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Title page

Novel Targeting Of COX-2 pre-mRNA Using Antisense Morpholino Oligonucleotides Directed To The 3' Acceptor And 5' Donor Splice Sites Of Exon 4: Suppression Of COX-2 Activity In Human Amnion-Derived WISH And Myometrial Cells

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Running Title Page

Running Title: Suppression of COX-2 by morpholino oligonucleotides

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Discussion: 1,083 words
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Non-standard abbreviations: TMPD (*N,N,N*^{1},*N*^{1} –tetra-methyl-*p*-phenylenediamine)

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Abstract

Increased expression of COX-2 has been implicated in the onset of both term and preterm labour. In this context both selective and non-selective COX-2 inhibitors have been used in clinical trials to determine their efficacy in delaying preterm labour. However recent evidence indicates that these tocolytics may have potential adverse fetal and maternal side effects. Consequently, the development of more specific and non-toxic agents to inhibit COX-2 needs to be considered. We have evaluated whether antisense morpholino oligonucleotides have therapeutic potential in inhibiting COX-2 by specifically targeting both the 3' and 5 ' acceptor and donor sites of exon 4 of its pre-mRNA sequence. Confocal microscopy on 'live' cells illustrated high levels of penetrance of antisense morpholino oligonucleotides using the Endo-Porter formula (Gene-Tools, LLC) with delivery efficiencies of 82% and 78% respectively in amnion-derived WISH and myometrial cells. Substantial inhibition by the morpholino oligonucleotides of COX-2 expression, induced by LPS administration, was observed at both the mRNA and protein level. Loss of enzymic activity of COX-2 was confirmed using a sensitive COX enzyme activity assay which reflects the rate of conversion of arachidonic acid to $PGH_{2.}$ Our results indicate that antisense morpholino oligonucleotides significantly inhibit expression and activity of this enzyme in *in vitro* cultures of amnion-WISH and myometrial cells. The potential thus exists that a similar approach can be mimicked *in vivo* to produce a highly specific and non-toxic strategy to inhibit COX-2 activity with its subsequent effects on the better management of preterm labour and other inflammatory conditions.

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Introduction

Cyclo-oxygenase-2 (COX-2) is a key regulatory enzyme responsible for the catalysis of arachidonic acid to prostaglandins and other prostanoids. Prostaglandins participate in various biological processes including immune function, cancer as well as reproductive processes of ovulation, implantation and both term and preterm labour (Chakraborty at al,1996; Lim et al, 1997; Slater et al, 1999; Loudon et al, 2003; Olson, 2005). Under normal conditions expression of COX-2 is undetectable in most tissues. Induction of COX-2 occurs rapidly and transiently in response to a variety of stimuli, including lipopolysaccharides (LPS), cytokines, growth factors and tumour promoters, signifying that COX-2 is responsible for the biosynthesis of prostaglandins under acute Inflammatory conditions (Fu et al, 1990; Coyne et al, 1992; Dubois et al, 1998; Williams et al, 1999).

With respect to human pregnancy considerable effort has been expended in unravelling the molecular events that regulate the activity of the uterus during gestation and parturition and several key genes have so far been identified whose inappropriate expression may underlie the initiation of preterm delivery. In this context there is now growing evidence to indicate that both normal healthy term and preterm labour may involve an inflammatory mechanism by which increased production of cytokines such as TNFα, IL-1α, IL-1β, IL-6, IL-8 and up-regulation of COX-2 occurs within the uterus (Challis et al, 2000). This increase in COX-2 expression is initially observed in the fetal membranes (Slater et al 1995) resulting in increased prostaglandin production which is also augmented by the concurrent down-regulation of prostaglandin dehydrogenase which inactivates prostaglandins. In normal pregnancies both cytokines and prostaglandins promote cervical ripening and

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co-ordinate uterine contractions to expel the neonate at term (Challis et al, 2000). However, bacterial infection leading to stimulation of Toll-like receptors (Elovitz et al, 2003) may trigger their early increased synthesis in uterine tissues resulting in premature labour as well as the associated affects of cytokines on normal fetal cerebral development. Evidence has also accrued to indicate the differential expression of specific prostaglandin receptors as the activity of the myometrium switches from a quiescent state (expressing predominantly EP_2 and EP_4 receptors) to an active contractile state (expressing FP receptors) at term (Myatt et al, 2004).

Various non-steroidal anti-inflammatory drugs (NSAIDS) have been used to selectively and non-selectively inhibit COX-2 at sites of inflammation. However, evidence proposes that some of these COX-2 inhibitory drugs, such as Rofecoxib (Vioxx) and Celecoxib (Celebrex) may in the long term increase the risk of cardiovascular events Warner and Mitchell, 2005; Psaty and Furberg, 2005). Non-selective COX-2 inhibitors, such as Indomethacin and Sulindac have been used clinically in an attempt to delay preterm birth (Loudon et al, 2003; Olson, 2005), however, clinical evidence indicates that these tocolytics have potential adverse fetal side effects including closure of the ductus arteriosus, high blood pressure in the lungs, bleeding in the brain or heart and impaired renal function (reviewed in Loudon et al, 2003; Groom et al, 2005). Rofecoxib has been tested in a double-blind randomised controlled trial to assess its safety and efficacy in delaying preterm delivery in women at high risk (Groom et al, 2005). However the outcome from this study indicated that use of Rofecoxib also resulted in adverse fetal side effects, although reversible with discontinuation of treatment, did not reduce the incidence of preterm delivery at early gestation ages (<30 weeks) and moreover, its usage was

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also associated with an increased risk of premature delivery in women at high risk. These studies highlight that at present there are no effective therapeutic strategies in preventing early gestational deliveries or even later preterm labour not associated with infection. Consequently, the development of novel, specific and non-toxic approaches to inhibit pro-inflammatory genes, such as COX-2, needs to be considered.

Antisense oligonucleotides to specific cellular RNAs have shown great promise in both research and clinical studies as sequence specific agents able to modulate the expression of targeted genes. In principle the most important feature of antisense oligonucleotides is their ability to base-pair with the target RNA to either block its translation or primarily to mediate its destruction by RNase H, an enzyme that destroys RNA in a DNA/RNA duplex (Sazani and Kole, 2003). However many studies have also indicated that these oligonucleotides can have toxic side effects and may exert their effects non-specifically by binding directly to a number of proteins *in vivo* in a sequence-dependent rather than a sequence-specific manner (Sazani and Kole, 2003). This non-antisense mechanism of binding was shown to particularly occur with the most commonly used 2'-oligodeoxynucleoside phosphorothioate species which also appear to be sensitive to degradation by RNase H. Moreover *in vivo* cellular RNAs are nearly always complexed with proteins that may block the sites and/or change the secondary/tertiary structure of the targeted RNA such that oligonucleotide targeting is frequently a trial and error process (Sazani and Kole, 2003). Recently a new application of antisense oligonucleotides has been developed whereby these agents have been used to modify the splicing pattern of pre-mRNA in contrast to down-regulation of gene expression by targeting mRNA (Sierakowska et

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al, 1999; Schmajuk et al , 1999). Morpholino-oligonucleotides have been designed to bind to specific *cis*-elements within target precursor mRNA to correct aberrant splicing brought about by disease mutations an example of which is the β-globin gene in thalassemic patients (Sierakowska et al, 1996). In contrast the potential also exists to use this methodology to selectively delete individual exons of specific pre-mRNA species and thus increase production of variant mRNAs which are translated in to proteins devoid of functional domains. In light of this a similar strategy could be adopted to knock out specific exons within pre-mRNA of uterine pro-labour genes, such as COX-2 (which we report here) resulting in decreased expression of functionally active protein species.

Materials And Methods

Morpholino oligonucleotides and nuclear delivery systems

All Morpholino antisense oligonucleotides used in this study were synthesised by Gene Tools, LLC, USA. Two nuclear delivery systems were applied, the EPEI system which delivers morpholinos using ethyoxylated polyethylenimine complexed ionically with morpholino oligonucleotides pre-annealed with DNA and the recently developed Endo-Porter system which delivers 'bare' oligonucleotides also by an endocytosis-mediated process. Both systems preferentially deliver oligonucleotides to the nucleus to affect pre-mRNA splicing as alluded to in the introduction. The morpholino oligonucleotides were designed to splice out exon 4 (which encodes for part of the catalytic domain) of the pre-mRNA transcript of COX-2 by targeting the splice site boundaries flanking exon 4 (shown in Figure 1). The sequences for the 3' acceptor site and 5' donor site blocking morpholino-oligonucleotides were TGTGATCTGGCTGAAATTTTCAAAG and GATTCTTCTTACTCACCTTTCACAC

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respectively. As a control, invert antisense morpholino-oligonucleotides with the same sequences but in a reverse orientation were used. Special delivery fluoresceinated standard control morpholino oligonucleotides and bare fluoresceinated standard control morpholino oligonucleotides were included in this study to determine and optimise the transfection efficiency of the two delivery systems in both cell types. The sequence for the FSC oligonucleotides was CCTCTTACCTCAGTTACAATTTATA. Confirmation of delivery was measured by confocal microscopy using a Leica TCS SP2 UV with a X40 NA 0.8 HCX water dipping lens. Delivery efficiencies were determined by calculating the number of positive cells with a fluorescence signal within defined fields. Note, live, unfixed cells were used as fixing cells may give a false positive result.

Cell culture and delivery of morpholino oligonucleotides

Both human amnion derived WISH cells and myometrial cells were used in this study. WISH cells (clone CCL-25) purchased from ATCC-LGC (USA) and liquid N_2-frozen stocks of human pregnant myometrial cells (prepared from samples collected from women undergoing elective caesarean sections with local research ethical committee approval from the Newcastle upon Tyne Hospital Trust at the Royal Victoria Infirmary) were cultured in DMEM-Glutamax™ medium (Gibco-Life Technologies) containing 10% fetal calf serum, penicillin (1U/ml), and streptomycin (1ng/ml). WISH and myometrial cells were transfected at 80-90 % sub-confluency in OptiMEM medium (Invitrogen), in the absence of antibiotics, and the effect of individual concentrations of morpholino-oligonucleotides assayed 24 or 48 hours after nuclear delivery using the two systems detailed above. Briefly, 1 ml of tissue-culture grade water was combined with 33.6 μl Morpholino/DNA stock (0.5mM), 33.6

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μl EPEI and 5.4 ml serum-free OptiMEM and and 1.5 ml of the above EPE1 /morpholino complex solution applied to cell cultures in 6 well plates in the presence of LPS (1μg/ml) for 24 or 48 hrs. After 3 hours the medium was removed and replaced with complete DMEM medium (in the absence of antibiotics). Experiments applying the Endo-Porter delivery system used 6-8 μl of Endo-Porter solution per ml of OptiMEM medium (in the absence of antibiotics) with 3-10% fetal calf serum and 3-10μl of the 0.5mM bare morpholino preparations. Note all transfection experiments were carried out in triplicate and repeated 3 times.

RT-PCR analysis

Confirmation of a COX-2 mRNA spliced variant with exon 4 skipped due to targeting with antisense morpholino-oligonucleotides was achieved by RT-PCR using COX-2 specific sense and antisense primers spanning exon 4. This procedure provides a quick and simple assay to both confirm and quantify the level of inhibition. RT-PCR was performed using total RNA extracted from individual experiments using the SV total RNA isolation kits as recommended by the manufacturer (Promega) and first strand cDNA synthesised from 1μg RNA using 20 units of Superscript III reverse transcriptase (Invitrogen) with 100ng of oligo$(dT)_{16}$ as primer. PCR amplification was carried out with 2μl cDNA using COX-2-specific sense and antisense oligonucleotide primers which amplify COX-2 mRNA spliced variants with and without the exon 4 sequence. DNA sequences for the PCR primers were CTACATACTTACCCACTTCAAGG (sense exon 3) and GTAGATCATCTCTGCCTGAGTATC (antisense exon 6). PCR was performed under standard conditions with an initial hot start cycle at 94^{o}C (4 min) 55^{o}C (30 s) and 72 ^{o}C (1min) followed by 25-28 cycles at 94^{o}C (1 min) 55^{o}C (30 s) and 72 ^{o}C (1min). PCR products representing the spliced COX-2 mRNA variants (472bp with

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exon 4 and 328bp without exon 4) were then analysed by gel electrophoresis followed by densitometric scanning using a UMAX scanner coupled to the intelligent quantifier software from BioImage. GAPDH primers were also included as control primers for use in RT-PCR with each cDNA sample. DNA sequences for the GAPDH primers were CTGCCGTCTAGAAAAACC (sense) and CCACCTTCGTTGTCATACC (antisense).

Western Immunoblotting

The effectiveness of the morpholino-oligonucleotides in repressing functional protein expression was determined by immunoblotting. Protein lysates from morpholino-oligonucleotide and LPS treated WISH and myometrial cells were prepared and resolved by 10% SDS-PAGE as previously described (Pollard et al, 2000). Recombinant COX-2 protein was also included as a positive control. Immunoblotting was then performed using a monoclonal COX-2 antibody (Upstate Biotechnology) at 1:1000 dilution overnight at 4 ^{0}C. All membranes were re-probed with a Gβ control antibody to confirm equal loading. Immunoreactive bands were detected by enhanced chemiluminescence (Amersham Biosciences) followed by densitometric scanning using a UMAX scanner coupled to the intelligent quantifier software from BioImage.

COX-2 Enzyme Activity Assay

Loss of enzymic activity of COX-2, as a consequence of morpholino inhibition, was measured using a COX enzyme activity assay (Cayman Chemicals). This procedure measures COX-2 activity by oxidation of the peroxidase co-substrate TMPD (*N,N,N*^{1},*N*^{1} –tetra-methyl-*p*-phenylenediamine) in 96 well plates and has been shown to accurately reflect the rate of conversion of arachidonic acid to PGH_2. Briefly, WISH

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and myometrial cells were cultured to 90% confluency in T75^{cm} flasks and treated with LPS/ Morpholino oligonucleotides as described in the Figure legends. Cells were then rinsed twice in PBS to remove all traces of DMEM medium, sonicated in 400 μl cold 0.1ṂTris (Ph7.8) containing 1 mM EDTA and freeze dried to to 200 μl. The assay mixture containing 140 μl assay buffer,10μl heme, 10μl of sample (or protein standard or inactivated protein prepared by boiling lysates for 5 minutes) and 10 μl of the COX-1 inhibitor *SC-560* to eliminate COX-1 activity (or as a control reaction the COX-2 inhibitor *DuP-687* to eliminate COX-2 activity) was incubated for 5 min at 25^{0}C and 20 μl of TMPD added. The reactions were then initiated by adding 20μl arachidonic acid to all wells and plates gently shaken and incubated for 5 min at 25^{0}C. The absorbance was then read at 590nM using a Molecular Devices Spectra MAX 190 plate reader. COX-2 activity was then calculated using the following formula whereby 1 unit is defined as the amount of enzyme to oxidise 1 nmol of TMPD per min at 25^{0}C. COX-2 activity = ((Δ_{590}/5min/0.00826 μ M^{-1}) x (0.21ml/0.01ml)) / 2* = nmol/min/ml (U/ml). *It takes two molecules of TMPD to reduce PGG_2 to PGH_2. Inhibition of COX-2 activity, as a consequence of morpholino inhibition was then measured and normalised to transfection efficiency.

Statistical Analysis

Data were compared using an unpaired, two-tailed *t* test, P<0.05 was considered statistically significant. All experiments were performed three times in triplicate and results are expressed as the mean ± SEM.

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Results

Delivery of Morpholino oligonucleotides within live amnion-derived WISH and myometrial cells

To first determine the optimal conditions for delivery of the oligonucleotides we compared the relative efficacy of the EPEI and Endo-Porter delivery systems under different experimental conditions, as described, using fluorescein tagged morpholino oligonucleotides. Delivery efficiencies were determined by confocal microscopy calculating the number of positive cells, in several fields, with a fluorescence signal. Note, live, unfixed cells were used. Results clearly indicated that the most effective delivery method in our test system was the Endo-Porter formula. Cell cultures treated with the morpholino oligonucleotides (5μM) and Endo-Porter (8μM) for 48 hours resulted in a transfection efficiency of 82% for WISH cells and 78% for myometrial cells and (Figure 2); whereas using the EPEI formula transfection efficiencies were 46% and 42% for WISH cells and myometrial cells respectively (data not shown). When cells were treated for 24 hours the delivery efficiencies using Endo-Porter were 66% for WISH cells and 60% for myometrial cell cultures (data not shown).

24 hour effect of morpholino oligonucleotides on LPS-induced COX-2 mRNA expression

Morpholino oligonucleotides were designed to target the 3' acceptor and 5' donor splice site boundaries flanking exon 4 of the COX pre-mRNA sequence (Figure 1). The steric blocking of these splice site sequences in theory blocks the binding of proteins that regulate splice-site selection and prevents inclusion of exon 4 into COX-

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2 mRNA transcripts (Figure1). Confirmation of a COX-2 spliced variant with exon 4 deleted as a consequence of morpholino inhibition was observed by RT-PCR using COX-2 specific primers spanning exons 3 and 6, respectively. As shown in Figure 3A, COX-2 expression was induced by LPS in both cell types, as described in the material & Methods. GAPDH mRNAs were also included as internal controls. Treating WISH and myometrial cells with the two antisense morpholino oligonucleotides for 24 hours, in the presence of LPS, resulted in a significant reduction in the expression of COX-2 mRNAs containing exon 4 as reflected by the decrease in the intensity of the 472bp PCR product and the appearance of a new PCR band of smaller size 328bp representing COX-2 spliced variants with the 144bp exon 4 skipped out. Note, only the 472 bp PCR product was observed when the invert morpholino oligonucleotides were used hence confirming that the control oligonucleotides have no effect. Levels of inhibition were normalised to the calculated delivery efficiencies of 66% for WISH cells and 60% for myometrial cells. The residual 472bp band observed in antisense morpholino oligonucleotide treated cells represents those cells that have not been transfected.

24 hour effect of morpholino oligonucleotides on LPS-induced COX-2 protein expression

The ability of morpholino oligonucleotides to inhibit the production of full length COX-2 protein was then assessed by Western immunoblotting using protein lysates prepared from WISH and myometrial cells treated with the two morpholino oligonucleotides for 24 hours. Quantification demonstrated that levels of 72kDa COX-2 were significantly lower ($p < 0.01$) in samples treated with the inhibitory COX-2 morpholino oligonucleotides when compared to the intensity of the 72kDa protein

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bands generated from the cells treated with LPS and the control morpholino oligonucleotides (Figure 4). The appearance of a novel band of smaller size of less than 65kDa was observed only in samples treated with the morpholino oligonucletides indicating translation of a truncated COX-2 protein. The predicted molecular weight of a COX-2 protein with the 144bp exon 4 spliced out, based on its amino acid content, was calculated to be 63kDa (www.bioinformatics.org/sms/prot_mw.html). Note, the reduction in 72kDa COX-2 protein levels, as a consequence of morpholino oligonucleotide inhibition correlated with the preceding decrease in full length COX-2 mRNA (Figure 3). The residual 72kda band observed in antisense morpholino oligonucleotide treated cells represents those cells that have not been transfected

48 hour effect of morpholino oligonucleotides on LPS-induced COX-2 mRNA and protein expression

We then evaluated whether the activity of the morpholino oligonucleotides could be prolonged to further reduce the levels of LPS-induced COX-2 expression. WISH and myometrial cell cultures were incubated for 48 hours with the morpholino oligonucleotides and the RT-PCR and Western immunoblotting analyses repeated as previously described. We observed that using the two morpholino oligonucleotides targeted to both the 3' acceptor and 5'donor splice sites of COX-2 pre-mRNA for this extended time period further increased the inhibitory effect of these oligonucleotides on COX-2 expression at both mRNA and protein levels (see Figure 5) which correlates with the calculated delivery efficiencies of 82% for WISH cells and 78% for myometrial cells observed at this time.

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Antisense-morpholino oligonucleotides suppress COX-2 enzyme activity

An important aspect of this present study was to ascertain whether morpholino oligonucleotides could significantly suppress COX-2 activity. To address this we tested the effect of the morpholino oligonucleotides after 48 hours on the enzymic activity of COX-2 by utilising a sensitive COX enzyme assay which accurately reflects the rate of conversion of arachidonic acid to PGH_2. SC-560, a potent and selective inhibitor of COX-1 was also included in the assay to eliminate all COX-1 activity and ensure that the enzymic activity was specifically that of COX-2. Note each reaction was carried out in triplicate and the assay repeated 3 times. As shown in Figure 6, antisense morpholino oligonucleotides targeted to COX-2 pre-mRNA significantly inhibited the activity of C0X-2 in cultures of amnion-WISH and myometrial cells.

Discussion

In this study we have taken the novel approach of evaluating the use of two antisense morpholino oligonucleotides directed to both the 3' acceptor and 5' donor splice sites of exon 4 of COX-2 pre-mRNA in suppressing expression and activity of COX-2 in human amnion derived WISH and myometrial cells. Our results indicate that these antisense morpholino oligonucleotides can significantly inhibit expression and activity of COX-2 in *in vitro* cultures of both cell types. Initial confocal microscopy on 'live' unfixed amnion-WISH and myometrial cells illustrated high levels of penetrance of antisense morpholino oligonucleotides using the recently developed non-toxic Endo-Porter system from Gene Tools LLC with delivery efficiencies of 66% and 60% after 24 hrs and 82% and 78% after 48 hrs respectively in WISH and myometrial cells. Substantial inhibition by the morpholino oligonucleotides of COX-2 expression, induced by LPS administration, was observed at both the mRNA and

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protein level employing RT-PCR and Western immunoblotting. Loss of enzymic activity of COX-2 was also confirmed using a highly sensitive COX enzyme activity assay which accurately reflects the rate of conversion of arachidonic acid to PGH_2.

Evidence from previous studies show that antisense oligonucleotides targeted to pre-mRNA splice sites redirect the splicing machinery to adjacent consensus splice sites or cryptic splice sites and alter the splicing pattern of the pre-mRNA (Sierakowska et al, 1999; Schmajik et al, 1999; Sazani et al, 2002). Oligonucleotides used to modify splicing result in products that are readily detectable with null or very low background contamination, whereas the effects of oligonucleotides directed to mRNA may easily be overlooked due to high background of the pre-existing mRNA. Moreover, since splicing takes place in the nucleus the shift in the pattern of splicing of the target gene can only be due to the intranuclear activity of the oligonucleotides (Sierakowska et al, 1999; Schmajik et al, 1999). The requirements for oligonucleotides that shift splicing are different to those required for down-regulation of mRNA. Essentially they must not activate RNase H which would destroy the pre-mRNA target prior to splicing and must be able to effectively compete with splicing factors for access to target pre-mRNA (Sierakowska et al, 1999). Synthetic morpholino-oligonucleotides having phosphordiamidate internucleotide linkages fit these requirements since they are RNase H inactive with high affinity for target pre-mRNA and are nuclease resistant as well as having the ability to cross cell membranes relatively easily (Schmajik et al, 1999; Sazani et al, 2002; Heasman, 2002). In several *in vitro* test systems antisense oligonucleotides have proven to be highly successful in correcting aberrant splicing brought about by disease mutations such as the β-globin gene in thalassemia and the CFTR gene (Friedman et al, 1999). Until now only two published studies have

used this antisense approach to target pre-mRNA and selectively splice out specific exons (Karras et al, 2000; Ittig et al 2004). The Karras et al study reported that constitutive/alternative splicing of murine interleukin-5 receptor-α (IL-5Rα) chain pre-mRNA can be modulated in cells using specific antisense morpholino-oligonucleotides directed to specific 3'/5'splice sites such that individual exons may be selectively deleted from mature transcripts. This study used a range of different morpholino oligonucleotides designed to target either 3' acceptor or 5' donor sites of the IL-5Rα chain pre-mRNA. Interestingly, in some cases specific oligonucleotides redirected splicing events to nearby cryptic splice sites resulting in novel IL-5Rα chain mRNA transcripts. What appeared to be effective in our *in vitro* test system was the use of two morpholino oligonucleotides targeted to both the 3' acceptor and 5' donor sites of the pre-mRNA sequence of COX-2.

The results from this present study provide the first evidence to indicate that antisense morpholino-oligonucleotides can be utilised, *in vitro,* in amnion-WISH and myometrial cell cultures to produce a functionally inactive protein and that this approach may therefore have therapeutic potential in the better management of preterm labour if it can be reproduced *in vivo.* In this respect a recent study by Luu et al using the mouse model (Luu et al, 2004) provides direct evidence to show the efficacy of morpholino oligonucleotides *in vivo.* This study reported that morpholino oligonucleotides were successful in down-regulating calbindins in the mouse uterus and that the effect of the morpholinos remained localised within the uterus of the mouse. Moreover, the study highlighted that administration of morpholino oligonucleotides by intrauterine injection using the less potent EPEI delivery system

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(Gene Tools, LLC) provided a highly effective technique in specifically targeting uterine genes *in vivo*.

Antisense morpholino-oligonucleotides have major advantages over other antisense gene silencing systems in that: i) they are DNA analogues which are not susceptible to enzymatic degradation and thus have increased biological stability (Hudziak et al, 1996), ii) whereas targeted mRNA with conventional oligonucleotides is continually being replaced by new transcription requiring continued treatment, morpholino-oligonucleotide targetting of pre-mRNA requires a single dose, iii) they have been shown to have greater specificity than siRNA and other phosphorothioate based oligonucletides (Summerton, 1999) and hence have no off-target toxic antisense effects and iv) they have a high 'loss-of-function' affect which has been shown to be up to 4 days (Braat et al, 2001; Dutton et al, 2001). Since antisense morpholino-oligonucleotides provide greater stability, nuclease-resistance, long term activity, low toxicity and excellent specificity when compared with alternative gene silencing reagents, they therefore may represent potential therapeutic tools within many fields of medicine including obstetrics. In this context there is extensive evidence to indicate increased expression of COX-2 at sites of inflammation and disease (Williams et al, 1999; Warner and Mitchell, 2005). For example expression of COX-2 is greatly increased in rheumatoid arthritic joints (Warner and Mitchell, 2005), plus clinical and experimental evidence suggests that COX-2 contributes to lesion formation in atherosclerosis (Paramo et al 2005). COX-2 over expression appears to be particularly prevalent in different cancers including gastric cancers (Saukkonen et al, 2001), esophageal cancer (Kaur and Triadafilopoulos, 2002), pancreatic cancer (Tucker et al,1999), lung adenocarcinoma (Wolff et al, 1998) and colon carcinomas

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(Gupta et al, 2001). In this light, suppression of COX-2 activity by antisense morpholino-oligonucleotides may also have potential in the better treatment inflammatory diseases and cancer.

In conclusion, this study has shown that antisense morpholino oligonucleotides designed to target both the 3' acceptor and 5' donor sites of exon 4 of the pre-mRNA sequence of COX-2 results in a substantial suppression of COX-2 activity in cultured human amnion-derived WISH and myometrial cells. Consequently, the possibility exists that a similar approach can be mimicked *in vivo* to produce a highly specific and non-toxic strategy to inhibit COX-2 activity.

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Footnotes

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Legends For Figures

Figure 1 *Targeting pre-mRNA of COX-2 by antisense morpholino oligonucleotides*

A) Diagram to show mechanism of action of morpholino oligonucleotides in targeting nuclear pre-mRNA. Binding of morpholino oligonucleotides to the splice sites blocks assess to the splice sites by splicing factors and redirects the splicing machinery to skip out the exon. This results in a shorter mRNA and a truncated protein or no protein. B)Two splice-site blocking antisense morpholino oligonucleotides were designed to target the 3' acceptor and 5' donor sites of exon 4 of the precursor mRNA sequence of COX-2 resulting in skipping of exon 4.

Figure 2 *Endo-Porter delivery of fluoroscein tagged morpholino oligonucleotide(s) in human amnion-derived WISH and myometrial cells*

Endo-Porter delivery of morpholino oligonucleotides in cells was as described in Methods and Materials. Delivery efficiencies were determined by confocal microscopy using a Leica TCS SP2 UV with the X40 NA 0.8 HCX water dipping lens. (A) Human amnion-derived WISH and (B) myometrial cells. Fluorescence signal showing delivery of the oligonucleotides inside 'live' non-fixed cells and also the high level of transfection efficiency for both cell types after 48 hours (82% WISH cells and 78% myometrial cells), 24 hour incubations resulted in efficiencies of 66% for WISH and 60% for myometrial cells (data not shown). (i) Fluorescence (ii) Bright field same slide and (iii) overlay field.

Figure 3 *24 hour effect of antisense morpholino oligonucleotides on LPS induced COX-2 mRNA expression*

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A) Control LPS induction of COX-2 mRNA expression in amnion-derived WISH and primary myometrial cell cultures. Cells were stimulated with LPS 24 hours (1μg/ml) to induce COX-2 mRNA expression. RT-PCR using COX-2 specific primers spanning exons 3 and 6 and total RNA isolated from cells treated with LPS resulted in a PCR product of 472bp representing untruncated full-length COX-2 compared to untreated cells. Two further experiments gave similar results. B) Amnion derived WISH and myometrial cells were administered antisense and control invert morpholino oligonucleotides for 24 hrs in the presence of LPS. RT-PCR using COX-2 specific primers spanning exons 3 and 6 resulted in PCR products of 472bp and 328bp representing COX-2 mRNAs with exon 4 included or skipped out respectively. The 472bp bands representing full-length COX-2 were scanned and quantified using a UMAX scanner coupled to the intelligent quantifier software from BioImage. Data were normalised to delivery efficiencies for each cell type. Note that the residual 472bp band in antisense treated cells represents COX-2 expression in untransfected cells. Data are means ± SEM (n= 6), **P* <0.01 (t test) in antisense compared to invert morpholino oligonucleotide treated cells. C) GAPDH house-keeping RT-PCR was used in each case to confirm equal loading.

Figure 4 *24 hour effect of antisense morpholino oligonucleotides on LPS-induced COX-2 protein* expression

A) Control LPS induction of COX-2 protein expression in amnion-derived WISH and primary myometrial cell cultures. Cells were stimulated with LPS for 24 hours (1μg/ml) to induce COX-2 protein expression. LPS treatment resulted in a 72kDa protein as detected by Western-blotting compared to untreated cells. Recombinant COX-2 protein (rec-COX-2) was used as a positive control. Two further experiments

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gave similar results. B) Amnion derived WISH and myometrial cells were administered antisense and control invert morpholino oligonucleotides for 24 hrs in the presence of LPS. The 72kDa bands representing full-length COX-2 were scanned and quantified using a UMAX scanner coupled to the Intelligent quantifier software from BioImage. Data were normalised to delivery efficiencies for each cell type. Note that the residual 72kDa band in antisense treated cells represents COX-2 expression in untransfected cells. Data are means ± SEM (n= 6) *P<0.01 (t test) in antisense compared to invert morpholino oligonucleotide treated cells. C) All membranes were re-probed with a Gβ control antibody to confirm equal loading.

•

Figure 5 *48 hour effect of morpholino oligonucleotides on LPS-induced COX-2 mRNA and protein expression*

A) Amnion derived WISH and myometrial cells were administered antisense and control invert morpholino oligonucleotides for 48 hrs in the presence of LPS. RT-PCR using COX-2 specific primers spanning exons 3 and 6 resulted in PCR products of 472bp and 328bp representing COX-2 mRNAs with exon 4 included or skipped out respectively. The 472bp bands representing full-length COX-2 were scanned and quantified using a UMAX scanner coupled to the intelligent quantifier software from BioImage. Data were normalised to delivery efficiencies for each cell type. Note that the residual 472bp band in antisense treated cells represents COX-2 expression in untransfected cells. Data are means ± SEM (n= 6) *P<0.001 (t test) in antisense compared to invert morpholino oligonucleotide treated cells. B) GAPDH house-keeping RT-PCR was used in each case to confirm and normalise equal loading. C) Amnion derived WISH and myometrial cells were administered antisense and control invert morpholino oligonucleotides for 48 hrs in the presence of LPS. The 72kDa

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bands, as detected by Western-blotting, representing full-length COX-2 were scanned and quantified using a UMAX scanner coupled to the intelligent quantifier software from BioImage. Data were normalised to delivery efficiencies for each cell type. Note that the residual 72kDa band in antisense treated cells represents COX-2 expression in untransfected cells. Data are means ± SEM (n= 6) **P*<0.001 (t test) in antisense compared to invert morpholino oligonucleotide treated cells. D) All membranes were re-probed with a Gβ control antibody to confirm equal loading.

Figure 6 *48 hour effect of antisense-morpholino oligonucleotides on COX-2 enzyme activity*

Loss of enzymic activity of COX-2, as a consequence of antisense morpholino inhibition, was measured using a COX enzyme activity assay. This procedure measures COX activity by oxidation of the peroxidase co-substrate TMPD (*N,N,N*^{1}*,N*^{1} –tetra-methyl-*p*-phenylenediamine) and reflects the rate of conversion of arachidonic acid to PGH_2. A) Reactions using recombinant COX-1 and COX-2 protein with and without specific COX inhibitors *SC-560* or *DuP-687,* to eliminate COX-1 and C0X-2, activity respectively were used as controls. B) Amnion derived WISH and myometrial cells were administered antisense and control invert morpholino oligonucleotides for 48 hrs in the presence of LPS. SC-560 was used to eliminate all COX-1 activity and inactivated protein from transfected cell lysates also used as a control. The absorbance was read at 590nM using a Molecular Devices Spectra MAX 190 plate reader. COX-2 activity was calculated using the following formula COX-2 activity = (($\Delta\tilde{}_{590}$5min/0.00826 □M^{-1}) x (0.21ml/0.01ml)) / 2* = nmol/min/ml (U/ml) and the % inhibition then measured. Data were normalised to delivery efficiencies for each cell

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type. Data are means ± SEM (n= 6) *P<0.01 (t test) in antisense compared to invert morpholino oligonucleotide treated cells.

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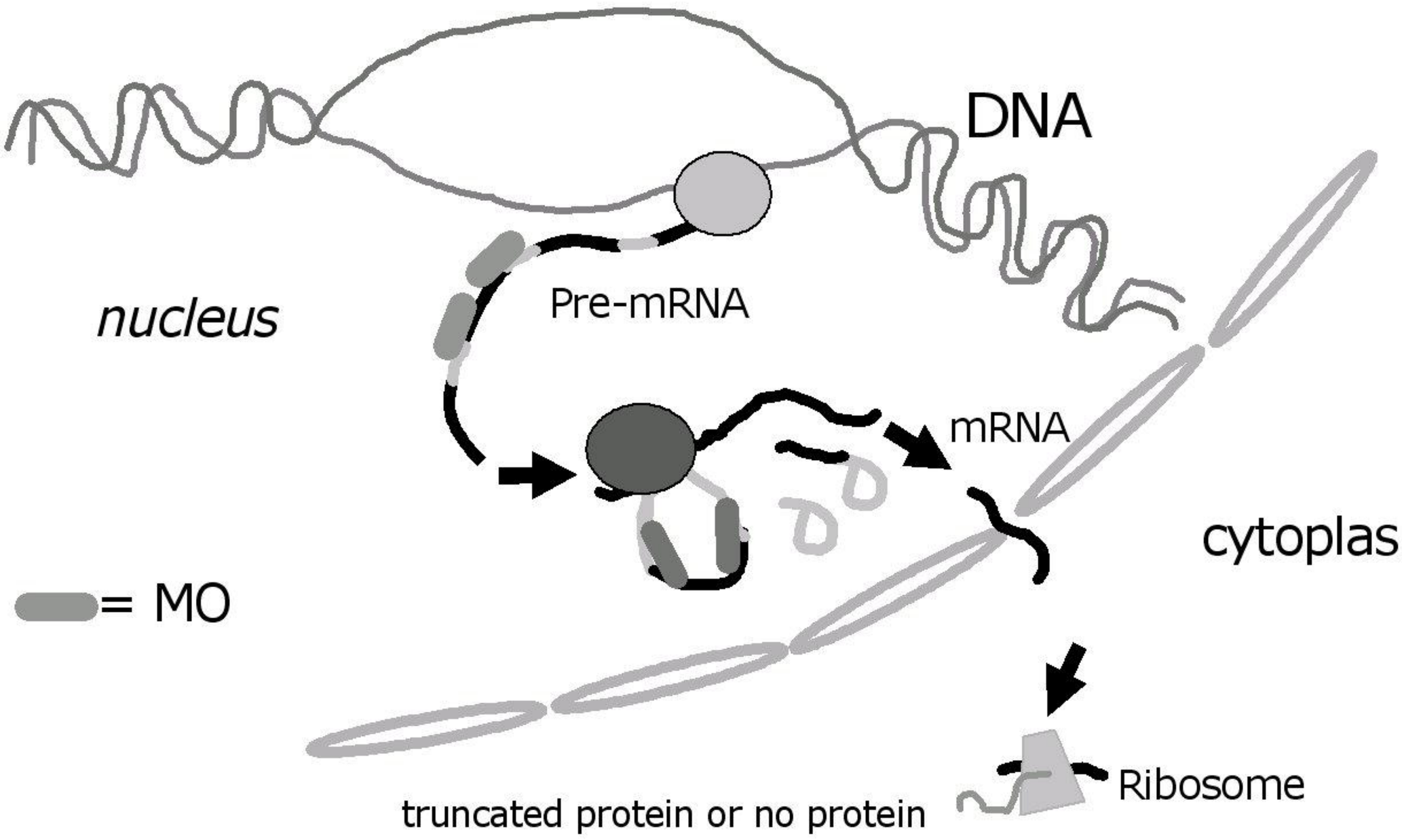

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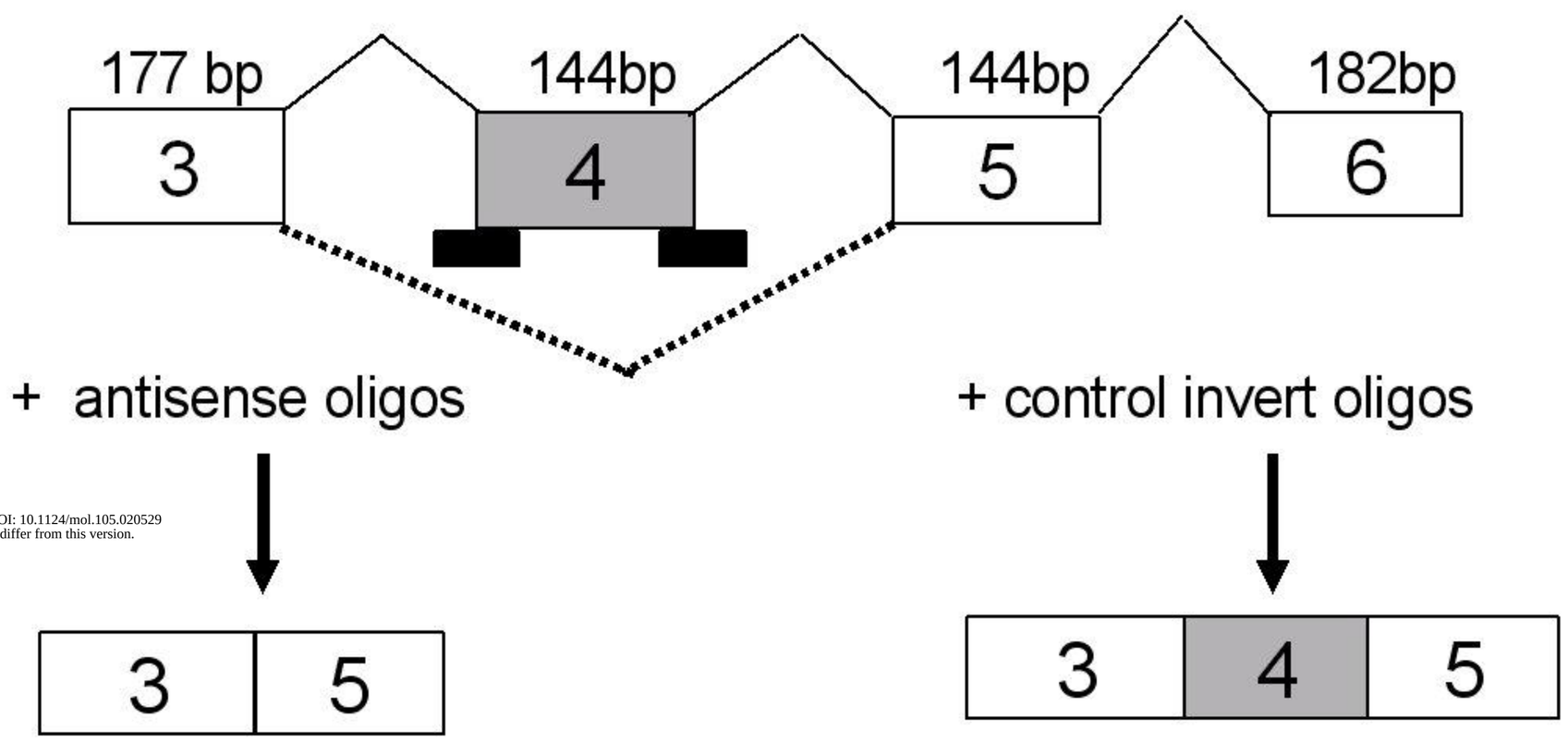

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Figure 1

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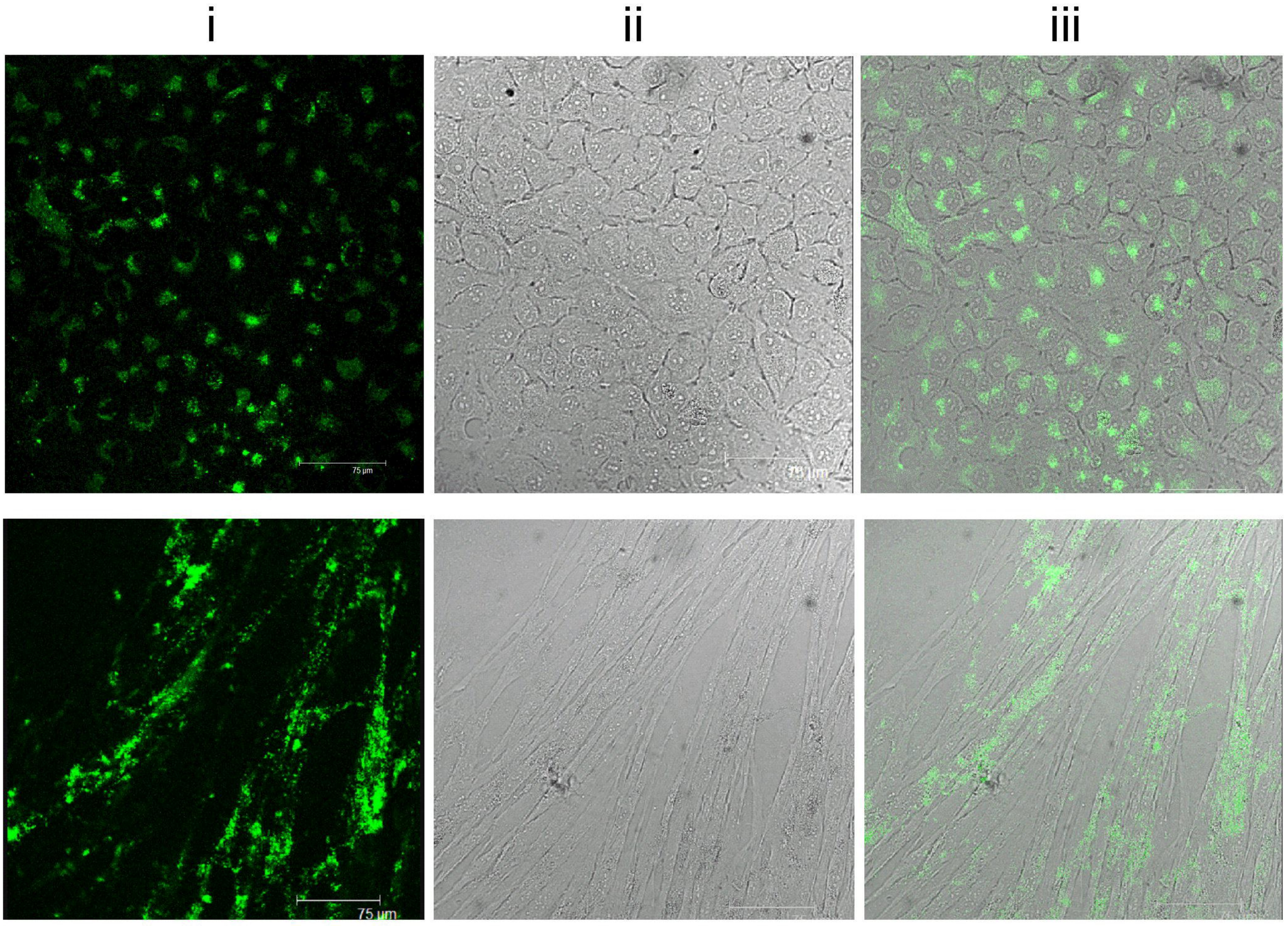

Figure 2

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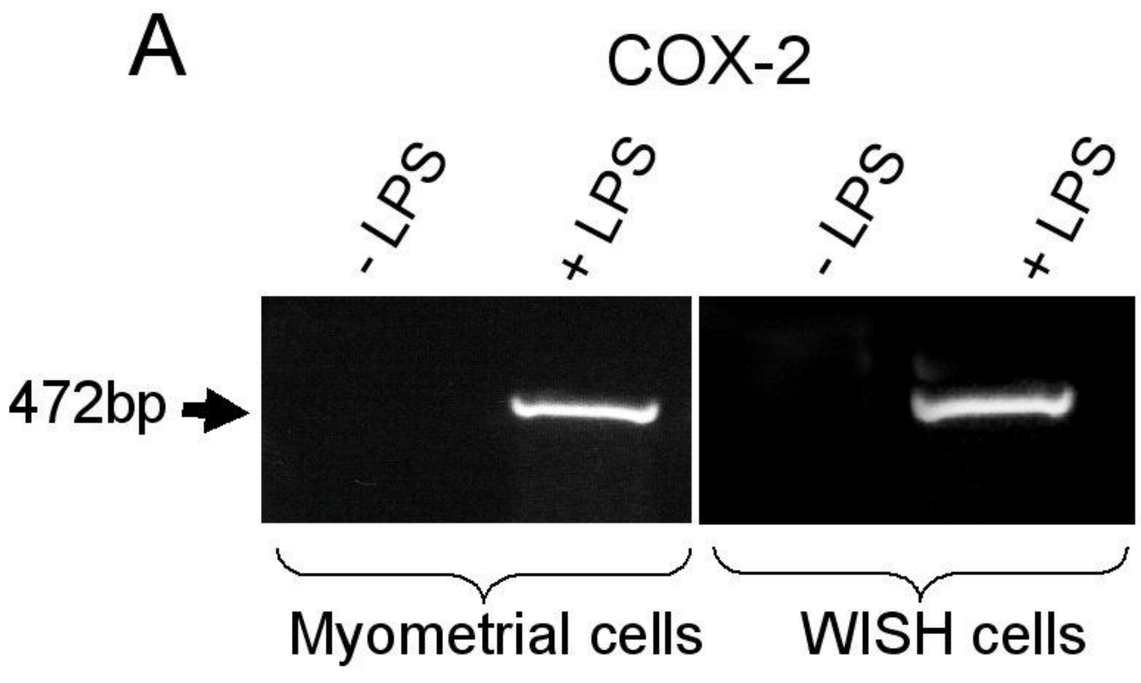

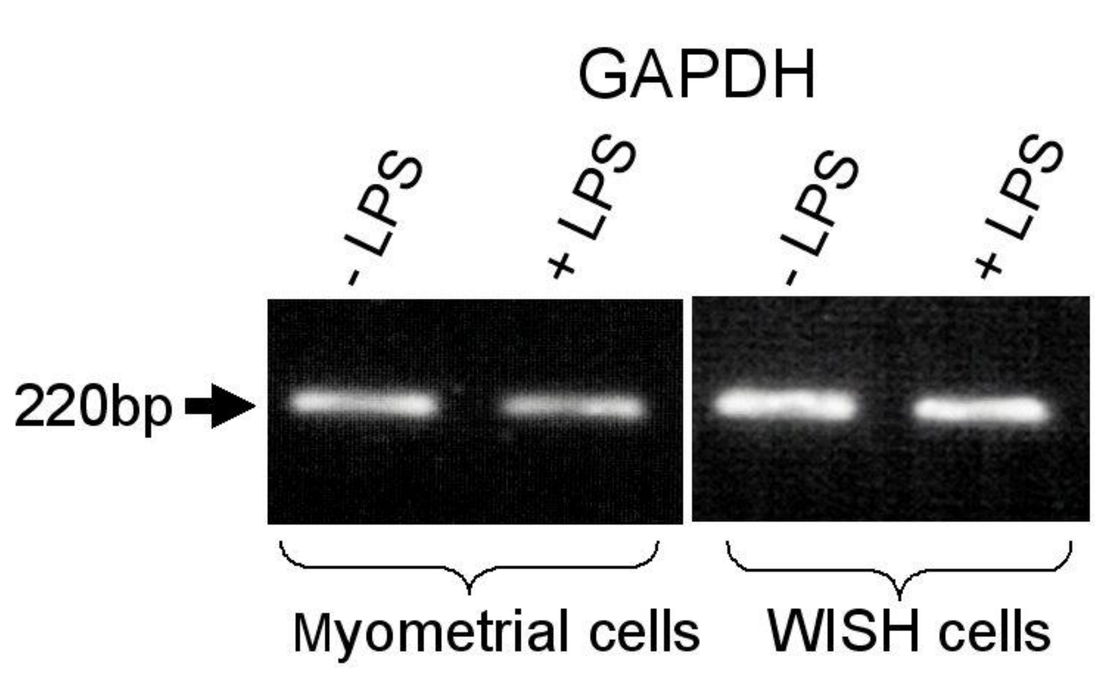

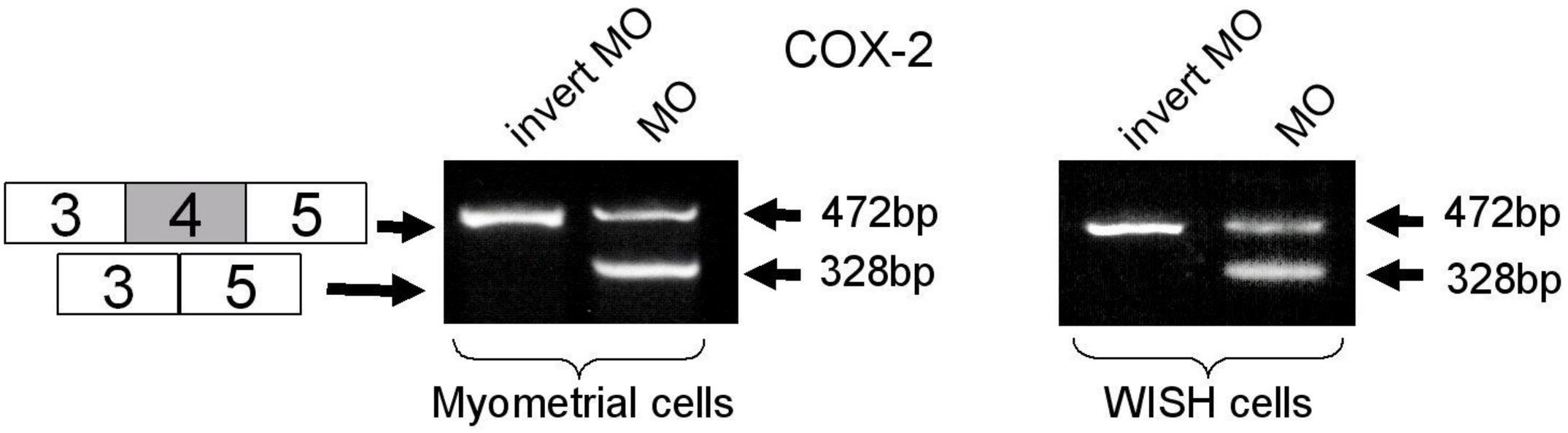

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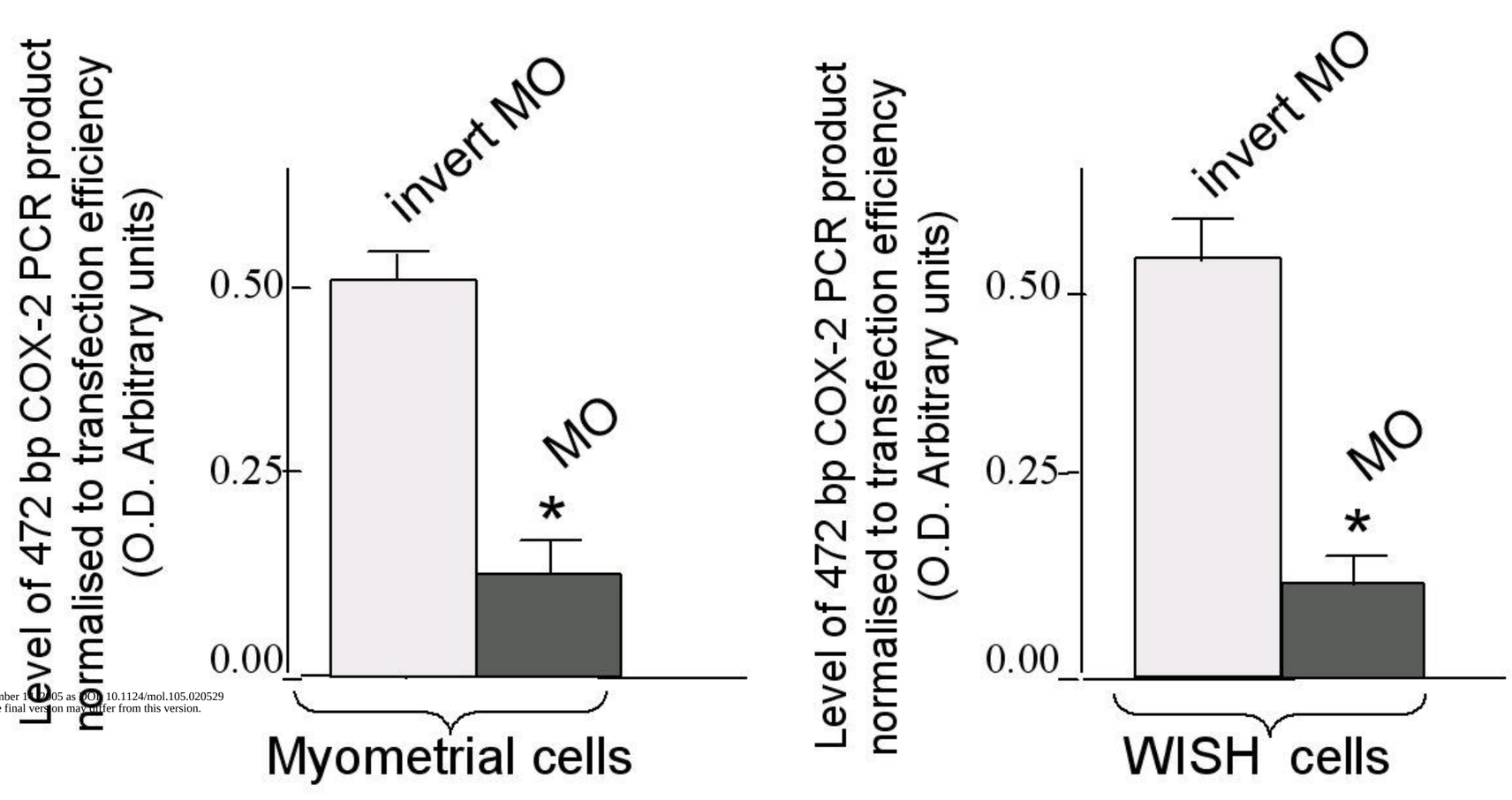

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Figure 3

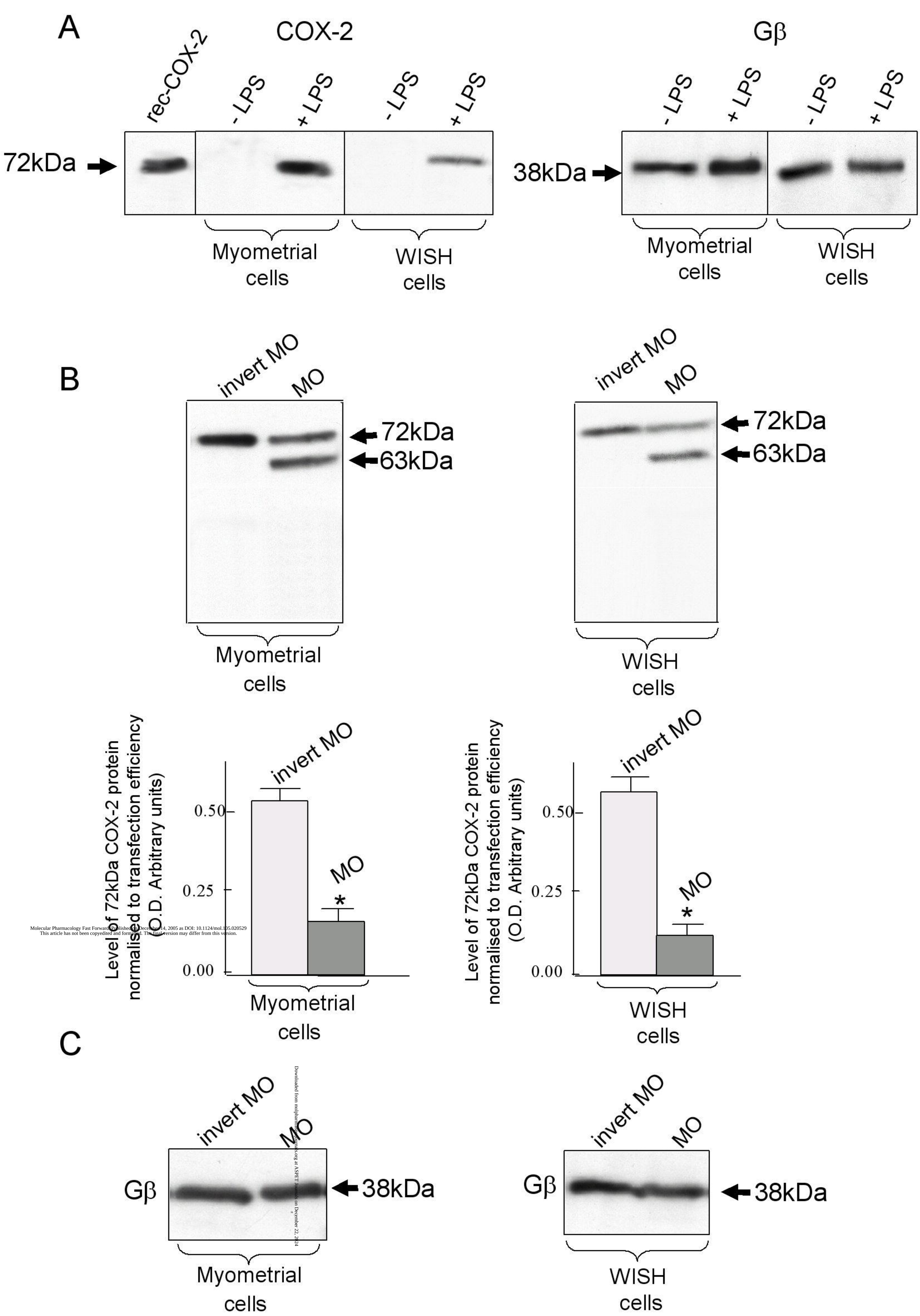

Figure 4

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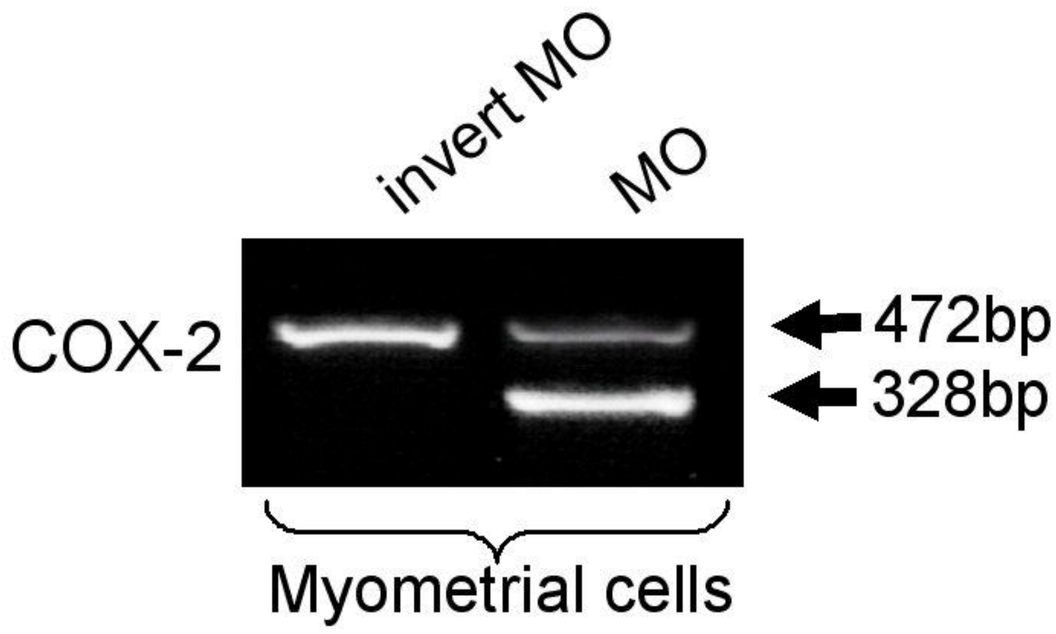

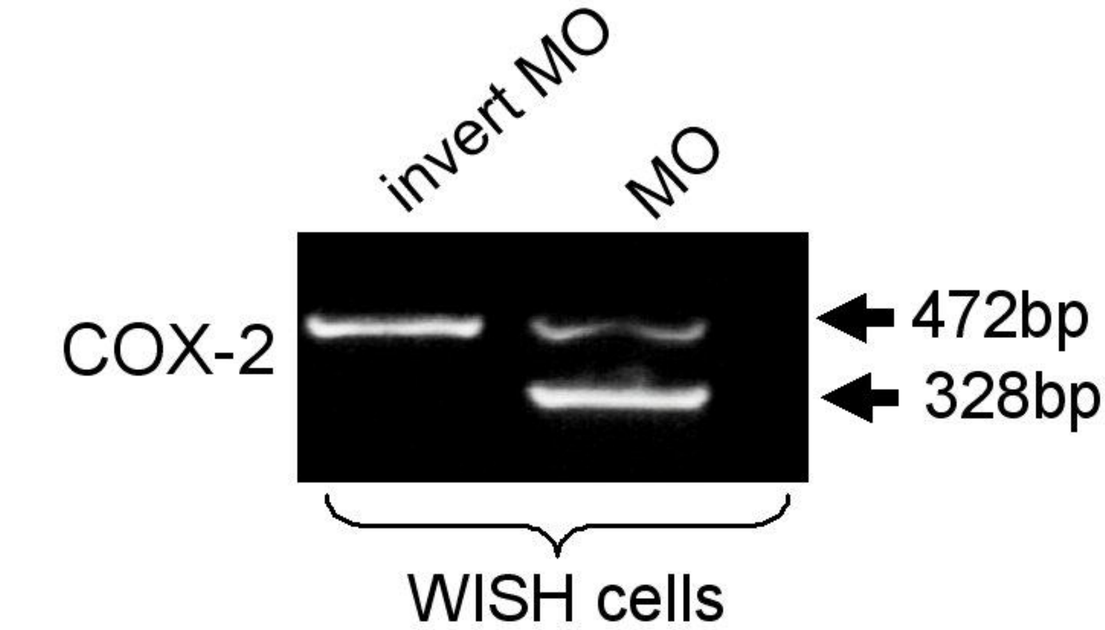

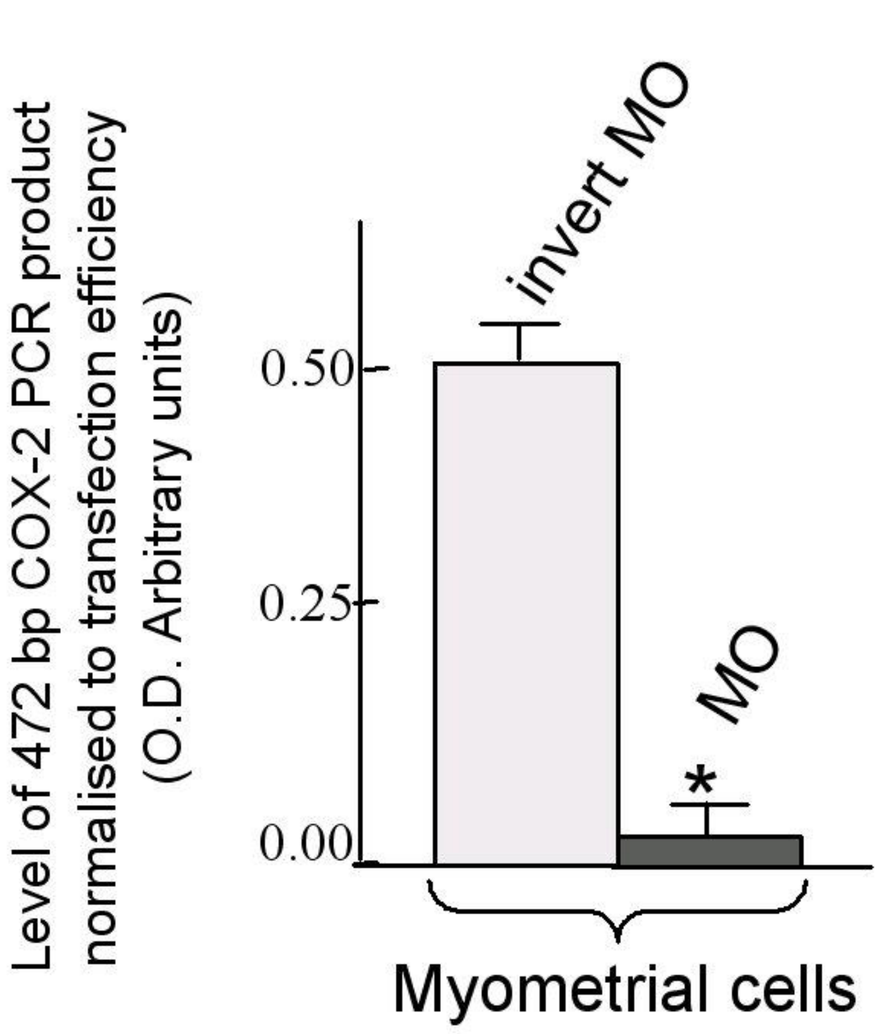

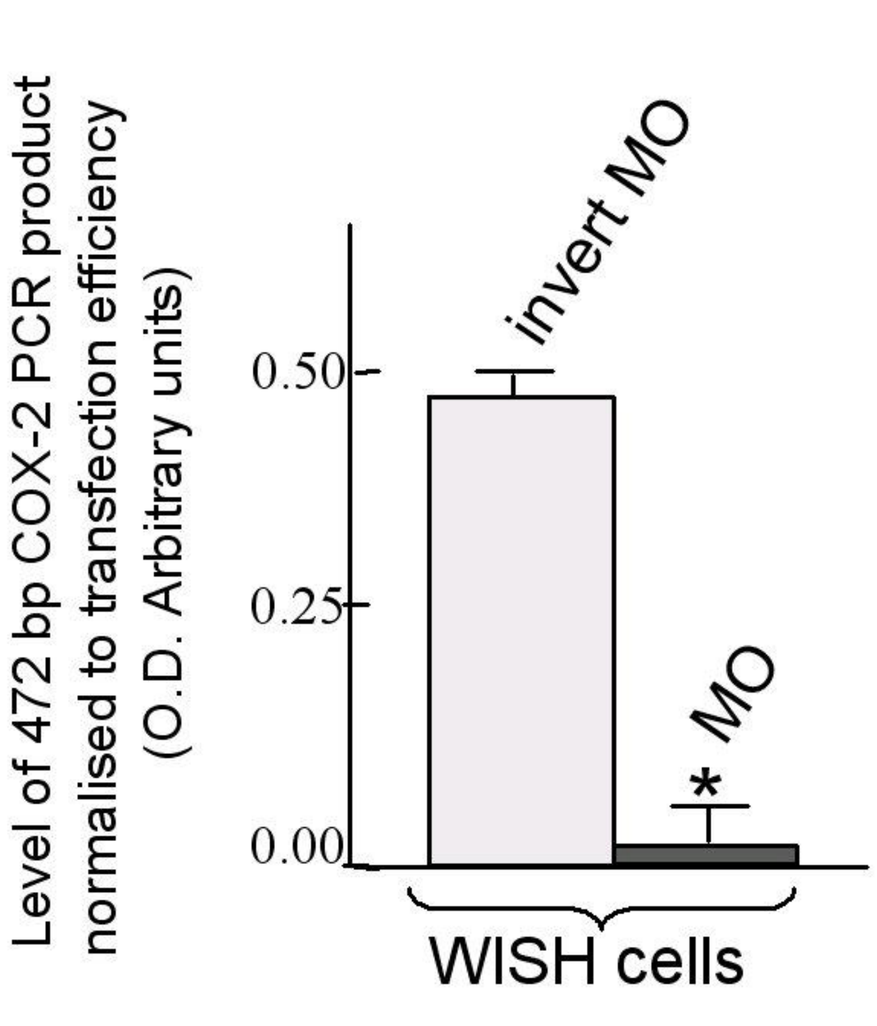

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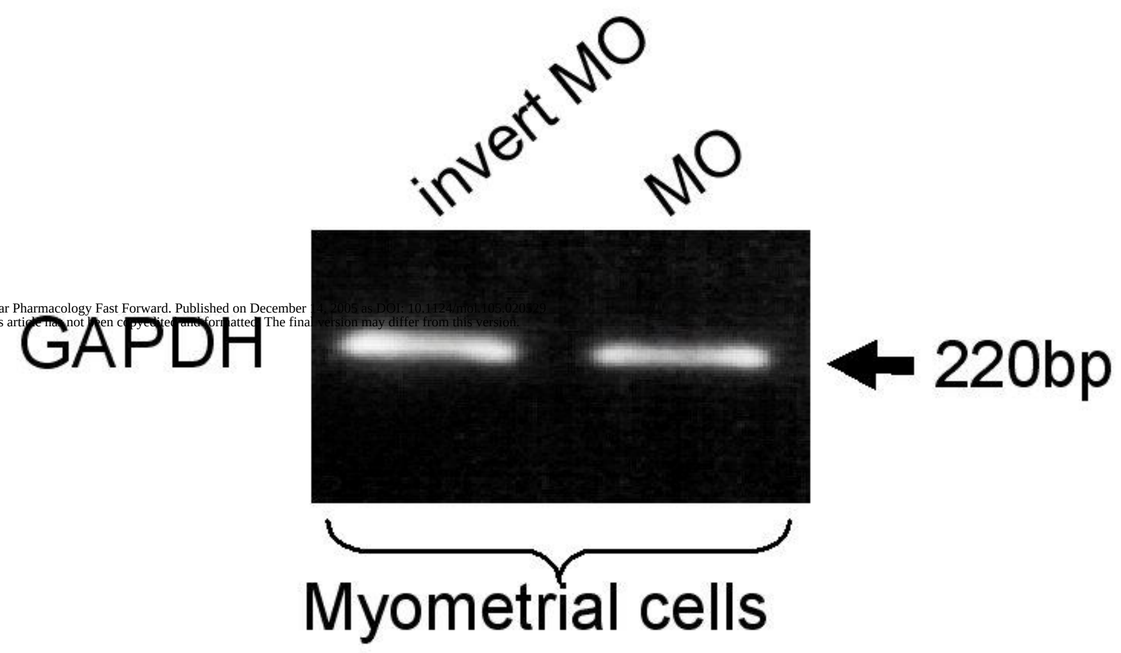

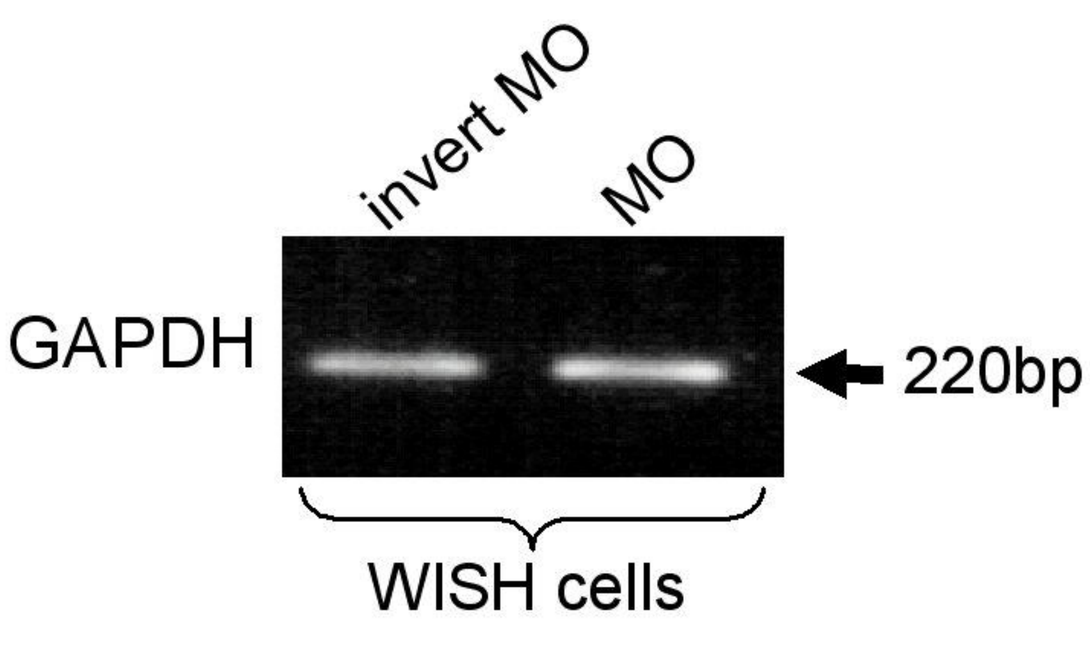

Figure 5

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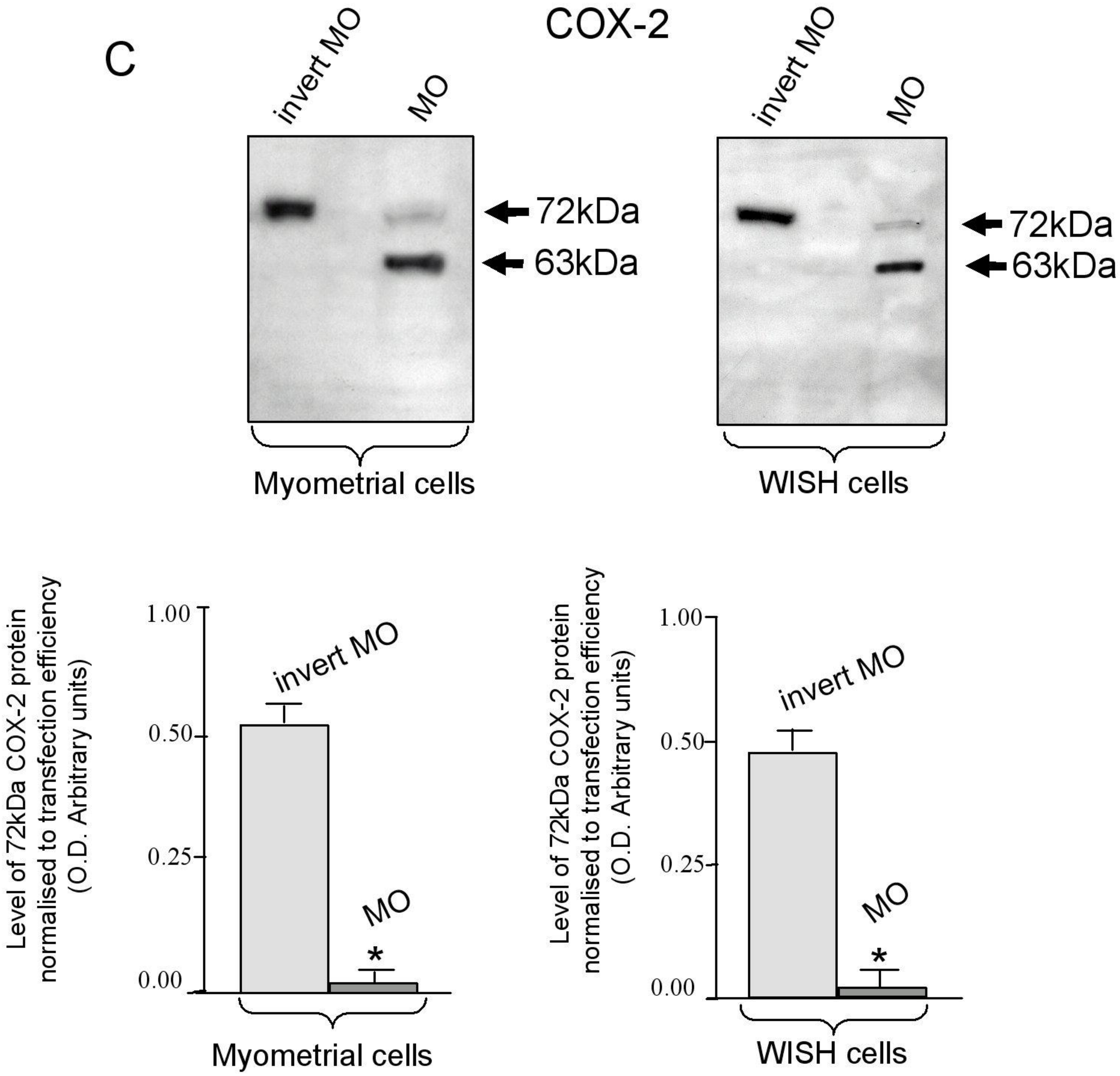

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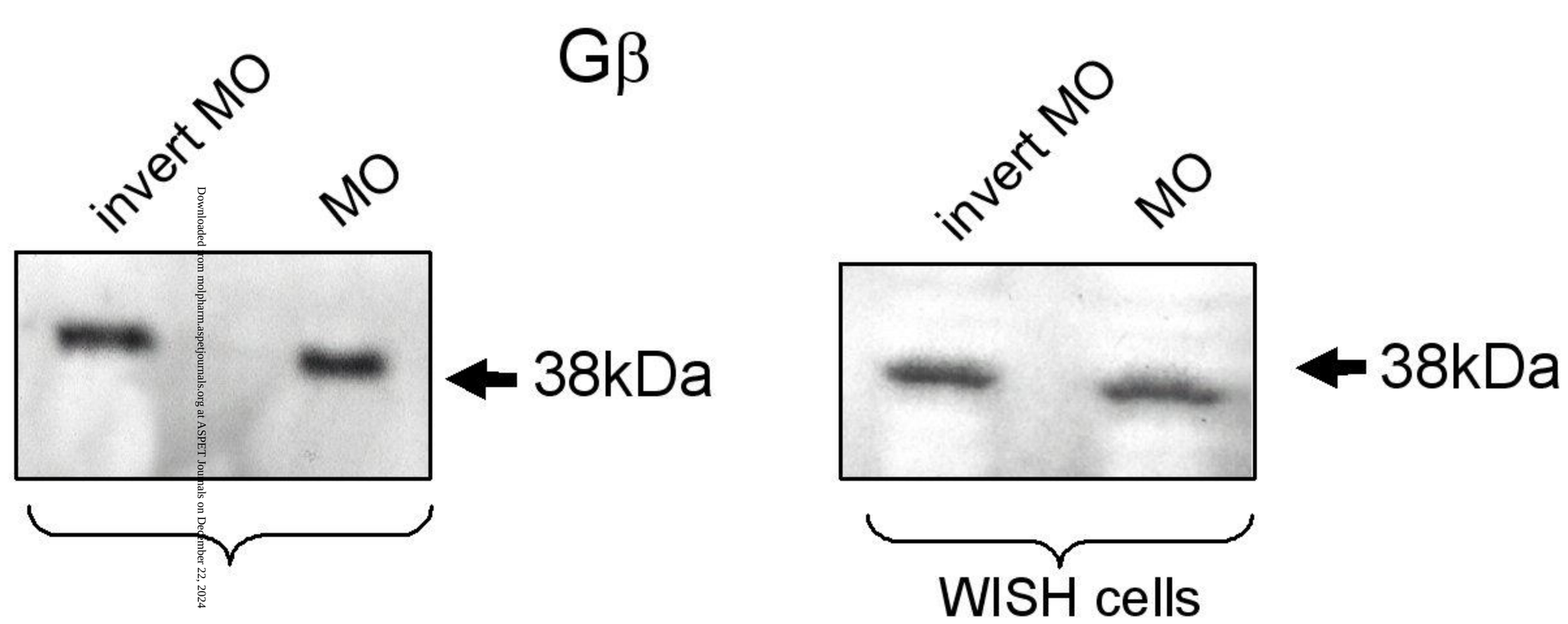

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Figure 5

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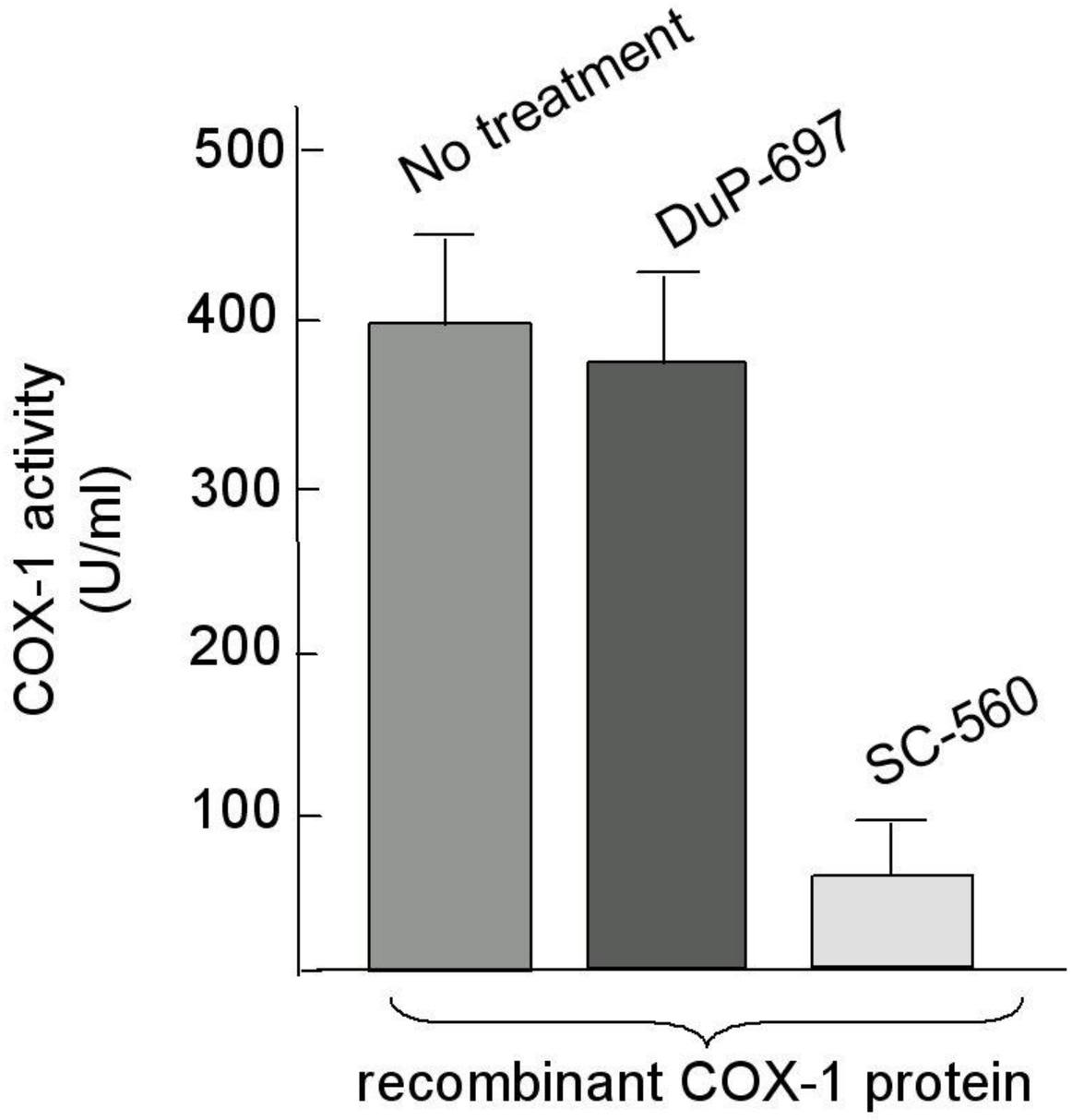

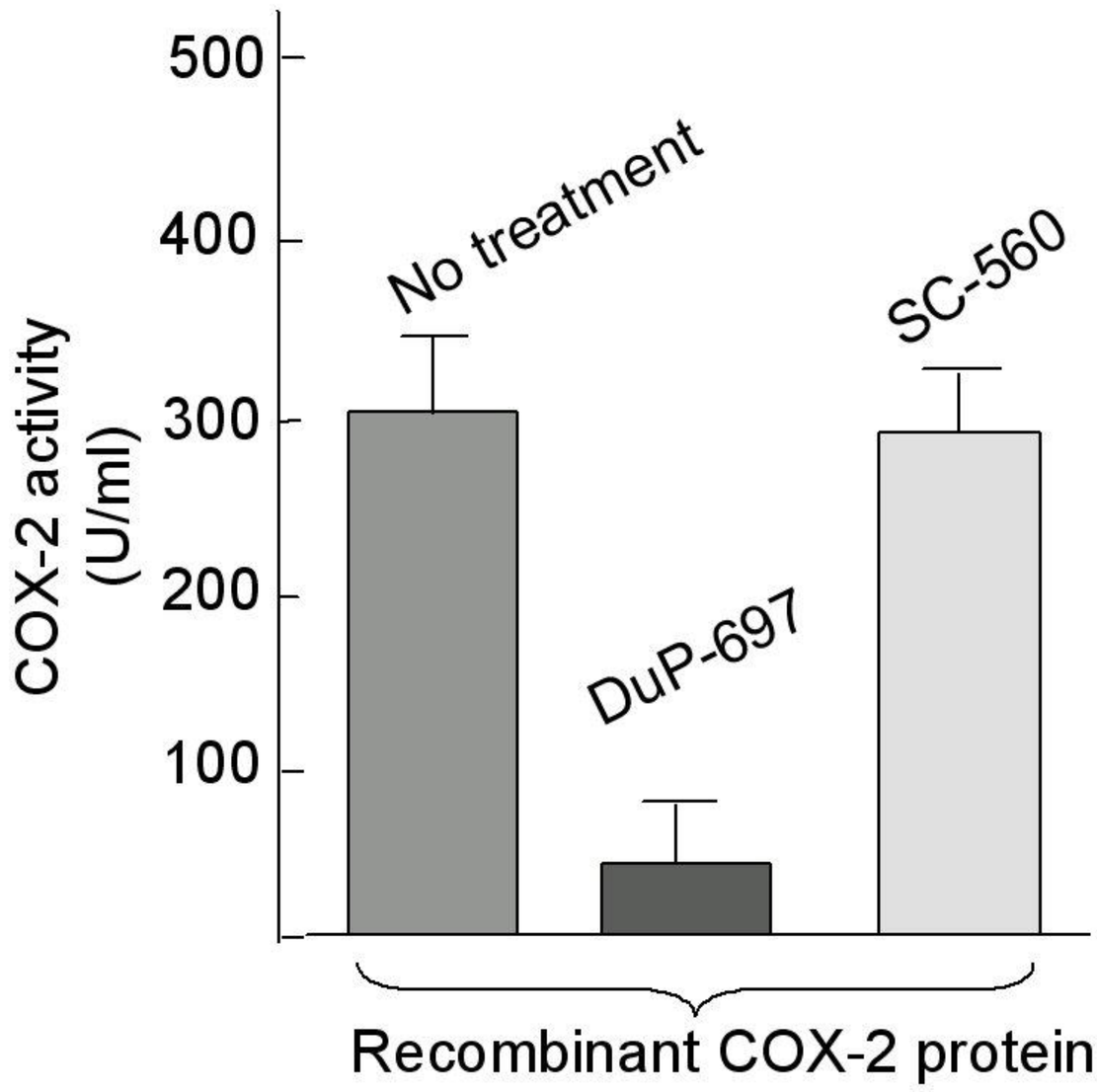

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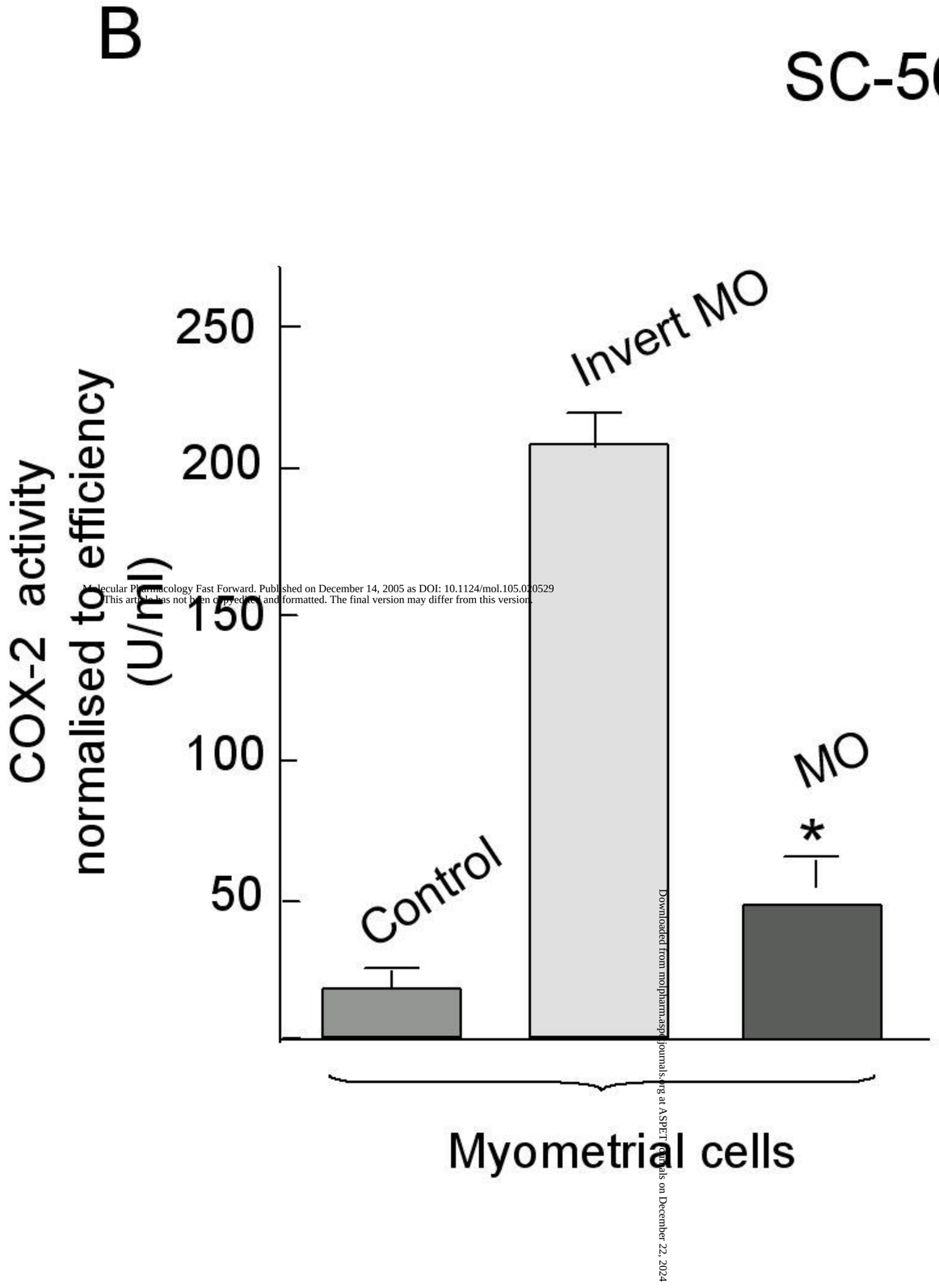

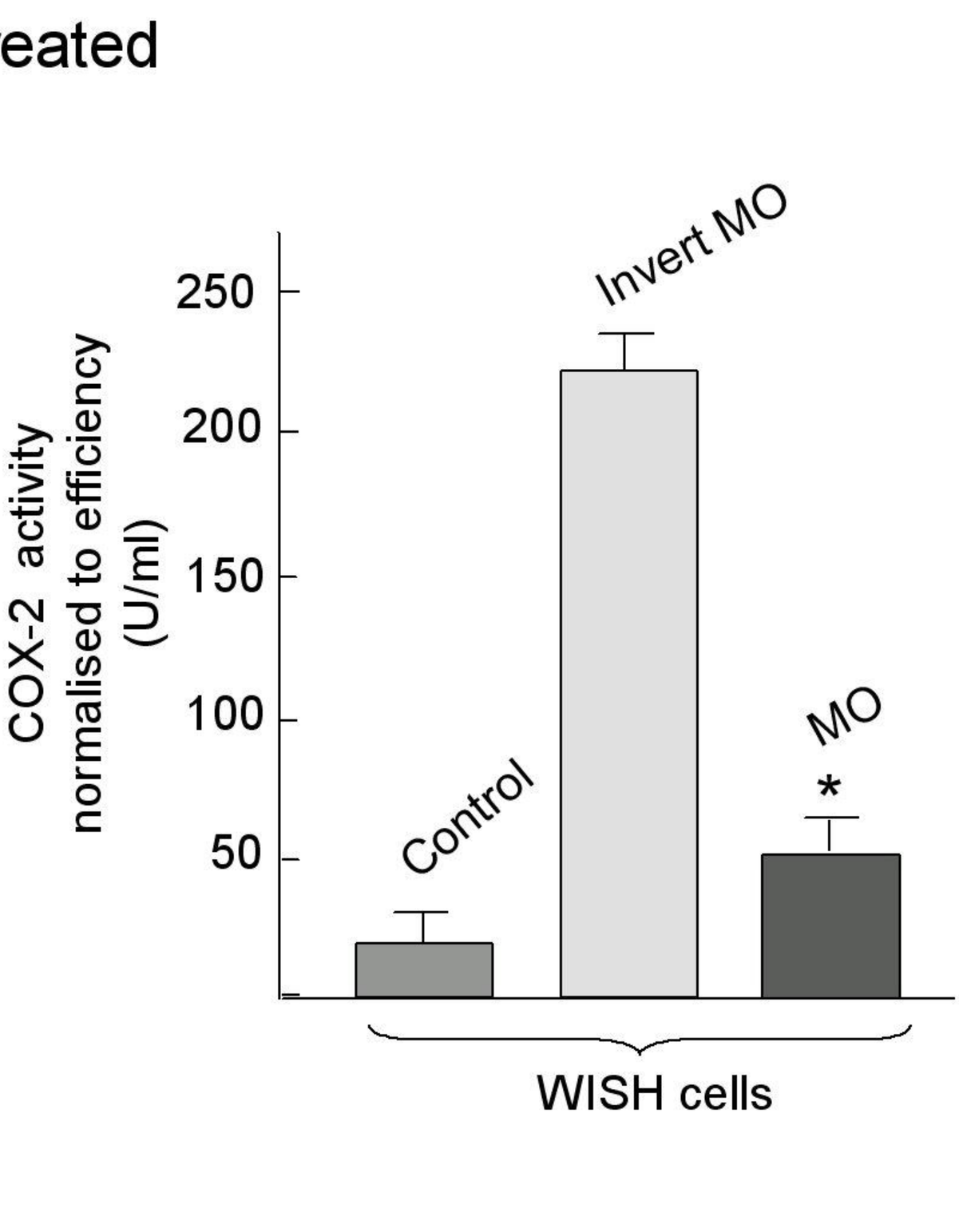

Figure 6