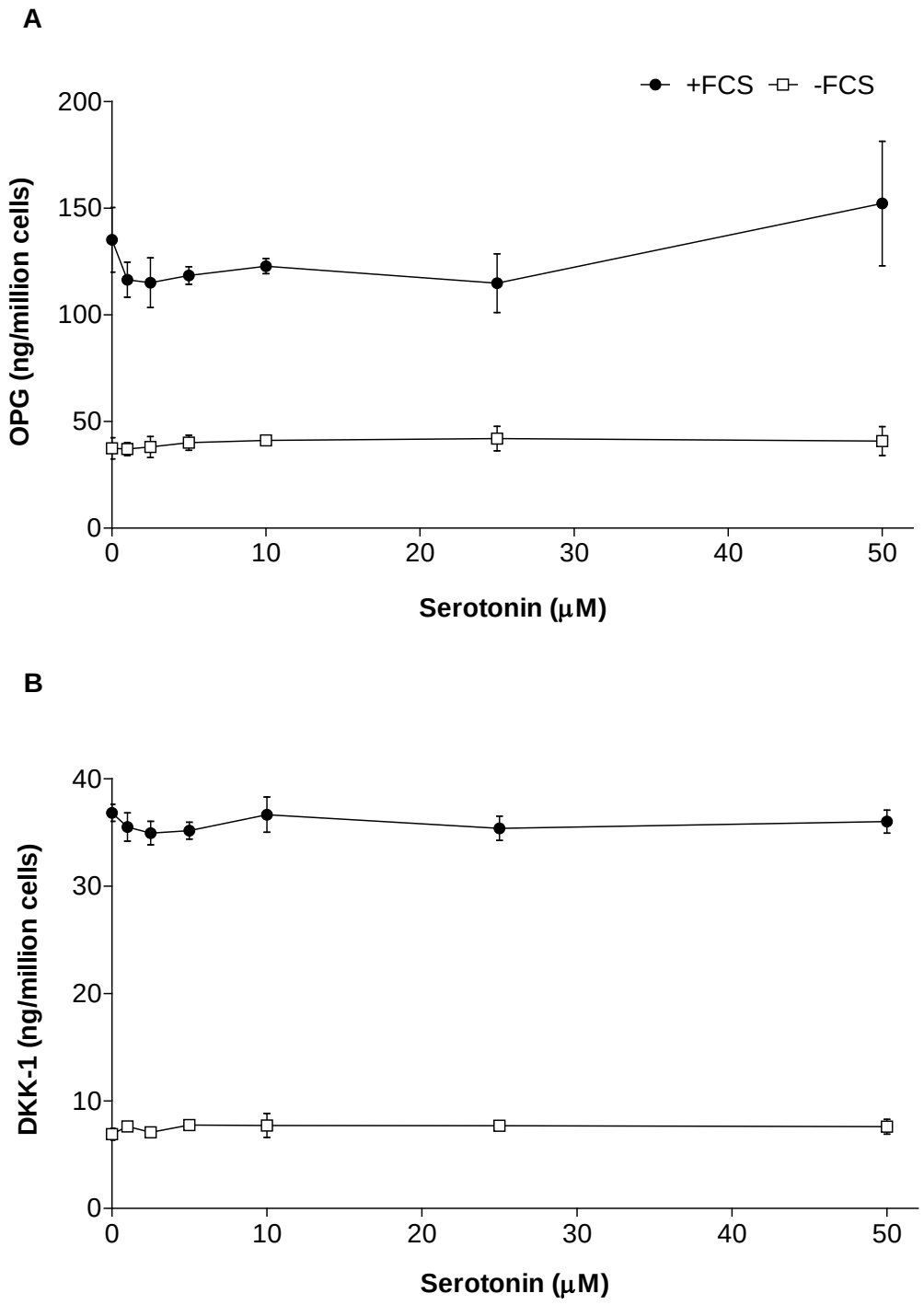

Fig 3.26 The effect of serotonin on OPG and DKK-1 production in osteoblastic cells. MG63 cells were stimulated for 24 hours with 0-50μM serotonin with or without FCS. Culture medium was removed and assayed for A) OPG and B) DKK-1. Data are presented as mean ± SD, n=4.

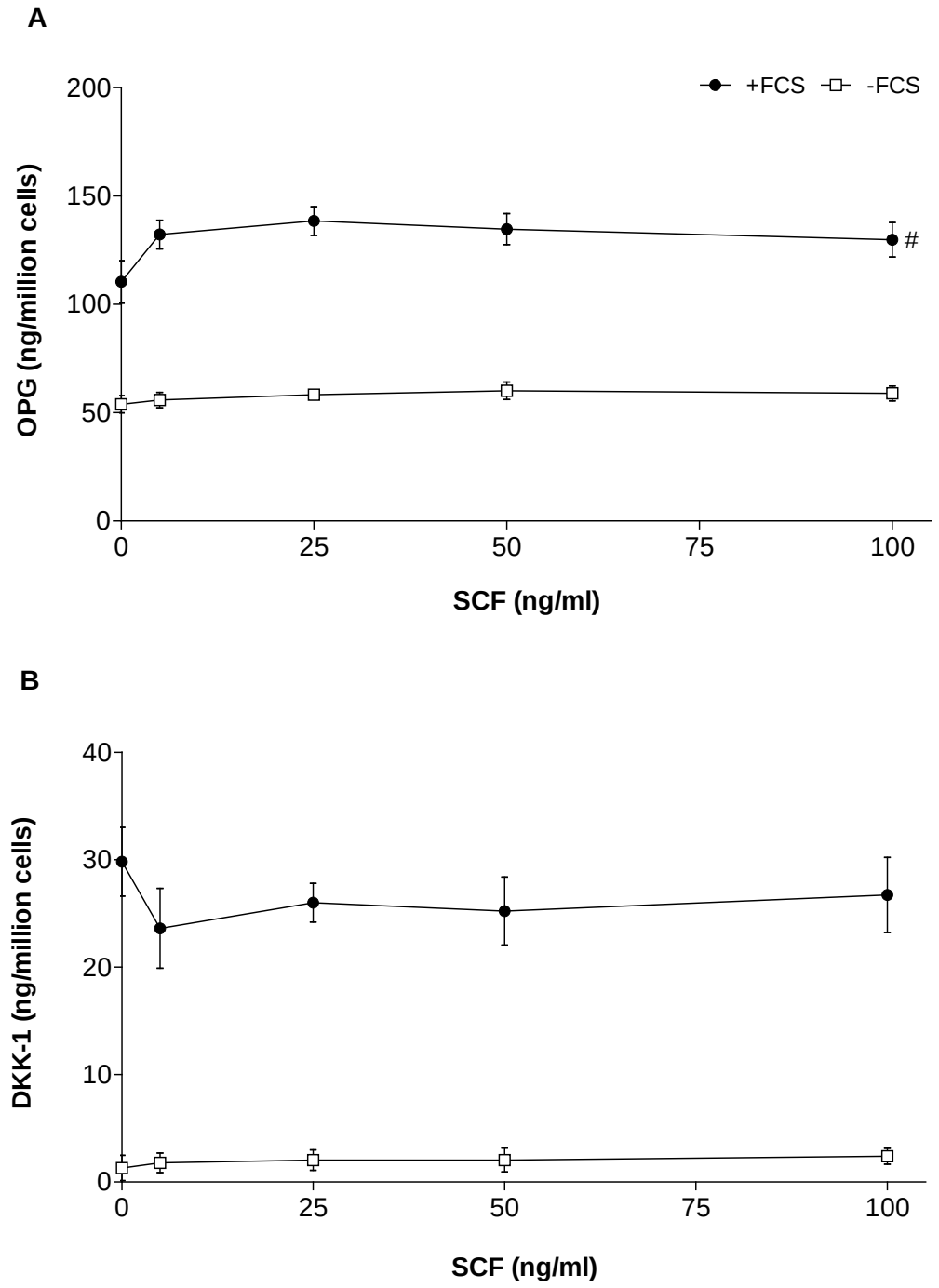

Fig 3.27 The effect of SCF on OPG and DKK-1 production in osteoblastic cells. MG63 cells were stimulated for 24 hours with 0-100ng/ml SCF with or without FCS. Culture medium was removed and assayed for A) OPG and B) DKK-1. Data are presented as mean ± SD, n=4. #P<0.01 versus control (0ng/ml SCF, one-way ANOVA using Dunnett's multiple comparison test).

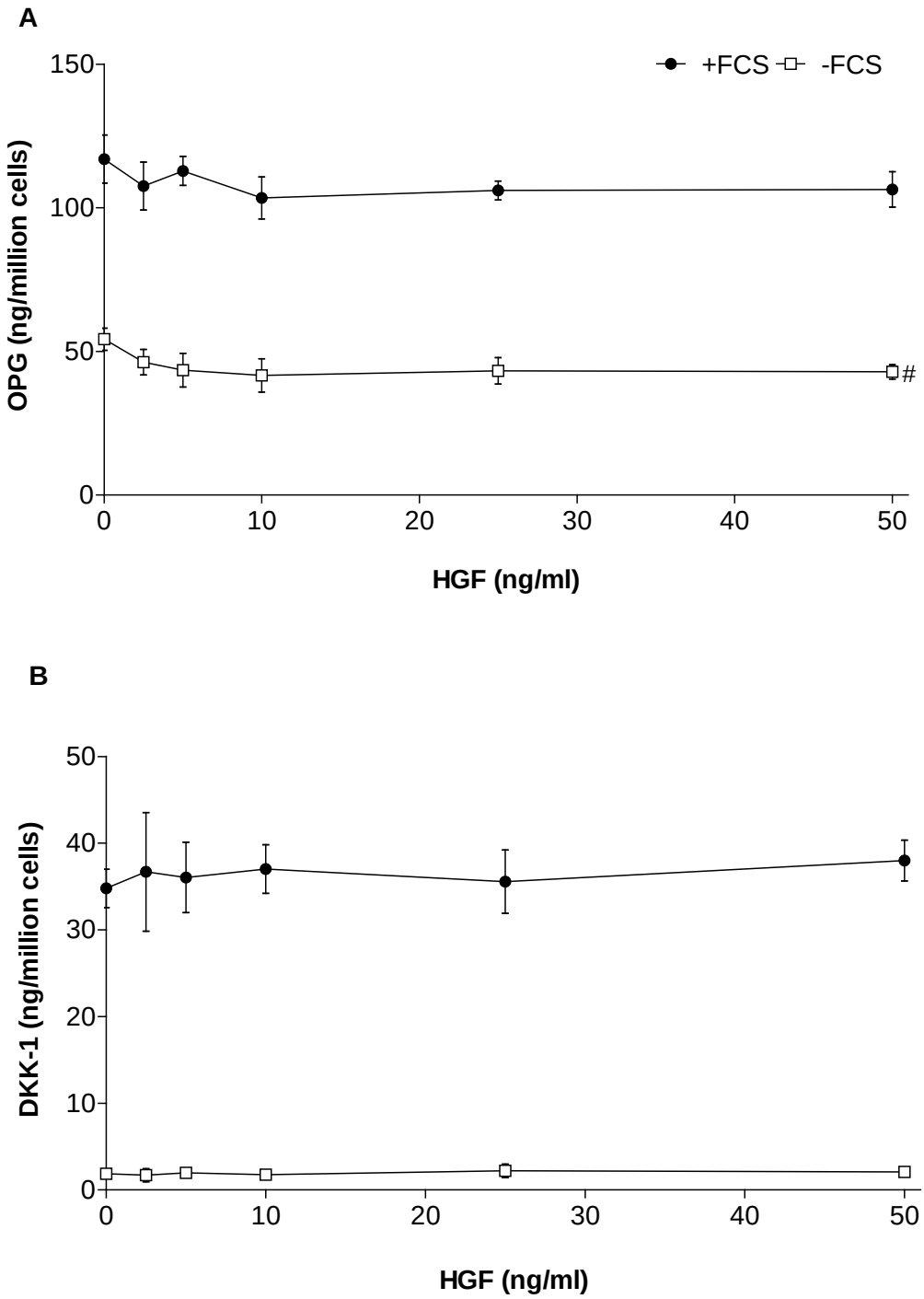

Fig 3.28 The effect of HGF on OPG and DKK-1 production in osteoblastic cells. MG63 cells were stimulated for 24 hours with 0-50ng/ml HGF with or without FCS. Culture medium was removed and assayed for A) OPG and B) DKK-1. Data are presented as mean ± SD, n=4. #P<0.05 versus control (0ng/ml HGF, one-way ANOVA using Dunnett's multiple comparison test).

3.3.10 The effect of Bradykinin on OPG and DKK-1 production

The effect of Bradykinin on OPG and DKK-1 production was investigated. MG63 cells were stimulated for 24 hours with 0-1000nM Braydkinin either with or without FCS. Culture medium was assayed for OPG and DKK-1 after the 24 hour period. In the absence of FCS, OPG production in MG63 cells was significantly increased in a dose-dependent manner ($P<0.001$, Fig 3.29A). The lowest concentration of Bradykinin to exhibit a significant response was 5nM. A maximal effect was observed at 1000nM where OPG production was significantly increased by 67% compared to control ($P<0.001$). There was no significant effect on OPG production in MG63 cells in response to Bradykinin in the presence of FCS.

DKK-1 production in MG63 cells was significantly increased by Bradykinin in the presence of FCS in a dose-dependent manner ($P<0.05$, Fig 3.29B). The lowest concentration of Bradykinin to exhibit a significant response was 50nM. A maximal effect was also observed at 50nM Bradykinin where DKK-1 production was stimulated by 29% compared to control ($P<0.05$). There was no significant effect on DKK-1 production in MG63 cells in response to Bradykinin in the absence of FCS.

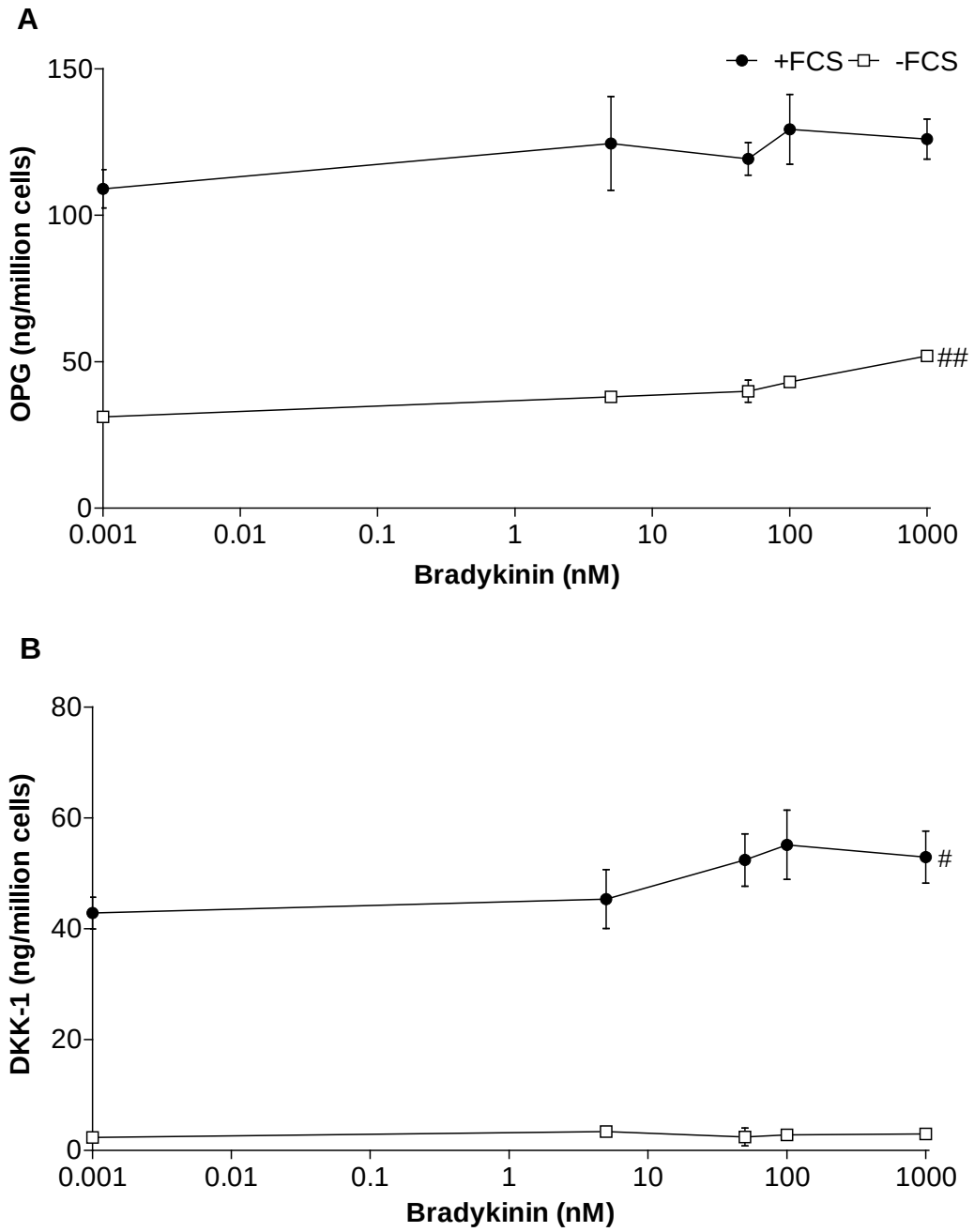

Fig 3.29 The effect of bradykinin on OPG and DKK-1 production in osteoblastic cells. MG63 cells were stimulated for 24 hours with 0-1000nM Bradykinin with or without FCS. Culture medium was removed and assayed for A) OPG and B) DKK-1. Data are presented as mean ± SD, n=4. #P<0.01 and ##P<0.001 versus control (0nM Bradykinin, one-way ANOVA using Dunnett's multiple comparison test).