CHAPTER 5

Mechanisms of DKK-1 production

5.1 The effect of simulating canonical Wnt signalling on DKK-1 production and Wnt expression

It is unclear from the literature whether DKK-1 is a product of canonical Wnt signalling, non-canonical Wnt signalling, or some other signalling pathway or combination of pathways. It has already been demonstrated in chapter 3 that DKK-1 production can be modulated by FCS and the addition of various cytokines and growth factors. Therefore the mechanisms of DKK-1 production in response to agents that modulate the Wnt signalling pathway were investigated.

The effect of the manipulation of canonical Wnt signalling on the production of DKK-1 in MG63 cells was investigated. BIO is an inhibitor of GS3Kβ and therefore simulates canonical Wnt signalling by preventing the phosphorylation and subsequent degradation of β-Catenin. MG63 cells were treated with 0-10μM BIO in complete medium for 24 hours. Culture medium was removed after 24 hours and assayed for DKK-1. BIO inhibited the production of DKK-1 in a dose-dependent manner ($P<0.001$, Fig 5.1A). The lowest effective concentration of BIO to exhibit a significant response was 0.1μM. A maximal effect was observed at 10μM BIO where DKK-1 production was inhibited by 78% compared to control ($P<0.001$, Fig 5.1A).

Wnt expression in the presence of BIO was investigated. MG63 cells were stimulated for 24 hours in complete medium with or without 10μM BIO. RNA was extracted and reverse transcribed. Expression of *Wnt3a, Wnt4, Wnt5a, Wnt7b Wnt11* and *DKK-1* was analysed by RT-PCR using *GAPDH* as a housekeeper. BIO significantly up-regulated the expression of *Wnt5a* 7-fold compared to control ($P<0.001$, Fig 5.1B). Expression of *DKK-1* was significantly down-regulated by 80% compared to control in response to 10μM BIO ($P<0.01$). Expression of *Wnt3a, Wnt4, Wnt7b* and *Wnt11* were not significantly affected by BIO.

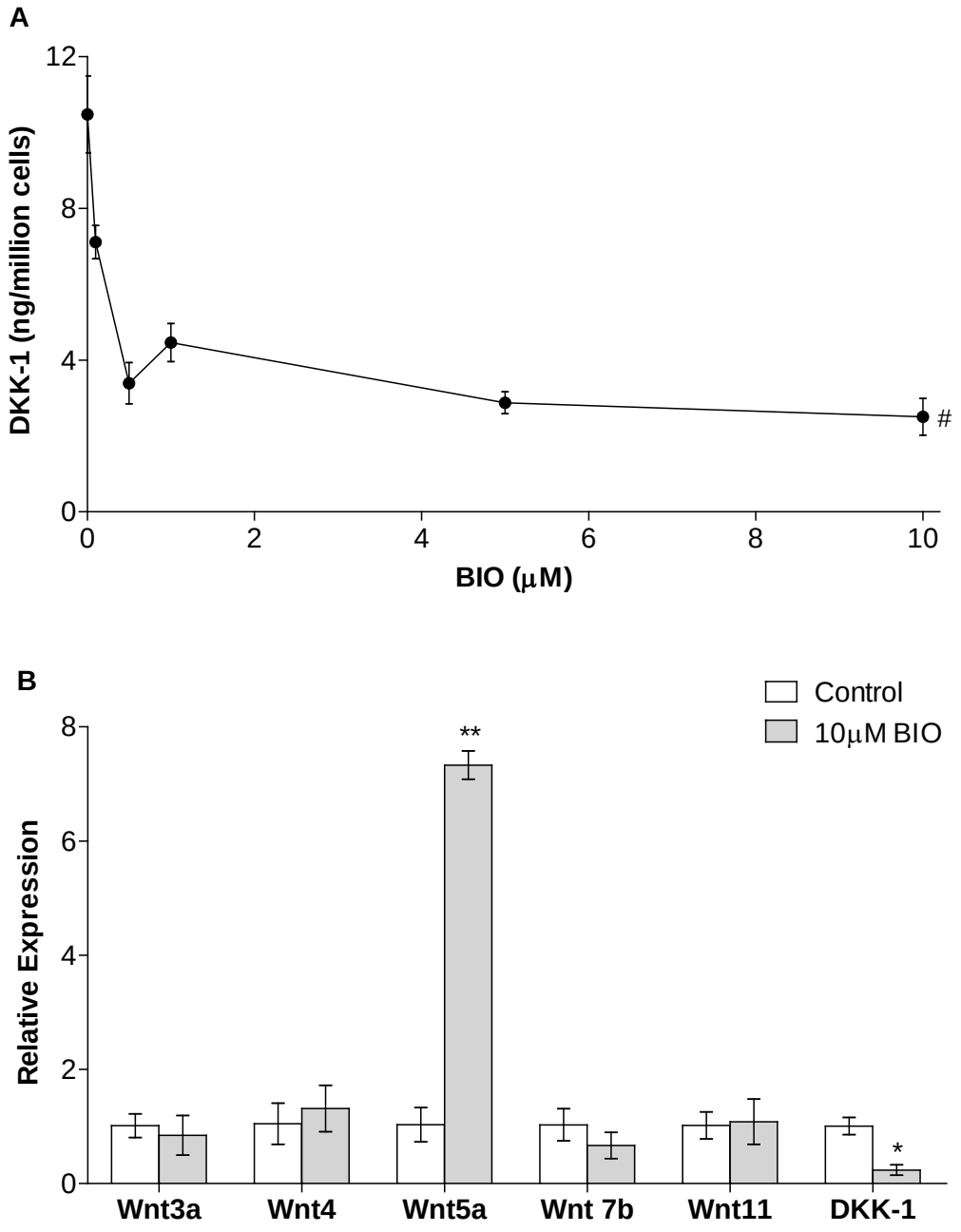

Fig 5.1 The effect of BIO on DKK-1 production and Wnt expression in MG63 cells. A) MG63 cells were stimulated 24 hours in complete medium with 0-10μM BIO. Culture medium was removed and assayed for DKK-1. Data are presented as mean ± SD, n=4. #P<0.001 versus control (0μM BIO, one-way ANOVA using Dunnett's multiple comparison test). B) MG63 cells were stimulated 24 hours in complete medium with or without 10μM BIO. RNA was extracted and mRNA assayed using RT-PCR. Data shown are expressed relative to control (24 hours culture in complete medium) normalised to *GAPDH* housekeeper expression (mean ± range, n=3). *P<0.01 and **P<0.001 versus own mRNA control (unpaired t-test).

5.2 The effect of sFRP-1 on DKK-1 production and Wnt expression

The effect of secreted frizzled-related protein 1 (sFRP1) on DKK-1 production was investigated. sFRP-1 is a soluble inhibitor of Wnt signalling by binding with free Wnts and preventing them from complexing with Fzd and/or LRP5/6. MG63 cells were stimulated for 24 hours in complete medium with 0-10µg/ml sFRP-1. Culture media was removed after 24 hours and assayed for DKK-1. sFRP-1 significantly increased the production of DKK-1 in a dose-dependent manner ($P<0.001$, Fig 5.2A). The lowest concentration of sFRP-1 to exhibit a significant response was 0.1µg/ml. A maximal effect of sFRP-1 was observed at 2µg/ml where DKK-1 production in MG63 cells was significantly stimulated by 18% compared to control ($P<0.001$).

The effect of sFRP-1 on the expression of various Wnts was investigated. MG63 cells were stimulated for 24 hours in full media with or without 2µg/ml sFRP-1. RNA was extracted and reverse transcribed. Expression of *Wnt3a, Wnt4, Wnt5a, Wnt7b, Wnt10, Wnt11, Wnt16* and *DKK-1* was analysed. sFRP-1 significantly up-regulated the expression of *Wnt7b* almost 2-fold compared to control ($P<0.05$, Fig 5.2B) whereas expression of *DKK-1* was not significantly different to control in response to sFRP-1. Expression of *Wnt3a, Wnt4, Wnt5a, Wnt10, Wnt11 and Wnt16* were not affected by sFRP-1.

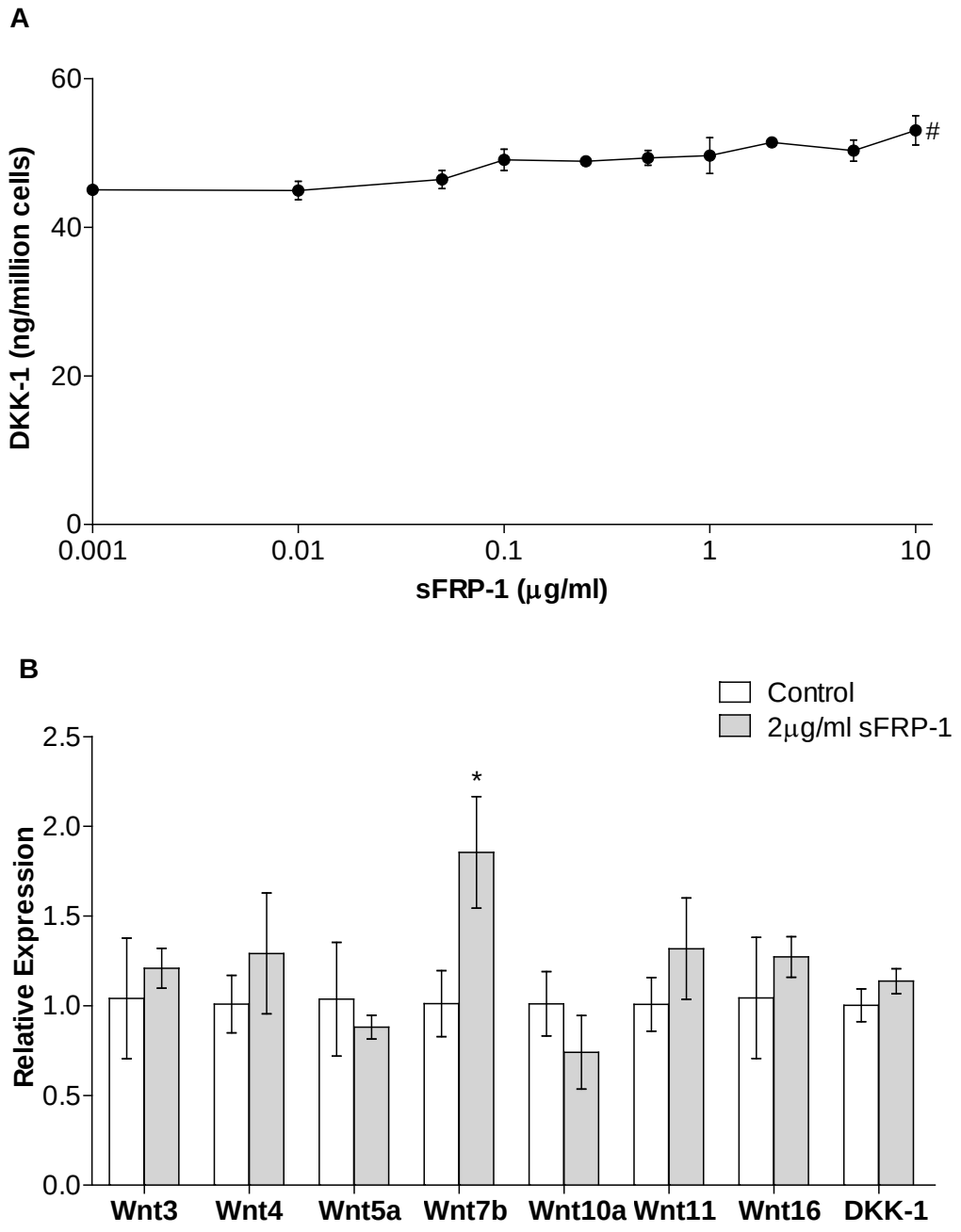

Fig 5.2 The effect of sFRP-1 on Wnt expression and DKK-1 production in MG63 cells. A) MG63 cells were stimulated 24 hours in full medium with 0-10µg/ml sFRP-1. Culture medium was removed and assayed for DKK-1. Data are presented on a log scale as mean ± SD, n=4. #P<0.001 versus control (0µg/ml sFRP-1, one-way ANOVA using Dunnett's multiple comparison test). B) MG63 cells were stimulated for 24 hours in full medium with or without 2µg/ml sFRP1. 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<0.05 versus own mRNA control (unpaired t-test).

5.3 The role of PKC in DKK-1 production and Wnt expression

Non-canonical Wnt signalling has often been reported to signal via PKC. BIM and STS, previously used in Chapter 4, are inhibitors of PKC. To investigate the involvement of PKC in the production of DKK-1, MG63 cells were stimulated for 24 hours in complete medium with 0-10µM BIM or 0-50nM STS. Culture media was removed after 24 hours and assayed for DKK-1. Both BIM and STS inhibited DKK-1 production in MG63 cells in a dose-dependent manner ($P<0.001$, Fig 5.3). The lowest effective concentration of BIM and STS to exhibit a response were 1µM ($P<0.001$) and 5nM ($P<0.001$) respectively. Maximal inhibitory effects were observed at 10µM BIM and 50nM STS. At 10µM BIM, DKK-1 production was significantly inhibited by 85% compared to control ($P<0.001$, Fig 5.3A). STS significantly inhibited DKK-1 production by 78% and 89% compared to control at 10nM and 50nM respectively ($P<0.001$, Fig 5.3B).

Wnt expression in the presence of BIM and STS was investigated. MG63 cells were stimulated for 24 hours in complete medium with or without 10µM BIM or 10nM STS. RNA was extracted and reverse transcribed. Expression of *Wnt3a, Wnt4, Wnt5a, Wnt7b Wnt11* and *DKK-1* was analysed. BIM significantly up-regulated the expression of *Wnt5a* 4.4-fold compared to control ($P<0.01$) and significantly down-regulated expression of *DKK-1* 70% compared to control ($P<0.05$, Fig 5.4). Expression of *Wnt3a, Wnt4, Wnt7b* and *Wnt11* were not significantly affected by BIM.

STS significantly down-regulated the expression of *DKK-1* by 77% compared to control ($P<0.01$, Fig 5.4). In contrast to BIM, STS down-regulated the expression of Wnt5a by 51% compared to control ($P<0.05$). STS did not have any significant effect on the expression of *Wnt3a, Wnt4, Wnt7b* or *Wnt11*.

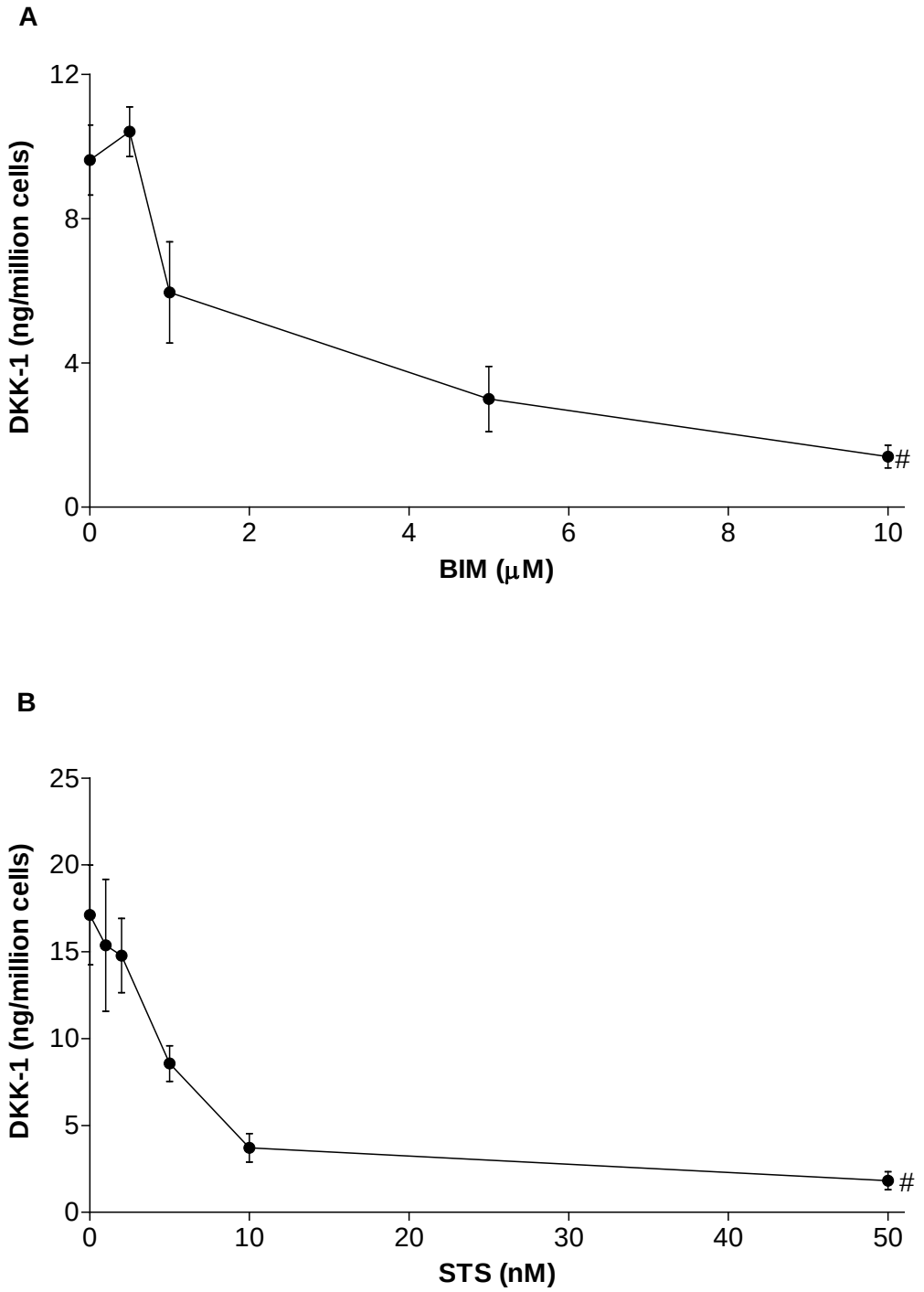

Fig 5.3 The effect of BIM and STS on DKK-1 production in MG63 cells. MG63 cells were stimulated for 24 hours in complete medium with A) 0-10μM BIM or B) 0-50nM STS. Culture medium was removed and assayed for DKK-1. Data are presented as mean ± SD, n=4. #P<0.001 versus control (no inhibitor, one-way ANOVA using Dunnett's multiple comparison test).

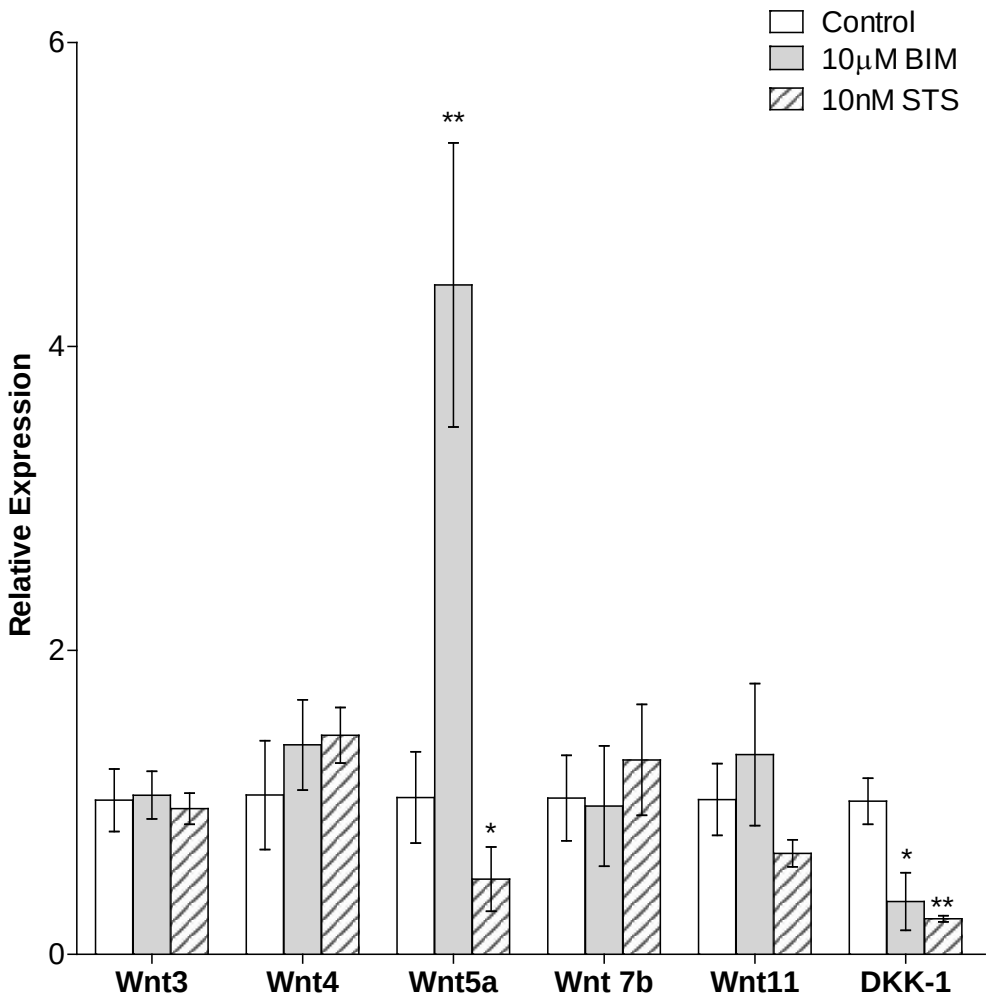

Fig 5.4 The effect of BIM and STS on Wnt and DKK-1 expression in MG63 cells. MG63 cells were stimulated for 24 hours in full media with or without either 10μM BIM or 10nM STS. RNA was extracted and mRNA assayed using RT-PCR. Data shown are expressed relative to control (24 hours culture with FCS) normalised to *GAPDH* housekeeper expression (mean ± range, n=3). *P<0.05 and **P<0.01, versus own mRNA control (one-way ANOVA using Dunnett's multiple comparison test)

5.4 The effect of phorbol ester on DKK-1 production and Wnt expression

The previous results of inhibitors have demonstrated that PKC is involved in the regulation of DKK-1 production. Phorbol ester (PE) is a known activator of PKC so the effects of PE were investigated for its ability to modulate DKK-1 production and Wnt expression. MG63 cells were cultured under standard conditions for 24 hours in complete medium with 10nM PE (optimum concentration as determined by preliminary experiments) and either 10µM BIM or 10nM STS. Culture medium was assayed for DKK-1 after 24 hours. RNA was extracted and reverse transcribed. Expression of *Wnt3a, Wnt4, Wnt5a, Wnt7b, Wnt11, Wnt16* and *DKK-1* was analysed.

DKK-1 production was significantly stimulated 160% compared to control in response to 10nM PE ($P<0.001$, Fig 5.5A). As expected, both 10µM BIM and 10nM STS significantly inhibited the production of DKK-1 in MG63 cells. Although PE significantly stimulated the production of DKK-1 in the presence of BIM by 800% compared to BIM alone ($P<0.001$), levels of DKK-1 remained significantly less than with no treatment ($P<0.001$, Fig 5.5A). STS blocked the ability of PE to stimulate DKK-1 production in MG63 cells.

10nM PE significantly up-regulated *DKK-1* expression 3-fold compared to control ($P<0.01$) and was inhibited by the addition of both BIM and STS (Fig 5.5B). *Wnt4* and *Wnt7b* were significantly up-regulated 2-fold compared to control in response to 10nM PE ($P<0.05$) while *Wnt16* was significantly up-regulated 4-fold compared to control when treated with 10nM PE ($P<0.01$, Fig 5.5B). PE-induced up regulation of *Wnt4, Wnt7b* and *Wnt16* was inhibited by the addition of 10µM BIM but not 10nM STS. There was no significant effect of PE on the expression of *Wnt3a, Wnt5a* or *Wnt11* although PE did inhibit the previously observed BIM-induced up-regulation of *Wnt5a* and in the presence of STS, expression of *Wnt5a* appears to be increased a little (Fig 5.5B).

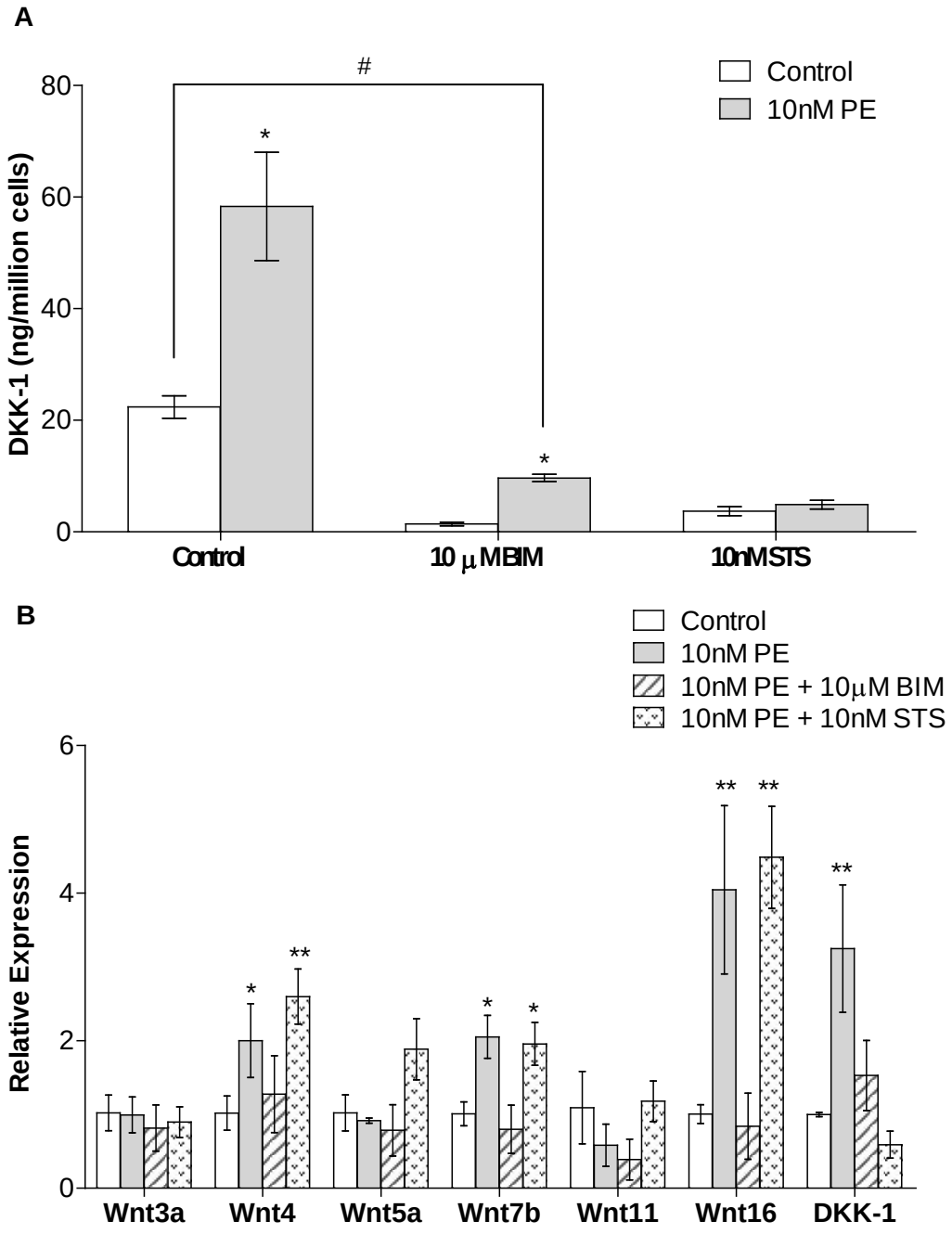

Fig 5.5 The effect of BIM and STS on PE-induced Wnt and DKK-1 expression in MG63 cells. MG63 cells were stimulated for 24 hours in full media with 10nM PE and either 10μM BIM or 10nM STS. A) Culture medium was removed and assayed for DKK-1. Data are presented as mean ± SD, n=4. *P<0.001 versus respective treatment control and #P<0.001 versus no treatment control (unpaired t-test). B) RNA was extracted and various Wnt and DKK-1 mRNA assayed using RT-PCR. Data shown are expressed relative to own mRNA control (24 hours culture with FCS) normalised to *GAPDH* housekeeper expression (mean ± range, n=3). *P<0.05 and **P<0.01 versus own mRNA control (one-way ANOVA using Dunnett's multiple comparison test).