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CYCLOOXYGENASE-2 INHIBITORS DECREASE VASCULAR ENDOTHELIAL GROWTH FACTOR EXPRESSION IN COLON CANCER CELLS BY ENHANCED DEGRADATION OF Sp1 AND Sp4 PROTEINS

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Running Title: COX-2 inhibitors induce Sp protein degradation

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Non-Standard Abbreviations:
AP-1, activating protein-1
ATCC , American Type Culture Collection
Cel, celecoxib, 4-[5-(4-methylphenyl)-3-(trifluoromethyl)-1H pyrazol-1-yl]benzenesulfonamide
COX-2, cyclooxygenase 2
Coxib, COX-2 inhibitors
EMSA, electrophoretic mobility shift assay
FAP, familial adenomatous polyposis coli
FITC, fluorescein isothiocyanate-conjugated
GL2, luciferase
HIF-1α, hypoxia-inducible factor -1α
HRE, hypoxia responsive element
NM, nimesulfide, N-(4-nitro-2-phenoxyphenyl)methanesulfonamide
NS, NS-398, N-[2-(cyclohexyloxy)-4-nitrophenyl]methanesulfonamide
NSAIDs, non-steroidal anti-inflammatory drugs
PPARγ, peroxisome proliferator-activated receptor γ
RIPA , radioimmunoprecipitation assay
Sp, specificity protein
VEGF, vascular endothelial growth factor
VEGFR, vascular endothelial growth factor receptor

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ABSTRACT

Cyclooxygenase 2 (COX-2) inhibitors, such as celecoxib (Cel), nimesulfide (NM) and NS-398 (NS), and other non-steroidal anti-inflammatory drugs inhibit colon cancer growth and angiogenesis; however, the mechanism of this response is not well defined. Treatment of SW-480 colon cancer cells with Cel, NS-398 or NM decreased vascular endothelial growth factor (VEGF) mRNA and immunoreactive protein expression. This was also accompanied by decreased transactivation in cells transfected with constructs containing VEGF gene promoter inserts. Deletion analysis of the VEGF promoter indicated that decreased VEGF expression by COX-2 inhibitors was associated with the proximal -131 to -47 GC-rich region of the VEGF promoter which binds Sp proteins. Treatment of SW-480 cells with Cel, NM and NS also decreased Sp1 and Sp4, but not Sp2 or Sp3 protein expression. Similar results were observed in RKO, HT-29 and DLD colon cancer cells demonstrating comparable responses in COX-2 expressing and non-expressing colon cancer cell lines. COX-2 inhibitors do not affect Sp1 or Sp4 mRNA levels in SW-480 cells; however, decreased expression of both proteins was accompanied by increased protein ubiquitination and inhibited by the proteasome inhibitor gliotoxin. These results suggest that the antiangiogenic activity of COX-2 inhibitors in colon cancer cells is linked to activation of proteasome-dependent degradation of Sp1 and Sp4 proteins.

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INTRODUCTION

Vascular endothelial growth factor (VEGF) is an angiogenic protein that plays an important role in cellular homeostasis and is a key regulator of embryogenesis, the menstrual cycle, and wound repair (Shifren et al., 1996; Torry and Torry, 1997; Li et al., 2003; Carmeliet, 2003). VEGF and VEGF-like proteins stimulate formation of new blood vessels or neovascularization, and this process is also essential in carcinogenesis for tumor growth and metastasis (Folkman, 1995; Carmeliet, 2003; Bergers and Benjamin, 2003). VEGF and related angiogenic factors are overexpressed in multiple tumor types and often serve as negative prognostic indicators for disease-free survival of cancer patients (Maeda et al., 1999; Mineta et al., 2000; Linderholm et al., 2001). Expression of VEGF in a cohort of breast cancer patients correlated with mutant p53 and poor prognosis for recurrence-free survival (Linderholm et al., 2001). Other studies show that high levels of VEGF correlate with poor prognosis for patients with pancreatic cancer, squamous cell carcinoma, gastric carcinoma, and colon cancer (Maeda et al., 1999; Mineta et al., 2000; Linderholm et al., 2001).

Five members of the VEGF family have been identified (VEGF-A, -B, -C, -D and -F) and these proteins induce angiogenic responses through binding VEGF receptor-1 (VEGFR-1) (or Flk-1), VEGFR-2 (KDR/Flk-1), or VEGFR-3 (Flt-4) which are tyrosine kinases that initiate intracellular kinase signaling pathways (Ferrara et al., 2003). Hypoxia is a major pathway for regulation of VEGF in tumor and non-tumor tissue (Shweiki et al., 1992; Rossler et al., 1999; Ziemer et al., 2001), and this is due, in part, to upregulation of hypoxia-inducible factor -1α (HIF-1α). HIF-1α forms a transcriptionally-active HIF-1α - HIF-1β complex that interacts with a distal hypoxia responsive element (HRE) in the VEGF gene promoter. Enhanced VEGF expression in cells/tissues has also been linked to other factors including treatment with

cytokines, mitogens, activation of kinase signaling pathways, oxidative stress, and hormone stimulation (Finkenzeller et al., 1997; Milanini et al., 1998; Fukuda et al., 2003; Schafer et al., 2003; Stoner et al., 2004). Interestingly, enhanced VEGF stimulation by some of these factors involves direct or indirect activation of members of the specificity protein (Sp) family bound to one or more GC-rich motifs located in the proximal region of the VEGF promoter (Finkenzeller et al., 1997; Milanini et al., 1998; Schafer et al., 2003; Stoner et al., 2004). Platelet-derived growth factor-enhanced expression of VEGF in NIH3T3 cells involves Sp1 and Sp3 bound to the -85 to -50 region of the VEGF promoter (Finkenzeller et al., 1997). Oxidative stress-induced activation of kinases enhance VEGF expression in gastric cancer cells through increased Sp1-dependent activation of the same proximal GC-rich sites (Schafer et al., 2003). Research in this laboratory showed that hormone-induced VEGF expression in ZR-75 breast cancer cells required ERα/Sp1 and ERα/Sp3 interactions with the proximal GC-rich VEGF promoter elements (Stoner et al., 2004).

Recent studies have demonstrated that Sp1 and other Sp family proteins are required for endogenous expression of VEGF in pancreatic cancer cells (Shi et al., 2001; Abdelrahim et al., 2004), and the cyclooxygenase 2 (COX-2) inhibitor celecoxib also decreased VEGF expression in these cells by suppressing Sp1 activity (Wei et al., 2004). COX-2 inhibitors and other non-steroidal anti-inflammatory drugs (NSAIDs) inhibit development and growth of colon cancer, and this is due, in part, to their antiangiogenic activity (Williams et al., 1999; Gately and Li, 2004). In this study, we have investigated the effects of three COX-2 inhibitors, celecoxib (Cel), NS-398 (NS), and nimesulide (NM) on VEGF expression in colon cancer cells. The results show that all three COX-2 inhibitors decreased VEGF protein, mRNA and reporter gene expression, and this was accompanied by downregulation of both Sp1 and Sp4 but not Sp3 or

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Sp2 proteins. The effects of the COX-2 inhibitors on VEGF could also be duplicated in colon cancer cells transfected with small inhibitory RNAs for Sp1 and Sp4 confirming the role of these proteins in mediating VEGF expression. These results and studies with the proteasome inhibitor gliotoxin suggest that the antiangiogenic activity of COX-2 inhibitors in colon cancer may be related to decreased Sp1/Sp4 expression due to activation of proteasomes and represent a novel mechanism of action for this class of antitumor agents.

MATERIALS AND METHODS

Cell Lines, Chemicals, Biochemical, Constructs and Oligonucleotides. SW-480, DLD, RKO, and HT-29 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). DME/F12 with and without phenol red, 100X antibiotic/antimycotic solution was purchased from Sigma. Fetal bovine serum was purchased from Intergen (Purchase, NY). [γ-^{32}P]ATP (300Ci/mmol) was obtained from Perkin Elmer Life Sciences. Poly (dI-dC) and T4 polynucleotide kinase were purchased from Roche Molecular Biochemicals (Indianapolis, IN). Antibodies for Sp1, Sp2, Sp3, Sp4, ubiquitin, COX-2, β-tubulin and VEGF proteins were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Celecoxib (Cel) was a kind gift from Dr. Robert Newman (M.D. Anderson Cancer Center, Houston, TX.). Nimesulide (NM) and NS-398 (NS) were purchased from Alexis Biochemicals (San Diego, CA). Gliotoxin was kindly provided by Dr. Alan Taylor, National Research Council of Canada (Halifax, N.S.). Lysis buffer and luciferase reagent were obtained from Promega Corp. (Madison, WI). VEGF promoter constructs have previously been described (Stoner et al., 2004; Abdelrahim et al., 2004). siRNA duplexes for Sp1, Sp2, Sp3, Sp4, COX-2, luciferase (GL2), and the scrambled

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siRNA were prepared by Dharmacon Research (Lafayette, CO). The sequences of these siRNAs are shown in Table 1.

Transfection of Colon Cancer Cells and Preparation of Nuclear Extracts. Cells were cultured in 6-well plates in 2 ml of DME/F12 medium supplemented with 5% fetal bovine serum. After 16-20 h when cells were 50-60% confluent, iRNA duplexes and/or reporter gene constructs were transfected using Oligofectamine Reagent (Invitrogen, Carlsbad, CA). The effects of the selective COX-2 inhibitors (Coxib) and Sp siRNAs on transactivation was investigated in SW480 cells cotransfected with (500 ng) different VEGF constructs. Briefly, iRNA duplex was transfected in each well to give a final concentration of 50 nM. Cells were harvested 48 h and luciferase activity of lysates (relative to β-galactosidase activity) was determined. For COX-2 inhibitor study, cells were treated with DMSO (control) or with the indicated concentration of Celecoxib, Nimesulide and NS-398 for 24-30 h. For EMSA assay, nuclear extracts from SW480 cells were isolated as previously described and aliquots were stored at -80°C until used (Stoner et al., 2004; Abdelrahim et al., 2004).

Western Immunoblot. Cells were washed once with PBS and collected by scraping in 200 μL of lysis buffer [50 mM HEPES, 0.5 M sodium chloride, 1.5 mM magnesium chloride, 1 mM EGTA, 10% (v/v) glycerol, 1% Triton X-100, and 5 μL/ml of Protease Inhibitor Cocktail (Sigma). The lysates from the cells were incubated on ice for 1 h with intermittent vortexing followed by centrifugation at 40,000 *g* for 10 min at 4°C. Equal amounts of protein (60 μg) from each treatment group were diluted with loading buffer, boiled, and loaded onto 10 and 12.5% SDS-polyacrylamide gel. For VEGF immunoblots, 100 μg of protein was used. Samples were electrophoresed and proteins were detected by incubation with polyclonal primary antibodies Sp1 (PEP2), Sp2 (K-20), Sp3 (D-20), Sp4 (V-20), COX-2 (H-62), VEGF (A-20) and β-tubulin

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(H-235) followed by blotting with appropriate horseradish peroxidase-conjugated secondary antibody as previously described (Abdelrahim et al., 2004). After autoradiography, band intensities were determined by a scanning laser densitometer (Sharp Electronics Corporation, Mahwah, NJ) using Zero-D Scanalytics software (Scanalytics Corporation, Billerica, MA).

Electrophoretic Mobility Shift Assay (EMSA). VEGF oligonucleotides were synthesized and annealed, and 5-pmol aliquots were 5'-end-labeled using T4 kinase and [γ-^{32}P]ATP. A 30-µL EMSA reaction mixture contained ~100 mM KCl, 3 µg of crude nuclear protein, 1 µg poly(dI-dC), with or without unlabeled competitor oligonucleotide, and 10 fmol of radiolabeled probe. After incubation for 20 min on ice, antibodies against Sp1, Sp2, Sp3 or Sp4 proteins were added and incubated another 20 min on ice. Protein-DNA complexes were resolved by 5% polyacrylamide gel electrophoresis as previously described (Stoner et al., 2004; Abdelrahim et al., 2004). Specific DNA-protein and antibody-supershifted complexes were observed as retarded bands in the gel. GC- and GT-box sequence used in gel shift analysis are given below:

Immunocytochemistry. SW480 cells were seeded in Lab-Tek Chamber slides (Nalge Nunc International, Naperville, IL) at 100,000 cells/well in DME/F12 medium supplemented with 5% fetal bovine serum. Cells were then treated with Celecoxib, Nimesulide or NS-398, and after 27 h the media chamber was detached and the remaining glass slides were washed in Dulbecco's PBS. The immunostaining for Sp2, Sp3, and Sp4 was determined essentially as previously described for Sp1 (Abdelrahim et al., 2004). Briefly, the glass slides were fixed with cold (-20°C) methanol for 10 min, and then slides were washed in 0.3% PBS/Tween for 5 min (2X) before blocking with 5% goat serum in antibody dilution buffer (stock solution: 100 ml of PBS/Tween, 1 g of bovine serum albumin, 45 ml of glycerol, pH 8.0) for 1 h at 20°C. After removal of the blocking solution, Sp1, Sp2, Sp3 or Sp4 rabbit polyclonal antibodies were added

in antibody dilution buffer (1:200) and incubated for 12 h at 4°C. Blank panels were incubated with antibody dilution buffer only with no primary antibody. Slides were washed for 10 min with 0.3% Tween in 0.02 M PBS (3X) and incubated with fluorescein isothiocyanate-conjugated (FITC) goat anti-rabbit antibodies (1:1000 dilution) for 2 h at 20°C. Slides were then washed for 10 min in 0.3% PBS-Tween (4X). Slides were mounted in ProLonged antifading medium (Molecular Probes, Inc., Eugene, OR), and cover slips were sealed using Nailslicks nail polish (Noxell Corp., Hunt Valley, MD). For VEGF staining, rabbit polyclonal antibody (A-20) was used and mounting media with DAPI was used for nuclear counterstaining. Fluorescence imaging was performed using Carlzeiss Axiophoto 2 (Calzeiss, Inc., Thornwood, NY) and Adobe Photoshop 5.5 was used to capture the images.

Ubiquitinated Sp proteins immunoprecipitation. SW480 cells were seeded into 150-mm tissue culture plates in maintenance medium and allowed to grow to approximately 90% confluence. Cells were treated with Me_2SO, 30 μM Cel or 60 μM NS for 7 h. Whole cell extracts for each treatment group were obtained using radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCL, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA) with the addition of protease inhibitor cocktail. Duplicate aliquots of 500 μg were used for the experiments. Cell extracts were diluted in ice-cold PBS containing protease inhibitor cocktail to a final volume of 1 ml, followed by the addition of 30 μL of protein A/G PLUS-agarose beads (Santa Cruz). The reactions were placed on a rocker at 4°C for 3 h, followed by centrifugation at 600 *g* at 4°C for 5 min. A 900 μl aliquot of supernatant from each sample was transferred into a new Eppitube on ice. Rabbit polyclonal anti-Sp1 (1 μg), Sp4 (1 μg), Sp3 (1 μg), or normal rabbit IgG (1 μg) was added to either treatment set, followed by the addition of 30 μl of protein A/G PLUS-agarose beads. The samples were then placed on a rocker at 4°C for 12 h, followed

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by centrifugation at 600 *g* at 4°C for 5 min. The supernatant was removed by aspiration and the immunoprecipitates were washed with two cycles of 1 ml of ice-cold RIPA buffer followed by 1 ml of ice-cold PBS using centrifugation at 600 x *g* at 4°C for 5 min. The agarose pellet was resuspended in 50 µl of loading buffer, boiled, and centrifuged. The supernatant was separated by SDS-10% PAGE, electrophoresed to PVDF membrane. The PVDF membrane probed with ubiquitin antibody (P4D1), then stripped and reprobed with Sp1 or Sp3 antibodies. The same membrane was then stripped and reprobed with Sp4 antibody and visualized by ECL as described above.

Semiquantitative Reverse Transcription-PCR Analysis. SW480 cells were treated with DMSO (control) or with the indicated concentration of Celecoxib, Nimesulide and NS-398 for 30 h before total RNA collection. Total RNA was obtained with RNAzol B (Tel-Test, Friendswood, TX) according to the manufacturer's protocol. RNA concentration was measured by UV 260:280 nm absorption ratio, and 200 ng/µL RNA were used in each reaction for reverse transcription-PCR. RNA was reverse transcribed at 42°C for 25 min using oligo d(T) primer (Promega) and subsequently PCR amplified of reverse transcription product using 2 mmol/L $MgCl_2$, 1 µmol/L of each gene-specific primer, 1 mmol/L dNTPs, and 2.5 units AmpliTaq DNA polymerase (Promega). The gene products were amplified using 22 to 25 cycles (95°C, 30 s; 56°C, 30 s; 72°C, 30 s). The sequence of the oligonucleotide primers used in this study was as follows.

VEGF forward 5' – CCA TGA ACT TTC TGC TGT CTT – 3'
VEGF reverse 5' – ATC GCA TCA GGG GCA CAC AG – 3'

Sp1 forward 5' – GGAGTTGGTGGCAATAATGGG – 3'
Sp1 reverse 5' – TGGTTCTGTAAGTTGGGAGCGG – 3'

Sp4 forward 5' – CCAGCAGTAATAACGGGAGTGC – 3'

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Sp4 reverse 5' – AAGTAGTGGTGTTGGTGGGTGTG – 3'

GAPDH forward 5' – AATCCCATCACCATCTTCCA – 3'
GAPDH reverse 5' – GTCATCATATTTGGCAGGTT – 3'

Following amplification in a PCR express thermal cycler (Hybaid US, Franklin, MA), 20 μL of each sample were loaded on a 2% agarose gel containing ethidium bromide. Electrophoresis was performed at 80 V in 1xTAE buffer for 1 h, and the gel was photographed by UV transillumination using Polaroid film (Waltham, MA). VEGF, GAPDH, Sp1, and Sp4 band intensity values were obtained by scanning the Polaroid on a Sharp JX-330 scanner (Sharp Electronics, Mahwah, NJ); background signal was subtracted; and densitometric analysis was performed on the inverted image using Zero-D software (Scanalytics). Results were expressed as VEGF, Sp1 or Sp4 band intensity values normalized to GAPDH values and then by averaging three separate determinations for each treatment group.

Statistical Analysis. Statistical significance was determined by analysis of variance and Scheffe's test, and the levels of probability are noted. The results are expressed as means ± SD for at least three separate (replicate) experiments for each treatment.

RESULTS

SW-480 colon cancer cells express mutant K-ras and p53, and several COX-2 inhibitors inhibit growth and/or induce apoptosis in SW-480 colon cancer cell lines (Smith et al., 2000; Yamazaki et al., 2002). Although the effects of these compounds are variable, many of the same responses are observed in COX-2-expressing and non-expressing cell lines such as SW-480 cells (Williams et al., 1999; Smith et al., 2000; Yamazaki et al., 2002; Gately and Li, 2004). COX-2 inhibitors also inhibit angiogenesis in colon cancer cells (Gately and Li, 2004), and this study has

focused on the effects of the COX-2 inhibitors Cel, NS and NM on expression of the angiogenic protein VEGF in SW-480 cells. Results illustrated in Figure 1A show that Cel, NS and NM significantly decreased VEGF-A mRNA levels in SW-480 cells, and the effects of these compounds on VEGF protein were also investigated by immunostaining in the same cell line. Secreted VEGF exhibits a background cytosolic staining indicative of expression of this protein in SW-480 cells (Fig. 1B). However, after treatment with the COX-2 inhibitors for 27 h, immunostaining of VEGF in SW-480 cells was decreased by all three compounds. Western blot analysis also confirmed decreased expression of VEGF after treatment with Cel or NS (Fig. 1C). The VEGF promoter contains multiple *cis*-elements that may be involved in decreased VEGF expression in SW-480 cells treated with the COX-2 inhibitors and these include activating protein-1 (AP-1), AP-2, hypoxia responsive elements, and a cluster of proximal GC-rich sites at -131 to -52 that bind Sp proteins and Egr-1 (Finkenzeller et al., 1997; Stoner et al., 2004; Abdelrahim et al., 2004). Initial studies investigated the effects of Cel (30 μM), NS (60 μM) and NM (60 μM) on transactivation in SW-480 cells transfected with pVEGF-1, pVEGF-2 and pVEGF-3 constructs (Fig. 2A) which contain -2018 to +50, -131 to +54, and -66 to -54 VEGF gene promoter inserts (Figs. 2B - 2D). The COX-2 inhibitors decreased transactivation by 40-60% in cells transfected with the three constructs. The pattern of inhibition was similar for the three COX-2 inhibitors. A concentration-dependent decrease in luciferase activity in cells treated with the COX-2 inhibitors and transfected with pVEGF-2 indicated that their order of potency for decreasing transactivation was Cel > NS > NM (Fig. 2E).

Results of deletion analysis of the VEGF promoter indicated that luciferase activity was decreased by < 50% after deletion of the -2018 to -132 region of the promoter. In contrast, deletion of the cluster of proximal GC-rich sites -131 to -67 (i.e. pVEGF-2 vs. pVEGF-3)

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resulted in a > 85% decrease in luciferase activity and a similar decrease was observed (data not shown) after deletion of the -66 to -50 region which contains two additional GC-rich sites. Previous studies on VEGF/VEGF promoter expression in pancreatic cancer cells gave similar results and it was shown that Sp1, Sp3 and Sp4 were the major transcription factors required for VEGF expression (Abdelrahim et al., 2004). Moreover, a recent study indicated that Cel decreased VEGF expression in pancreatic cancer cells and this was linked to decreased Sp1 protein and phospho-Sp1/Sp1 protein ratios (Wei et al., 2004). Results in Figure 3A show that Sp1, Sp3 and Sp4 are expressed in SW-480 cells; moreover, we also identified Sp2 protein in SW-480 cells as previously reported in other cancer cell lines . All three COX-2 inhibitors significantly decreased Sp1 and p-Sp1 proteins; however, a significant decrease in p-Sp1/Sp1 ratio was only observed for NS. Sp4 protein expression was also decreased (Fig. 3E) in a manner parallel to that of Sp1, whereas levels of Sp2 and Sp3 were not affected by Cel, NS or NM (Figs. 3C and 3D). Nuclear extracts from SW-480 cells were incubated with ^{32}P-labeled (-66/-47) which contains the two proximal VEGF promoter GC-rich sites (Fig. 4A) and a series of retarded bands were formed (lane 2), whereas in the absence of protein, retarded bands were not observed (lane 1). Incubation with non-specific IgG (lane 3) or Sp2 antibodies (lane 5) did not affect the retarded band. Antibodies to Sp1 (lane 4), Sp3 (lane 6) and Sp4 (lane 7) supershifted specific retarded bands (as indicated by an arrow) and 100-fold excess unlabeled (-66/-47) decreased retarded band intensities (lane 8). This same ^{32}P-labeled oligonucleotide was incubated alone (lane 1) or with nuclear extracts from untreated SW-480 cells (lanes 2 and 6) and from cells treated with Cel (lanes 3 - 5), and NS (lanes 7 - 9). The only consistent decrease that was observed in gel mobility shift assays was the retarded band associated with Sp1 in the high dose treatment groups. This was due, in part, to the relatively intense (and overlapping) Sp3-

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DNA bands which are not affected by the COX-2 inhibitors. Confirmation that the COX-2 inhibitors downregulated Sp1 and Sp4, but not Sp2 and Sp3 proteins, was confirmed in immunostaining experiments in SW-480 cells (Fig. 5). The blank panels were stained only with the secondary antibody in the presence (right) or absence (left) of the DAPI nuclear stain. All four proteins exhibited nuclear staining and decreased staining intensities of Sp1 and Sp4 (but not Sp2 and Sp3) were observed after tretment with Cel, NS and NM.

These results suggest that COX-2 inhibitors specifically decrease Sp1 and Sp4 expression and previous studies in pancreatic cancer cells show that Sp1, Sp4 and Sp3 play a critical role in VEGF expression (Abdelrahim et al., 2004). This was further investigated in SW-480 cells transfected with siRNA for Sp1 (iSp1), Sp2 (iSp2), Sp3 (iSp3), and Sp4 (iSp4) (Fig. 6A) which resulted in > 50% knockdown of their targeted protein based on analysis of whole cell lysates of transfected cells. In cells transfected with siRNA for Sp proteins and pVEGF-2, it was apparent that decreased expression of Sp1, Sp3 and Sp4 decreased luciferase activity. These data confirmed that downregulation of Sp1 and Sp4 by the COX-2 inhibitors was responsible for decreased VEGF expression in SW-480 cells; moreover, overexpression of Sp1 or Sp4 induced transactivation in cells transfected with pVEGF-2 and this response was inhibited by Cel (Figs. 6C-6E). It was also shown that iSp3, but not iSp2, inhibited transactivation (Fig. 6B), and overexpression of Sp3 also enhanced luciferase activity and treatment with Cel decreased this response (Fig. 6E). These results confirm that Sp proteins play an important role in regulating VEGF expression in SW-480 cells and therefore, the antiangiogenic responses observed for COX-2 inhibitors is due to their inhibition of Sp1 and Sp4 protein expression in this cell line.

The effects of Cel and NS on Sp1, Sp3 and Sp4 protein levels were also determined in RKO, HT-29 and DLD cells which were treated for 30 h with 30 and 60 μM of the COX-2

inhibitors, respectively (Fig. 7). COX-2 was expressed in these cells (Fig. 7A). The role of Sp proteins in regulating VEGF in COX-2 overexpressing HT-29 cells was investigated in cells transfected with pVEGF-2, iScr (non-specific), iGL2 (luciferase), and iSp1, iSp2, iSp3 and iSp4 as well as iCOX-2 (Fig. 7B). The results were similar to those observed in SW-480 cells (Fig. 6A) and demonstrate that Sp1, Sp3 and Sp4 are critical transcription factors required for VEGF expression in HT-29 cells. iCOX-2 significantly decreased COX-2 protein in HT-29 cells but did not affect transactivation in cells transfected with pVEGF2. Moreover, both compounds also decreased Sp1 and Sp4 proteins and luciferase activity in RKO, HT-29 and DLD cells transfected with pVEGF-2 (Fig. 7C), and this was similar to data observed in SW-480 cells (Fig. 2). These results demonstrate that COX-2 inhibitors specifically decrease Sp1 and Sp4 protein expression in several colon cancer cell lines, and this also resulted in decreased VEGF expression.

The effects of these compounds on VEGF expression were not related to decreased p-Sp1/Sp1 ratios, and NS and NM did not decrease Sp1 or Sp4 mRNA levels (Figs. 8A and 8B), suggesting a post-transcriptional effect on Sp1 and Sp4 protein expression. Preliminary studies showed that proteasome inhibitors, such as MG-132 and lactacystin, were cytotoxic in SW-480 cells; however, gliotoxin, a fungal metabolite that inhibits chymotrypsin-like activity of the 20S proteasome, significantly inhibited NS-induced degradation of Sp1 and Sp4 (Fig. 8C) and also inhibited the NS-dependent decrease in luciferase activity in SW-480 cells transfected with pvEGF2 (Fig. 8D). Thus, the antioangiogenic activity of COX-2 inhibitors is associated with selective activation of proteasome-dependent degradation of Sp1 and Sp4 proteins.

Proteasome-dependent degradation of proteins is preceded by ubiquitination of the targeted protein or increased conjugation with an ubiquitin-like protein. The effects of Cel and

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NS on ubiquitination of Sp1, Sp3 and Sp4 were therefore investigated in SW-480 cells (Fig. 9) in which whole cell lysates from each treatment group were initially immunoprecipitated with IgG, or antibodies to Sp1, Sp3 or Sp4. Immunoprecipitated fractions were separated by SDS-PAGE and immunoblotted with ubiquitin antibodies (Fig. 9A), and multiple bands associated with ubiquitinated proteins were observed in lysates from NS or Cel-treated cells and immunoprecipitated with Sp1 or Sp4 (but not Sp3) antibodies. The multiple bands were also not observed in solvent (D) treated lysates; in addition an unidentified low molecular weight band was observed in the D, Cel and NS lysates immunoprecipitated with Sp1, Sp3 or Sp4 antibodies but not with IgG. The membrane was then stripped and probed first with Sp1 antibodies (Fig. 9B) and without stripping, the membrane was reprobed with Sp3 antibodies (Fig. 9C) which shows both Sp1 and Sp3 proteins. The membrane was then stripped and reprobed with Sp4 antibodies (Fig. 9D) which appears as primarily a single band on the gel. These latter experiments (Fig. 9B - 9D) were used as controls to confirm the initial Sp antibody immunoprecipitation. The results confirm that COX-2 inhibitor-induced degradation of Sp1 and Sp4 is accompanied by enhanced protein ubiquitination, and this is consistent not only with the observed degradation of these proteins, but the inhibition of this response by the proteasome inhibitor gliotoxin (Fig. 8C and 8D).

DISCUSSION

COX-2 is one of the rate limiting steps in the metabolism of arachidonic acid to prostaglandins and thromboxanes, and COX-2 inhibitors which include a broad spectrum of NSAIDs are extensively used as anti-inflammatory agents (Williams et al., 1999). COX-2 is also overexpressed in multiple tumor types including colon cancer, and both NSAIDs and COX-2

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inhibitors have been investigated for cancer chemoprevention and chemotherapy (Taketo, 1998a; Taketo, 1998b; Williams et al., 1999; Gately and Li, 2004). Several studies show that some COX-2 inhibitors including aspirin decrease the incidence and/or mortality rate of human colorectal cancer (Martinez et al., 1995; Giovannucci et al., 1995; Peleg et al., 1996). The COX-2 inhibitor, sulindac, has also been successfully used for treating patients with familial adenomatous polyposis coli (FAP), a hereditary colon cancer syndrome in which there is rapid and early development of polyps and tumors (Labayle et al., 1991; Nugent et al., 1993; Giardiello et al., 1993). COX-2 inhibitors are also highly effective chemotherapeutic agents for treating colon cancer in laboratory animals including carcinogen-induced and transgenic rodent models (Pereira et al., 1994; Boolbol et al., 1996; Chiu et al., 1997).

The mechanisms of COX-2 inhibitor-mediated cancer chemoprevention and chemotherapy are complex, and the inhibition of prostaglandin/thromboxane production (e.g. prostaglandin E2) contributes to these effects (Taketo, 1998a; Taketo, 1998b; Williams et al., 1999; Gately and Li, 2004). However, it is also clear that COX-2 inhibitors can be effective in cells with minimal COX-2 expression and many cell growth inhibitory responses induced by these compounds are COX-2-independent (Smith et al., 2000; Yamazaki et al., 2002; Liu et al., 2002). For example, sulindac sulfone induces apoptosis in Caco-2 cells by decreasing polyamine levels through activation of peroxisome proliferator-activated receptor γ (PPARγ)-dependent induction of spermidine/spermine N-acetyltransferase gene expression (Liu et al., 2002).

The cancer chemotherapeutic action of COX-2 inhibitors have also been linked to their antiangiogenic action and inhibition of VEGF expression (Williams et al., 1999; Gately and Li, 2004). COX-2 catalyzes the biosynthesis of PGE2 which induces HIF-1α; this transcription factor forms a heterodimer that interacts with the HRE in the VEGF promoter to activate VEGF

gene expression (Masferrer et al., 2000; Fukuda et al., 2003). In this study, we initially used SW-480 cells as a model for investigating the mechanisms of VEGF inhibition by COX-2 inhibitors in COX-2-negative (or under-expressing) cells. Cel, NS and NM clearly inhibited VEGF mRNA levels (Fig. 1A) and cellular VEGF (Fig. 1B), and this paralleled their inhibitory effects on luciferase activity in SW-480 cells transfected with VEGF constructs (pVEGF-1-3) (Fig. 2). Moreover, using the pVEGF constructs, we also observed comparable decreased transactivation in RKO, DLD and HT-29 cells (Fig. 7C), and these cell lines exhibited highly variable expression of COX-2 protein (Fig. 7B). The results demonstrate that COX-2 inhibitors downregulate VEGF and this response is associated with the proximal -131 to -47 GC-rich region of the VEGF promoter which binds Sp proteins (Stoner et al., 2004; Abdelrahim et al., 2004).

A recent study in pancreatic cancer cells reported that treatment of Panc-1 cells with Cel decreased VEGF expression *in vitro* and *in vivo*, and transactivation studies in cells transfected with VEGF constructs indicated that the proximal GC-rich sites were associated with this response (Wei et al., 2004). It was concluded that decreased Sp1 and pSp1/Sp1 ratios were important determinants for this antiangiogenic response in pancreatic cancer. Results of this study in colon cancer cell lines also confirms that COX-2 inhibitors downregulated Sp1 (Figs. 3 - 5) but do not consistently affect pSp1/Sp1 ratios (Fig. 3B). Moreover, it was also apparent that COX-2 inhibitors also induced downregulation of Sp4 protein in colon cancer cells, whereas Sp3 and Sp2 protein levels ere unaffected (Figs. 3 - 5). These results suggest that the effects of COX-2 inhibitors on VEGF expression in colon cancer cells was related to their downregulation of Sp1 and Sp4, and previous studies in pancreatic cancer cells have demonstrated that VEGF expression is regulated by Sp1, Sp4 as well as Sp3 (Sp2 was not investigated) (Abdelrahim et al.,

2004). This was also observed in colon cancer cells since RNA interference assays with iSp1, iSp2, iSp3 and iSp4 showed that transactivation in SW-480 and HT-29 cells transfected with pVEGF-2 was decreased by iSp1, iSp4 and iSp3, but not iSp2 (Figs. 6B and 7B). Moreover, overexpression of Sp1, Sp3 or Sp4 enhanced transactivation in SW-480 cells transfected with pVEGF-2 (± Cel) (Fig. 6C). These results show that VEGF expression in colon cancer cells is primarily dependent on Sp1, Sp4 and Sp3 as previously reported in pancreatic cancer cells (Abdelrahim et al., 2004), and the antiangiogenic activity of COX-2 inhibitors is linked to induced degradation of these transcription factors. Interestingly, treatment of SW-480 cells with NS or NM did not decrease Sp1 or Sp4 mRNA levels (Fig. 8), and gliotoxin, a specific inhibitor of the 20S proteasome complex (Kroll et al., 1999), inhibits NS-induced degradation of both Sp1 and Sp4 and transactivation in SW-480 cells transfected with pVEGF2 (Fig. 8). Moreover, whole cell lysates from SW-480 cells treated with Cel or NS also exhibit several ubiquitinated bands immunoprecipitated with Sp1 or Sp4 (but not Sp3) antibodies (Fig. 9A). These results now provide a mechanistic basis for the antiangiogenic activity of COX-2 inhibitors in colon cancer cells where these drugs selectively activate proteasome-dependent degradation of Sp1 and Sp4 proteins. Current studies are focused on potential applications of COX-2 inhibitors alone and in combination with other drugs for treatment of colon and possibly other cancers. In addition, we are also investigating other related compounds that also induce degradation of Sp3 protein and thereby enhance their antiangiogenic activities.

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FOOTNOTES

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Figure Captions

Figure 1. Effects of COX-2 inhibitors on VEGF expression in SW-480 cells. [A] VEGF mRNA levels. SW-480 cells were treated with DMSO, 30 μM Cel, 60 μM NS or 60 μM NM for 30 h and mRNA levels were determined by semiquantitative RT-PCR as described in the Materials and Methods. Significantly ($p < 0.05$) decreased mRNA levels are indicated (*), and results are expressed as means ± SD for three replicate determinations for each treatment group. [B] Immunostaining of VEGF. SW-480 cells were treated with COX-2 inhibitors (as described in [A]) for 30 h and cells were immunostained with VEGF antibody as described in the Materials and Methods. VEGF staining (green) was diffuse in the cytosol, and nuclei (blue) were stained with DAPI. [C] Western blot analysis. Cells were treated as described in [B] and VEGF was analyzed by Western blot analysis as described in the Materials and Methods. Significantly ($p < 0.05$) decreased VEGF protein in the treatment groups (relative to Me_2SO) is indicated by an asterisk, and results are expressed as means ± SD for 3 replicate determinations for each treatment group.

Figure 2. Effects of COX-2 inhibitors on transactivation in SW-480 cells transfected with different pVEGF constructs [A], namely, pVEGF-1 [B], pVEGF-2 [C] or pVEGF-3 [D]. Cells were transfected with pVEGF-1, pVEGF-2 or pVEGF-3, treated with DMSO, 30 μM Cel, 60 μM NM or 60 μM NS, and luciferase activity determined as described in the Materials and Methods. Significantly ($p < 0.05$) decreased activity is indicated (*), and results are expressed as means ± SD for three replicate determinations for each treatment group. [E] Concentration-dependent decrease in luciferase activity. Cells were transfected with pVEGF-2, treated with different

concentrations of COX-2 inhibitors, and luciferase activity determined as described in the Materials and Methods. Results are expressed as means ± SD as described above and significance ($p < 0.05$) induction was observed for Cel (≥ 20 μM), NS (≥ 40 μM) and NM (≥ 40 μM).

Figure 3. COX-2 inhibitors downregulate Sp1 and Sp4 proteins. [A] Western blot analysis of Sp proteins. SW-480 cells were treated with 30 μM Cel or 60 μM NM or NS for 30 h, and Sp proteins were analyzed by Western blot analysis of whole cell lysates as described in the Materials and Methods. Quantitation of Sp1 [B], Sp2 [C], Sp3 [D] and Sp4 [E] proteins. Levels of Sp proteins relative to DMSO were immunoquantitated from the Western blots and significantly ($p < 0.05$) decreased protein levels are indicated. Results are expressed as means ± SD for three replicate determinations of reach treatment group.

Figure 4. COX-2 inhibitor-dependent decrease of Sp1/Sp4-DNA binding. [A] Binding of Sp proteins to ^{32}P-VEGF(-66/-47) and antibody supershifts. ^{32}P-VEGF(-66/-47) was incubated with nuclear extract and Sp protein antibodies and separated by electrophoresis as described in the Materials and Methods. Sp1-DNA complexes and supershifted bands are indicated by arrows. [B] Assay of Sp protein-DNA complexes using nuclear proteins from SW-480 cells treated with COX-2 inhibitors. Nuclear extracts from various treatment groups were analyzed as described in [A] and Sp-DNA complexes are indicated (arrows).

Figure 5. Immunocytochemical analysis of Sp proteins. SW-480 cells were treated with DMSO, 30 μM Cel, or 60 μM NS or NM for 24 h, and immunostained with antibodies for Sp1, Sp2, Sp3 and Sp4 proteins as described in the Materials and

Methods. The blank panels represent staining only with the secondary antibody in presence (right) or absence (left) of DAPI. All Sp proteins were localized in the nucleus, and decreased Sp1 and Sp4 (but not Sp2 or Sp3) staining was observed in replicate experiments.

Figure 6. RNA interference decreases activation of VEGF and Sp protein expression. [A] Decreased Sp protein expression. SW-480 cells were transfected with iScr (non-specific), iSp1, iSp2, iSp3 or iSp4, and whole cell lysates were analyzed by Western blot analysis. Significantly ($p < 0.05$) decreased levels of Sp proteins compared to levels in cells transfected with iScr are indicated (*), and results are expressed as means ± SD for three replicate determinations. [B] Effects of RNA interference on transactivation of pVEGF-2. SW-480 cells were transfected with pVEGF-2 and small inhibitory RNA, and luciferase activities were determined as described in the Materials and Methods. Results are expressed as means ± SD for three replicate determinations for each treatment group, and significant ($p < 0.05$) inhibition of luciferase activity is indicated (*). Sp1 [C], Sp3 [D] and Sp4 [E] expression plasmids increase transactivation in cells transfected with pVEGF-2 and treated with DMSO or 30 μM Cel. Cells were transfected with pVEGF-2, different amounts of Sp expression plasmid, treated with DMSO or 30 μM Cel, and luciferase activity determined as described in the Materials and Methods. Sp1, Sp3 and Sp4 expression plasmids (≥ 5 μg) significantly ($p < 0.05$) increased activity and 30 μM Cel significantly decreased activity in cells transfected with 0 - 1 μg Sp expression plasmid. Results are expressed as means ± SD for three replicate determinations for each treatment group.

Figure 7. Effects of COX-2 inhibitors on VEGF/Sp proteins in RKO, DLD and HT-29 colon cancer cells. [A] Western blot analysis of COX-2 proteins. Cells were treated with DMSO (D), 30 μM Cel or 60 μM NS for 30 h, and whole cell lysates were analyzed for Sp proteins and COX-2 by Western blot analysis. [B] Effects of Sp and COX-2 protein knockdown by RNA interference on transactivation in HT-29 cells transfected with pVEGF-2. HT-29 cells were transfected with pVEGF-2 and small inhibitory RNAs, and luciferase activities were determined as described in the Materials and Methods. Results are expressed as means ± SD for three replicate determinations for each treatment group, and significant ($p < 0.05$) inhibition of luciferase activity is indicated (*). [C] COX-2 inhibitors decreased transactivation in colon cancer cells transfected with pVEGF2. Cells were transfected with pVEGF-2, treated with DMSO, 30 μM Cel or 60 μM NS for 30 h, and transactivation determined as described in the Materials and Methods. Sp protein expression in these cell lines was also determined by Western blot analysis. Results are expressed as means ± SD for three replicate experiments for each treatment group and significant ($p < 0.05$) inhibition of luciferase activity in the treatment groups compared to solvent control (Me_2SO) is indicated (*). N.S. represent non-specific proteins which are used as loading controls to compare intensities of the COX-2 bands.

Figure 8. COX-2 inhibitors activate proteasome-dependent degradation of Sp1 and Sp4. [A and B] Effects of NS and NM on mRNA levels. SW-480 cells were treated with 60 μM NS or NM for 20 h, and Sp1 and Sp4 mRNA levels were determined by semiquantitative RT-PCR as described in the Materials and Methods. Results are expressed as means ± SD for three replicate determinations for each treatment group.

[C] Gliotoxin inhibits downregulation of Sp1/Sp4 proteins by NS. SW-480 cells were treated with DMSO, 60 μM NS alone or in combination with 3 μM gliotoxin for 20 h, and whole cell lysates were isolated by Western blot analysis as described in the Materials and Methods. [D] Gliotoxin inhibits effects of NS on transactivation in SW-480 cells transfected with pVEGF-2. Cells were transfected with pVEGF-2, treated with DMSO, 60 μM NS alone or in combination with 3 μM gliotoxin, and luciferase activity determined as described in the Materials and Methods. Results in [C] and [D] are expressed means ± SD for three replicate determinations for each treatment group. A significant ($p < 0.05$) decrease in Sp1 or Sp4 proteins or luciferase activity by NS is indicated (*) and inhibition of these responses by gliotoxin is also indicated (**).

Figure 9. Cel and NS induce ubiquitination of Sp proteins. [A] Ubiquitination of Sp1 and Sp4. Whole cell lysates from different treatment groups were immunoprecipitated with IgG and antibodies to Sp1, Sp3 or Sp4, and immunoprecipitates were separated by SDS-PAGE and immunoblotted with ubiquitin antibodies as described in the Materials and Methods. Western blot analysis of Sp1 [B], Sp3 [C] and Sp4 [D]. After analysis for ubiquitinated proteins [A], the gel was stripped and reprobed for Sp1 and Sp3 without stripping as described in the Materials and Methods. The gel was then stripped and probed for Sp4 protein in Western immunoblot analysis as described in the Materials and Methods. This approach was required to ensure that the Sp1 proteins could be sequentially analyzed on the same gel. Total cell extracts were also analyzed separately for ubiquitinated proteins and Sp1, Sp3 and Sp4 proteins.

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Table 1. Summary of siRNAs used in this study.

Gene	siRNA Duplex
GL2	5' – CGU ACG CGG AAU ACU UCG ATT –3'
	3' – TT GCA UGC GCC UUA UGA AGC U – 5'
Scramble VIII	5' – ACU CUA UCU GCA CGC UGA CTT – 3'
	3' – TT UGA GAU AGA CGU GCG ACU G – 5'
Sp1	5' – AUC ACU CCA UGG AUG AAA UGA TT – 3'
	3' – TT UAG UGA GGU ACC UAC UUU ACU – 5'
Sp2	5' – GGA AAU AAC CUG CUC AUU GTT – 3'
	3' – TT CCU UUA UUG GAC GAG UAA C – 5'
Sp3	5' – GCG GCA GGU GGA GCC UUC ACU TT – 3'
	3' – TT CGC CGU CCA CCU CGG AAG UGA – 5'
Sp4	5' – GCA GUG ACA CAU UAG UGA GCTT – 3'
	3' – TT CGU CAC UGU GUA AUC ACU CG – 5'
COX-2	5' – CUG CUC AAC ACC GGA AUU UTT – 3'
	3' – TT GAC GAG UUG UGG CCU UAA A– 5'

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Figure 1

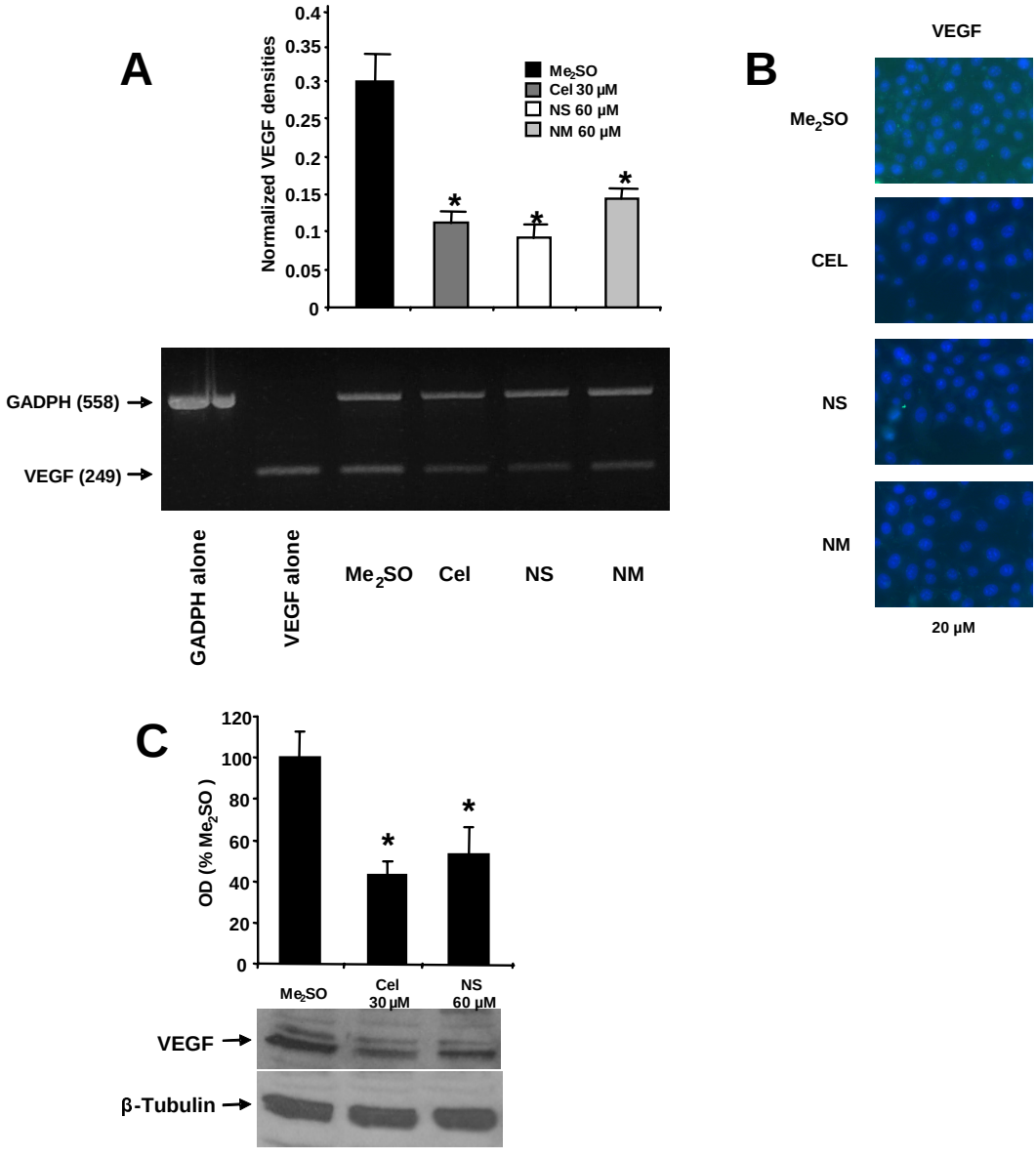

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Figure 2

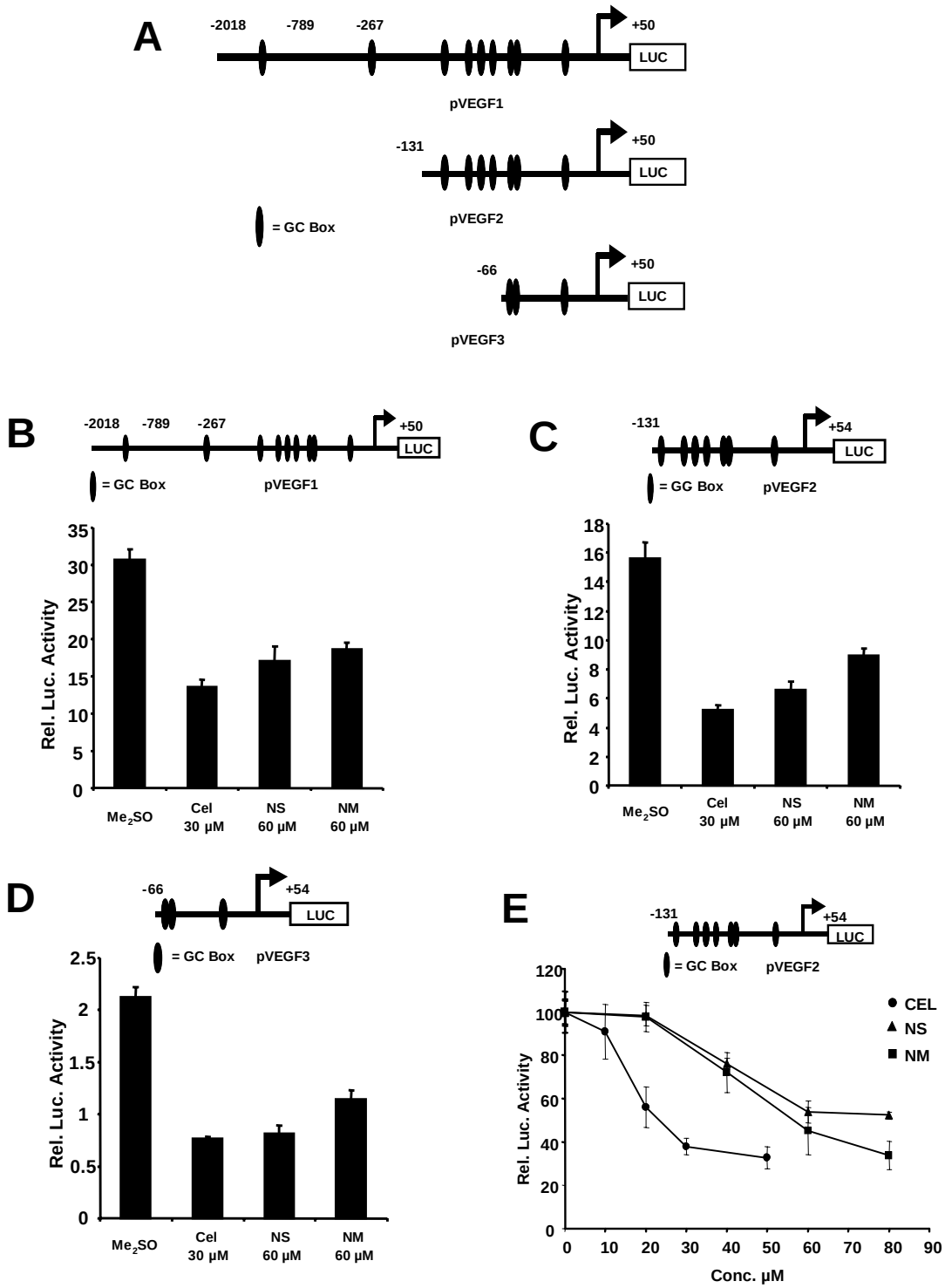

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Figure 3

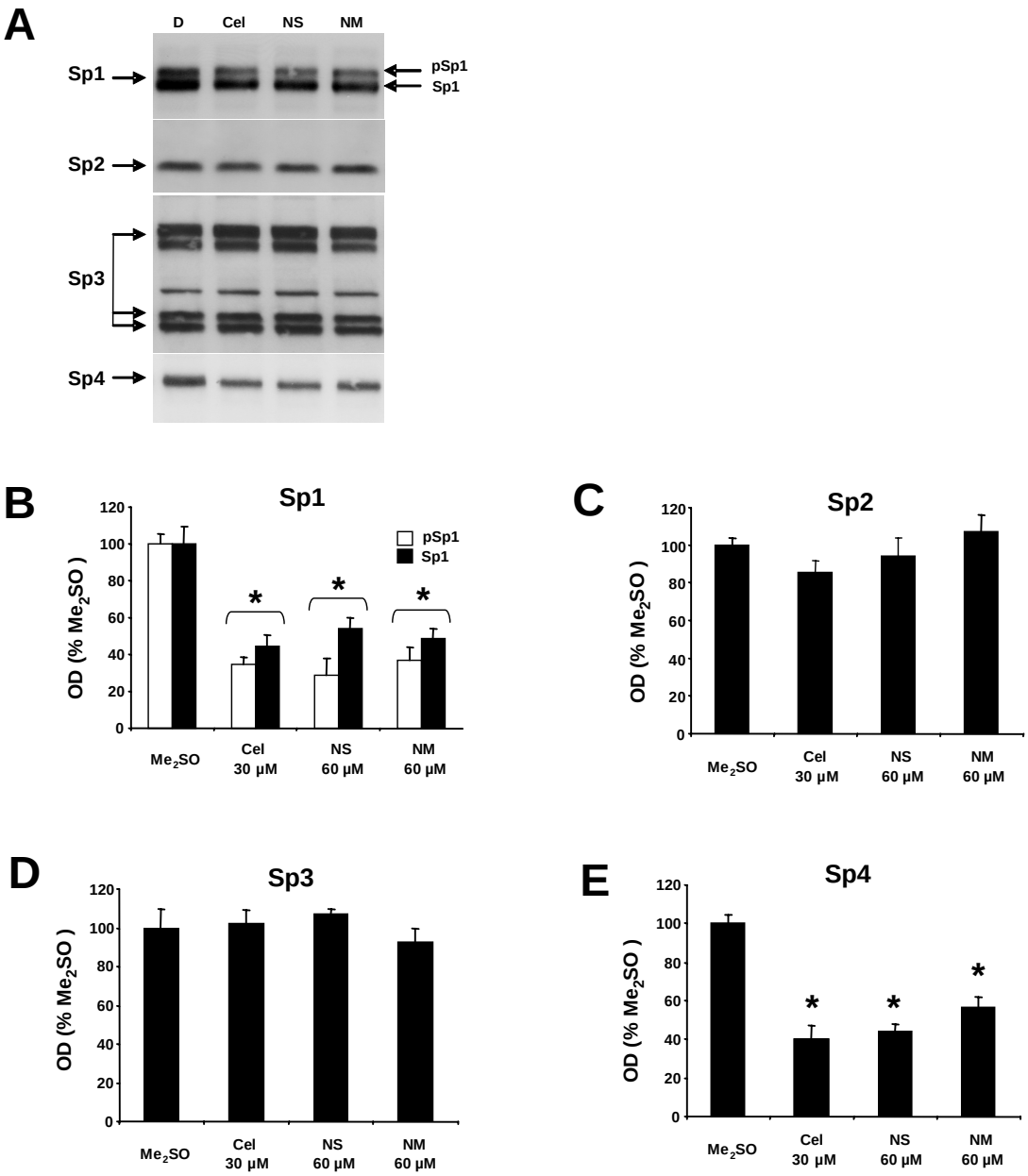

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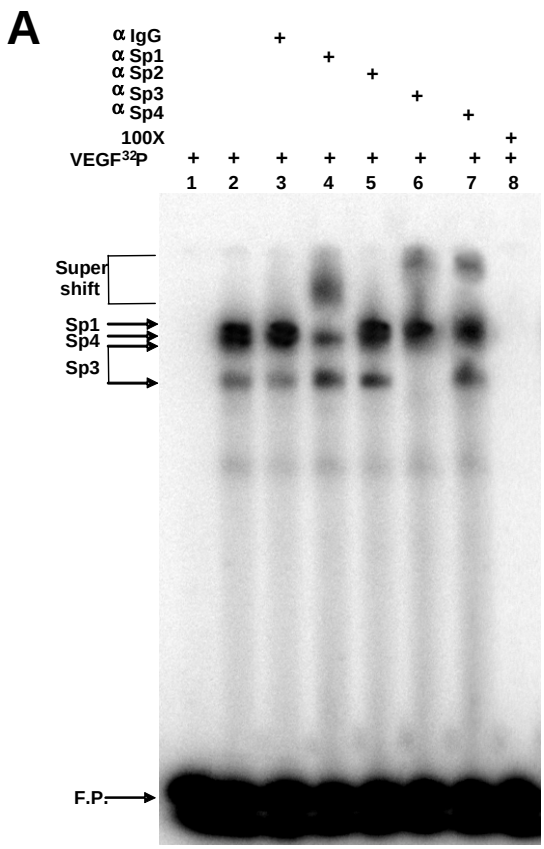
Figure 4



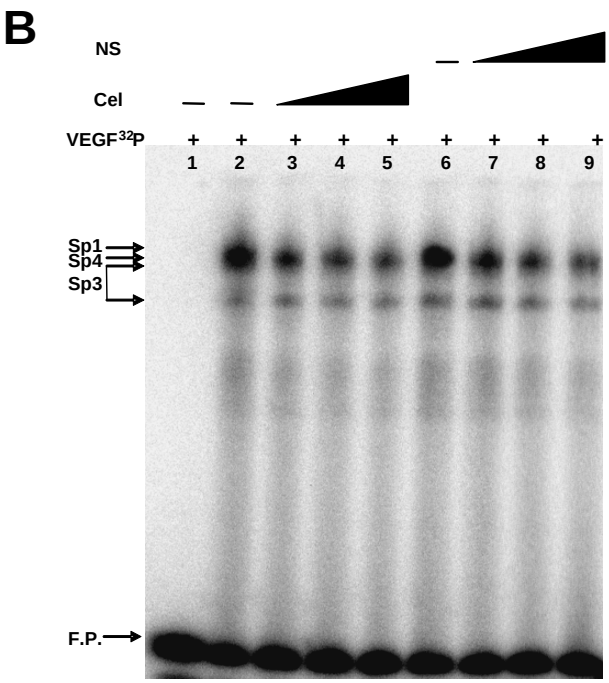


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Figure 5

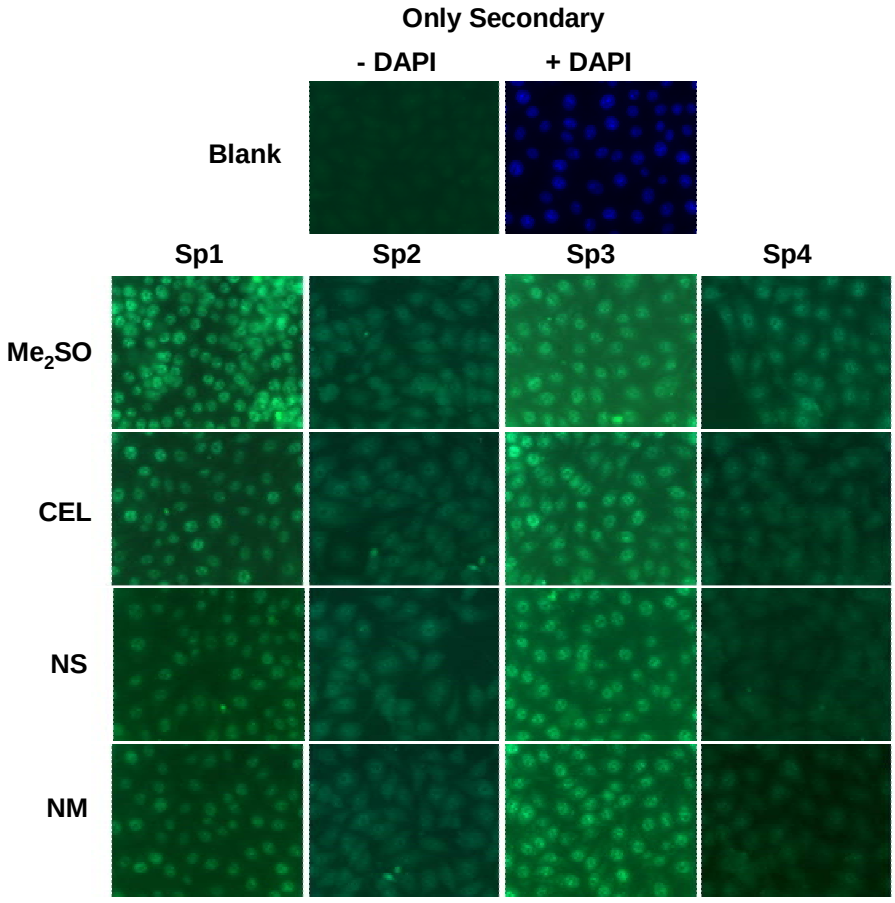

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Figure 6

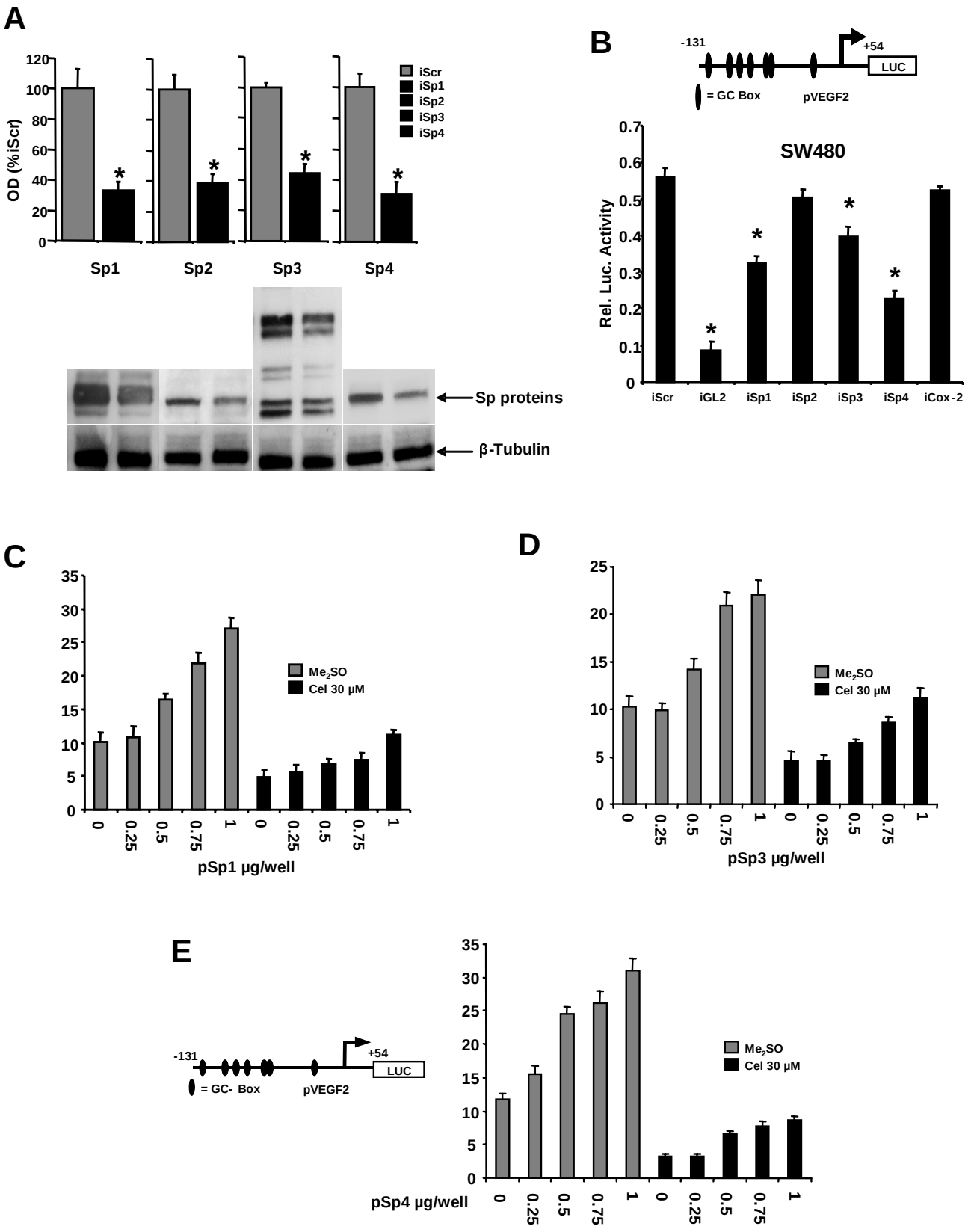

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Figure 7

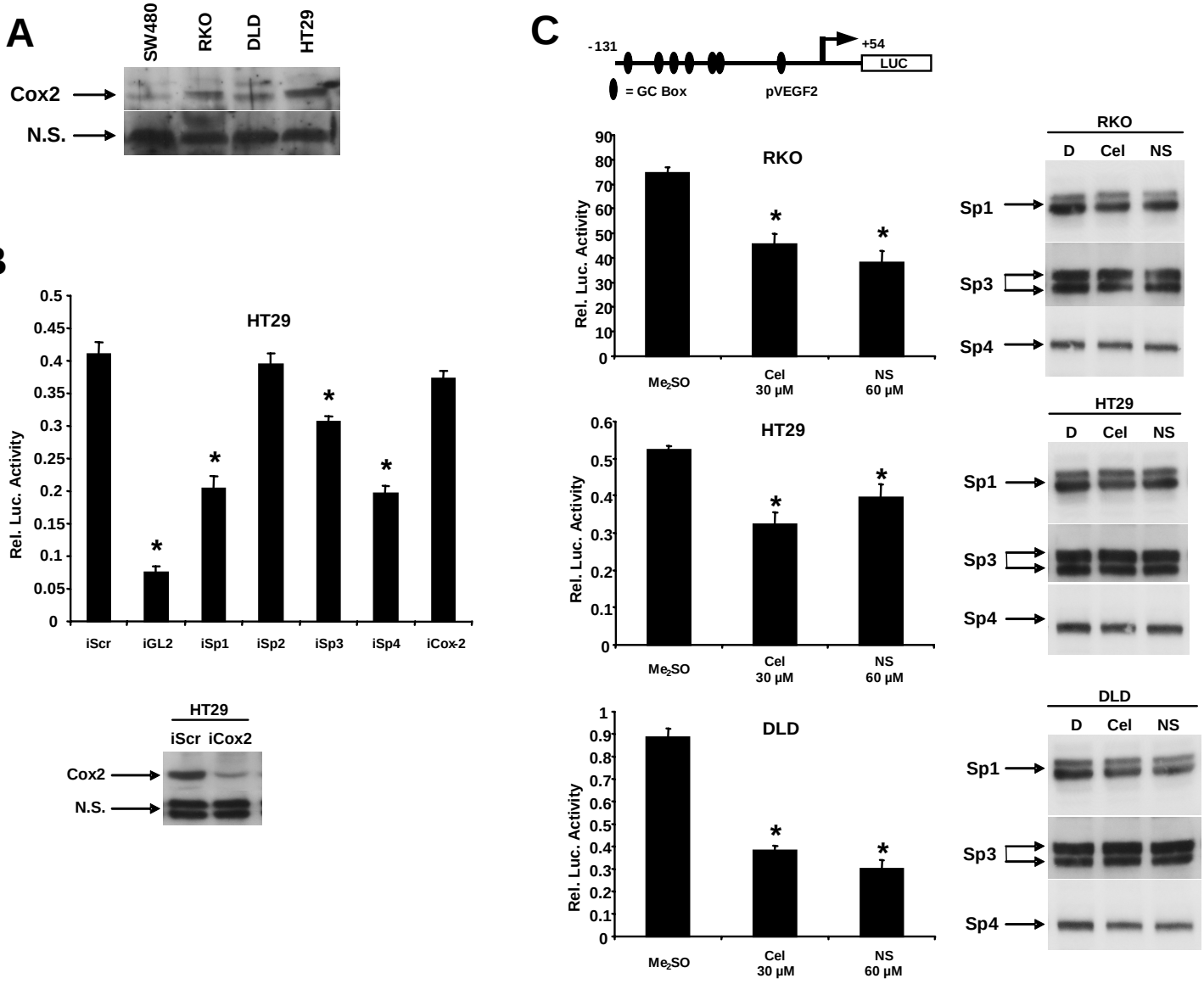

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Figure 8

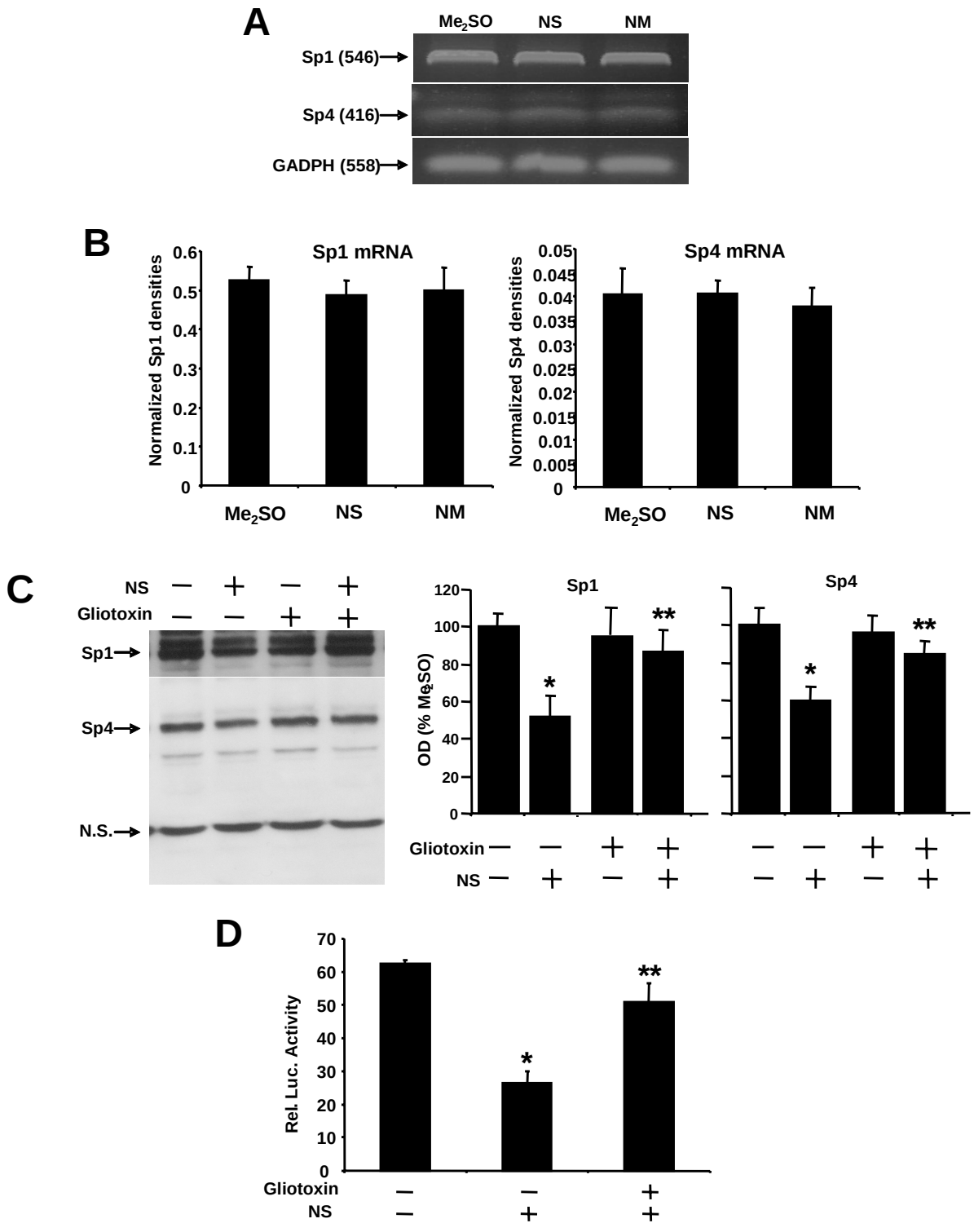

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Figure 9