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*J Immunol* 2007; 179:256-265; doi: 10.4049/jimmunol.179.1.256
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The Transcription Factor Wilms Tumor 1 Regulates Matrix Metalloproteinase-9 through a Nitric Oxide-Mediated Pathway

Marcelo Marcet-Palacios,* Marina Ulanova,† Florentina Dutu,* Lakshmi Puttagunta,‡ Samira Munoz,* Derrick Gibbings,* Marek Radomski,‡ Lisa Cameron,* Irvin Mayers,* and A. Dean Befus1*

Matrix metalloproteinase-9 (MMP-9) is released by human lung epithelial cells (LEC) in conditions such as asthma and chronic obstructive pulmonary disease and expression of MMP-9 correlates with the severity of these disorders. MMP-9 production has been reported to be regulated by a NO/soluble guanylate cyclase-dependent pathway. Transcriptional regulation of this enzyme, however, is poorly understood. Using phylogenetic analysis, we observed a highly conserved sequence in the 5′ flanking region of the MMP-9 gene containing binding sites for the transcription factor Wilms tumor 1 (WT1). We confirmed the presence of WT1 in human LEC and that treatment with TNF or a mixture containing LPS, PMA, and IFN-γ resulted in translocation of WT1 from the nucleus to the cytosol. This translocation coincided with increased expression of MMP-9 and could be blocked by inhibitors of the NO/soluble guanylate cyclase pathway. WT1 knockdown using small-interfering RNA up-regulated MMP-9 expression in human LEC and that treatment with TNF or a mixture containing LPS, PMA, and IFN-γ resulted in translocation of WT1 from the nucleus to the cytosol. This translocation coincided with increased expression of MMP-9 and could be blocked by inhibitors of the NO/soluble guanylate cyclase pathway. WT1 knockdown using small-interfering RNA up-regulated MMP-9 expression in the presence of the NO synthase inhibitor 1400W. Using either WT1 pulldown with probes for the conserved region of the MMP-9 promoter or chromatin immunoprecipitation, we confirmed WT1 binding to the MMP-9 promoter. These findings indicate WT1 is a repressor of MMP-9, regulated by a NO-mediated pathway in human LEC. To our knowledge, this is the first report of WT1 regulating MMP-9 expression. Further study is needed to determine whether clinical conditions exhibiting tissue remodeling, such as asthma and/or chronic obstructive pulmonary disease, demonstrate reduced levels of WT1 or its repressor activity. The Journal of Immunology, 2007, 179: 256–265.

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tatrix metalloproteinases (MMP)2 degrade collagens and other extracellular matrix proteins and are important to normal turnover or remodeling of extracellular matrix proteins (1). MMP also degrade other proteinases, as well as clotting factors, receptors, and cell adhesion proteins (2), thereby implicating this family in a multitude of homeostatic and pathogenic processes.

MMP-9, also called gelatinase-B, is a type IV collagenase present in low quantities in the healthy lung, while increased expression has been observed in several lung diseases including idiopathic pulmonary fibrosis, chronic obstructive pulmonary disease (COPD), and asthma (1). Indeed, MMP-9 expression in the lungs correlates with lung injury as well as with epithelial cell death (3). Furthermore, individuals with allergic asthma have elevated levels of MMP-9, both in the lung and the blood (4–6) and airway allergen challenge is associated with increased MMP-9 levels (6). MMP-9 knockout mice have heightened airway hyperresponsiveness to methacholine and expression of Th2 cytokines in response to OVA-induced lung inflammation (7). Several cell types within the lung express MMP-9, including structural and inflammatory cells (1), and in vitro studies indicate that it is produced by lung epithelial cells (LEC) stimulated with TNF and IFN-γ or by bacterial products such as LPS (8, 9). MMP-9 plays a role in dendritic cell recruitment during allergen-induced airway inflammation (10, 11) and its deficiency impairs cellular infiltration and bronchial hyperresponsiveness (12). Therefore, in addition to remodeling, MMP-9 may also be important in development of adaptive immune responses in the lung.

NO is an important regulator of MMP-9 (13–17) and we have previously shown this appears to be through a soluble guanylate cyclase (sGC)-dependent pathway (18). Moreover, mice with mutant inducible NO synthase (iNOS) produce significantly less MMP-9 than wild-type mice (19, 20). Despite an established role for MMP-9 in asthma and studies demonstrating proinflammatory stimuli such as cytokines, LPS, and NO inducing its expression, transcriptional regulation of MMP-9 is still not well-characterized.

In an effort to identify potential regulatory elements of MMP-9, we performed comparative genomic analysis. The most highly conserved noncoding sequence in >10 kb spanning the MMP-9 gene and flanking region was within the 300 bp 5′ of the transcription initiation site. This region contains a CA-repetitive element that is reported to be polymorphic, ranging from 14 to 28 repeats within a Caucasian population (21). Furthermore, one study indicates that MMP-9 transcription may be inversely associated with the length of the CA repeat (22). In silico analysis of
of exon 5, a known spliced exon that results in WT1 variants. The primer sequences used were developed based on the WT1 sequence of accession number NM_002446. The forward primer sequence was 5’-GAG ATG GCC AGT AGC CCC ATT AAA-3’ and the reverse primer sequence was 5’-TAT GTC TCC TCT TTT GGT TGT TTA-3’, which generated 412- and 361-bp long PCR products. MMP-9 primers were designed from the sequence of accession number NM_004994.2. The forward primer 5’-CTT CGC TAT CTC TTG TC-3’ and reverse primer 5’-CTA CGG CCA CTA CTG TGC CT-3’ were used, which generated a PCR product of 665 bp. β-actin was used as the internal positive control. β-actin primers were forward 5’-GGC ATC TTC ACC CTG AAG TA-3’ and reverse 5’-AGG GCA TAC CCC TCG TAG AT-3’ generated from the sequence NM_001101. These primers amplified a 325-bp PCR product. iNOS primers were forward 5’-CAC TGA GCT CAT CCC CT-3’ and reverse 5’-CAC CTT CTC TCG TAT CTG TAA-3’ generated from the sequence NM_15329.2, to generate a 428-bp PCR product. Primers were used analyzing the basic local alignment search tool sequencing program at GenBank to ensure unique complementation of Homo sapiens. PCR amplification was performed in cycles of 1 min at 94°C, 1 min at 60°C, and 2 min at 72°C, and a final cycle of 72°C for 10 min to complete polymerization. The number of cycles was optimized to be in the exponential phase of the reaction by performing the reaction at different cycles. Densitometric analysis of the gels was performed to select optimal PCR cycle numbers. MMP-9 and iNOS were run for 29 cycles, β-actin for 25, whereas WT1 was run for 30. PCR products were analyzed on a 2% agarose gel containing ethidium bromide.

Cloning and sequencing of PCR products

The amplified PCR products were cloned into the pCR2.1 plasmid vector using the T/A cloning kit (Invitrogen Life Technologies) as previously described (33). dsDNA sequencing was conducted using an ABI 373A automated sequencer (Applied Biosystems) and sequences were tested for homology using the basic local alignment search tool sequencing program at GenBank.

Comparative genomic analysis

Assessment of sequence conservation of MMP-9 across multiple species was performed using the VISTA program (Mayor; Bioinformatics) located at www.gsd.lbl.gov/vista/.

Antibodies

mAbs against WT1 were obtained from Santa Cruz Biotechnology (F-6) and from Oncogene (Ab-1; Oncogene Science). WT1 polyclonal Ab (N20) and its peptide blocker (N20 P) were obtained from Santa Cruz Biotechnology. Abs used in immunoprecipitation (ChiP), other than N20, were normal rabbit IgG and anti-acetyl-histone H3 (Upstate Cell Signalling Solutions). Other Abs included: peroxidase-conjugated goat anti-mouse IgG (BD Biosciences), donkey anti-mouse IgG, IRDye 800 conjugated (Rockland Immunonochemicals), mouse IgG1 isotype control (R&D Systems), peroxidase-conjugated affinity purified F(ab’2) goat anti-mouse IgG (H + L; Jackson Immunoresearch Laboratories) and Rhodamine Red-X goat anti-mouse IgG.

Immunoprecipitation

Following stimulation (see below), cells were harvested (10^7 cells) and washed twice in cold PBS and spun at 400 × g for 10 min. Cells were then resuspended in lysis buffer (10 mM HEPES, 2 mM MgCl2, 15 mM KCl, 0.1 mM Tris-EDTA, and 0.15% Nonidet P-40, containing a protease inhibitor mixture (Roche Molecular Biochemicals)) and precleared with 50 μl of protein A/G beads (Pierce Biotechnology). Ten micromgrams of Ab were added to lysate and incubated for 1 h. Then 50 μl of protein A/G beads were incubated for 1 h at 4°C. Collected beads were then used for Western blot analysis or 2-dimensional (2-D) electrophoresis. Cells were stimulated for 10 min with a mixture containing 10 μg/ml bacterial LPS, 0.1 ng/ml TNF-α, and 1 nM PMA, or with only 10 ng/ml human recombinant TNF (Sigma-Aldrich).

Isolation of WT1 using its DNA-binding site (DNA pulldown)

Streptavidin-coated paramagnetic particles (PMP) (Promega) were incubated for 3 h with a dsDNA oligo, corresponding to the WT1-binding site (tgctg CCACA CACAC ACAC ACAA CACAC ACAC CACAC CACAC CACAC CCctga) (34). Five additional nucleotides beyond the recognition site were included in the 5’ and 3’ ends of the sequence, shown above in small letters. The oligos were 5’ biotinylated to ensure strong binding to PMP (Invitrogen Life Technologies). The PMP-DNA complex was then collected and washed three times in 4°C PBS using a
magnet to precipitate the beads to wash unbound DNA. Total cell lysate was then added to the clean PMP-DNA pellet and incubated for 1 h at 37°C. The PMP-DNA-protein complex was then collected using a magnet and consequently washed three times in 4°C PBS. The supernatant was discarded and the pellet was resuspended in the ReadyPrep 2-D cleanup kit (Bio-Rad). The protein pellet was then resuspended in rehydration sample buffer.

Western blot analysis and 2-D electrophoresis

Cells were harvested and homogenized with lysis buffer (10 mM HEPES, 2 mM MgCl2, 15 mM KCl, 0.1 mM EDTA, and 0.15% Nonidet P-40), containing a protease inhibitor mixture (Roche Molecular Biochemicals). Samples were subjected to 7% SDS-PAGE followed by blotting and immunodetection with Abs. In some experiments, 2-D electrophoresis was conducted by loading 200 μl of sample onto 7-cm immobilized pH gradient strips (pH 3–10) and electrofocused using the Protean IEF Cell (Bio-Rad). Strips were then loaded onto Tris-HCl 4–15% gels and separated by electrophoresis. Gels were then analyzed by Western blot (18) or silver staining using the Amersham silver staining kit (Amersham Biosciences).

Molecular mass and isoelectric point (pI) estimation

To estimate molecular masses of spots detected by anti-WT1 Abs from Western blot analysis of 2-D gels, we used the Odyssey Imaging System (Li-cor Biosciences). 2-D electrophoresis was run with samples generated from either immunoprecipitation or DNA pulldown. Using molecular mass standards, the software estimates the molecular mass of a given spot. Using pI standards from Bio-Rad, specific to our immobilized pH gradient strips, a pI scale was designed as shown in Fig. 4C. The pI values for WT1 were estimated using this scale and also using the IgG H chain pI (Fig. 3CII) as a reference. Using molecular mass and pI data, mean ± SE values were estimated (n = 4).

Subcellular fractionation

Subcellular fractionation was performed according to Ref. 35. Briefly, following stimulation, A549 cells were harvested (15 × 106 cells), washed with cold PBS, and resuspended in lysis buffer, subjected once to a freeze-thaw cycle, and resuspended on ice for 15 min. The supernatant was centrifuged at 14,000 × g for 20 min and stored as cytoplasmic extract. The pellet was washed with lysis buffer and nuclei were resuspended in 100 μl of extraction buffer (20 mM HEPES, 1.5 mM MgCl2, 0.4 M NaCl, 20% glycerol, 0.2 mM EDTA, and 0.15% Nonidet P-40, with protease and phosphatase inhibitors) and left on ice for 30 min. The nuclear extract was recovered by centrifugation at 14,000 × g for 20 min and stored in aliquots at −80°C for use in Western blot analyses. The nuclear extracts were tested for purity by Western blot using Ab to the cytoskeleton protein α-tubulin and nuclear protein Oct-1.

Immunohistochemistry analysis

Histopathological material including sections of healthy adult lungs (n = 3) were obtained from the University of Alberta Hospital (Edmonton, Alberta, Canada). The study was approved by the Health Research Ethics Board, University of Alberta. Sample block preparation was performed by the Department of Laboratory Medicine and Pathology, University of Alberta Hospital. After fixation in 10% buffered formalin, lung tissue specimens were embedded in paraffin and sections were cut at 4 μm. All steps were performed at room temperature (−20°C) in a humidified container to prevent dehydration of tissues. Sections were deparaffinized and rehydrated to water. Endogenous peroxidase was blocked with 30% hydrogen peroxide-methanol (1:4) for 10 min. Sections were covered with a 1:1 dilution of protease or phosphatase inhibitor mixture and left on ice for 15 min. The supernatant was centrifuged at 63°C for annealing temperatures and 30 cycles.

WT1-regulates MMP-9 through a NO-mediated pathway

The NO dye 4-aminio-5-methylamino-2′,7′-difuoro-2′-7′difluoro-3′-amino-4′-methyl-6′-nitro-7-nitro-2′-1′-4′-triazol-3′-carboxyanilide (DAF-FM) was used to assess NO formation. A549 cells were cultured overnight in 96-well black plates (Corning). After stimulation with 10 ng/ml TNF and the NOS inhibitors 1-NAME, L-NAME, and 1400W (see Fig. 1 for concentrations; Cayman Chemical), cells were incubated with 10 μM DAF-FM for 30 min and washed twice with fresh medium. Cells were then allowed to rest for 30 min and fluorescence was measured using fluorescence plate reader FLX800 (Biotek Instruments) with excitation wavelength at 485 nm and detection at 516 nm.

WT1-small interfering RNA (siRNA) transfection

siRNA was used as previously described (36) with some modifications. WT1 mRNA was targeted using the sequence 5′-AAGGACUGUGAGAAAGGGUU-3′ in exon 8. The control sequence was generated by inverting two 4-nt-long regions, shown in small letters 5′-AAGG gtaa TGAA aage GAGGTTT-3′. A549 cells were transfected with siRNA using Lipofectamine 2000 (Invitrogen Life Technologies) according to the manufacturer’s instructions. Briefly, 106 cells were centrifuged with 3 μg of WT1-siRNA or control siRNA diluted in 10 μl of OPTI-MEM (Invitrogen Life Technologies) and 25 μg/ml Lipofectamine 2000. After a 4-h incubation of cells with siRNA-Lipofectamine 2000 complexes, medium was replaced with DMEM (Invitrogen Life Technologies), and cells were maintained for a total of 24 h. Harvested cells were used for RNA or protein extraction. In some experiments, cells were treated with 10 ng/ml TNF during the last 12 h of culture, and supernatants and medium were frozen at −80°C for WT1 and MMP-9 detection, respectively. Sham treatments contained 25 μg/ml lipofectamine.
NO regulates MMP-9 through a sGC- and cGMP-dependent path-
Using rat vascular smooth muscle cells, we previously showed that PKA and sGC are involved in expression and activity in human LEC.

These levels were significantly up-regulated (4.5-fold) after 12 h of TNF treatment (Fig. 1, A). A time-course analysis of MMP-9 expression showed significant up-regulation after 12 h of TNF treatment (Fig. 1B). A similar time-course analysis for iNOS protein and mRNA expression (10 min, 6, 12, 24, and 48 h after TNF treatment) demonstrated that mRNA and protein were elevated as early as 6 h and remained increased above sham treatment for at least 48 h (Fig. 1B). Following optimization of PCRs for the log phase of amplification (see Materials and Methods), we found that MMP-9 and iNOS mRNA levels were undetectable (30 cycles) in resting (sham) A549 cells (Fig. 1C). After 10 min of TNF stimulation, iNOS and MMP-9 mRNA signals were significantly up-regulated (Fig. 1C). Furthermore, 1400W blocked TNF-induced MMP-9 gene expression in a concentration-dependent manner (2 and 20 μM), but did not block iNOS mRNA expression. These data show that 1400W blocks NO activity, but not iNOS expression itself. Thus, NO activity is required for MMP-9 gene expression.

To investigate the role of NO activity in the induction of MMP-9 enzyme activity, gelatin zymography analysis of the medium (without FBS) used to grow A549 was performed. We detected low levels of MMP-9 activity in resting (sham) A549 medium. These levels were significantly up-regulated (4.5-fold) after 12 h of TNF treatment (Fig. 1, F and H). The NO inhibitors l-NNAME and 1400W significantly reduced MMP-9 activity in a concentration-dependent manner (Fig. 1, E and G). These data suggest that NO is an important up-regulator of MMP-9 gene expression and activity in human LEC.

PKA and sGC are involved in MMP-9 gene regulation
Using rat vascular smooth muscle cells, we previously showed that NO regulates MMP-9 through a sGC- and cGMP-dependent path-

Confocal microscopy
Cells were grown on glass coverslips overnight at 37°C. Cells were stimulated with TNF (10 ng/ml), washed with PBS, and fixed with 4% paraformaldehyde at room temperature. After permeabilization with 0.2% Triton X-100, nonspecific binding was blocked with 10% FBS plus 3% BSA. Cells were incubated overnight at 4°C with mAbs diluted 1/50 in blocking buffer, washed three times with PBS, and incubated with Rhodamine Red-X-conjugated secondary Ab (1/50) for 2 h at room temperature. 4′,6-diamidino-2-phenylindole (DAPI) was used as a nuclear marker. Coverslips were mounted on glass slides and cells were examined with a confocal microscope (Olympus Fluoview; FV 1000).

Transcription factor-DNA interaction in silico analysis
The MMP-9 promoter was studied using in silico tools: Transcription Element Search System (TESS; www.cbil.upenn.edu/tess/) and MatInspector (www.genomatix.de/). These software were also used to study the mucin genes MUC2, MUC5AC, and MUC5B.

Statistics
Results are means ± SE of at least three independent experiments. They were analyzed using one-way ANOVA, and when significant differences were found, the multiple comparison Tukey-Kramer test was used (GraphPad InStat). Values where p ≤ 0.05 were considered statistically significant. The n values in figure legends represent independent experiments.

Results
TNF induces iNOS-mediated MMP-9 expression and enzyme activity
Because we previously observed that NO mediates MMP-9 gene expression in rat vascular smooth muscle cells (18), we tested whether NO-mediated regulation of MMP-9 also occurs in human LEC. A549 cells were stimulated with TNF (10 ng/ml) and NO production, iNOS, and MMP-9 expression were investigated. TNF induced significant production of NO after 12 h (p < 0.05) and 24 h (p < 0.01) as detected by DAF-FM (Fig. 1A). This effect was blocked by the NO inhibitors 1400W (100 μM), l-NNAME (300 μM), and l-NMMA (300 μM) (Fig. 1A). Similarly, MMP-9 activity showed significant up-regulation after 12 h of TNF treatment (Fig. 1B). A similar time-course analysis for iNOS protein and mRNA expression (10 min, 6, 12, 24, and 48 h after TNF treatment) demonstrated that mRNA and protein were elevated as early as 6 h and remained increased above sham treatment for at least 48 h (Fig. 1B). Following optimization of PCRs for the log phase of amplification (see Materials and Methods), we found that MMP-9 and iNOS mRNA levels were undetectable (30 cycles) in resting (sham) A549 cells (Fig. 1C). After 10 min of TNF stimulation, iNOS and MMP-9 mRNA signals were significantly up-regulated (Fig. 1C). Furthermore, 1400W blocked TNF-induced MMP-9 gene expression in a concentration-dependent manner (2 and 20 μM), but did not block iNOS mRNA expression. These data show that 1400W blocks NO activity, but not iNOS expression itself. Thus, NO activity is required for MMP-9 gene expression.

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PKA and sGC are involved in MMP-9 gene regulation
Using rat vascular smooth muscle cells, we previously showed that NO regulates MMP-9 through a sGC- and cGMP-dependent path-

FIGURE 1. NO regulates MMP-9 gene expression in human A549. A, A549 cells were stimulated with TNF (10 ng/ml) in the presence of the NO inhibitors 1400W, l-NNAME, and l-NMMA. NO release was measured using the NO-specific dye DAF-FM (n = 4). B, Time course of treatment with TNF shows iNOS expression from 10 min to 48 h after treatment. Increased MMP-9 activity is statistically significant at 12–48 h (p = 0.007). C, Effect of 1400W on TNF-induced MMP-9, iNOS, and β-actin gene expression (n = 5). D, Densitometric analysis of C plotted as MMP-9 expression in arbitrary units (aU) corrected for β-actin loading. E and G, NO effects on TNF-induced MMP-9 expression. MMP-9 protein expression and activity was studied using gelatin zymography (n = 4). MMP-2 is a constitutive gelatinase. F and H, Densitometric analysis (for E and G) was performed and the ratio MMP-9/MMP-2 plotted as a percent of control of MMP-9 activity. Statistical significance: p < 0.05 (*); p < 0.01 (**).
the NO-dependent regulation of MMP-9, perhaps through PKA translocation to the nucleus and phosphorylation of transcription factors that regulate gene expression.

Comparative genomic analysis of MMP-9

Comparative genomic analysis is a powerful method for identifying sequence conservation over evolution and as such putative regulatory elements (38). Comparative analysis of the MMP-9 gene across several mammalian species was performed and demonstrated significant conservation of the 5′ region of the MMP-9 gene (data not shown). Indeed, −321/−21 (relative to the ATG) was highly identical from human to cow (75%, upper), rat (72%, middle), and mouse (67%, lower) (data not shown). Sequence alignment of these species revealed the presence of a CA repetitive element. Although in silico analysis using TESS (www.cbil.upenn.edu/tess/) and MatInspector (www.genomatix.de) revealed the presence of numerous putative transcription factor binding sites, the most highly conserved sequence was the [CA]21 repeat, which was predicted to contain multiple binding sites for a transcriptional repressor called WT1. These results indicated WT1 may be an important transcriptional regulator of MMP-9.

WT1 is expressed in human lung epithelial cells

Given that WT1 may participate in transcriptional regulation of MMP-9 and the fact that LEC are a major source of MMP-9, we investigated whether these cells express WT1. Using RT-PCR analysis, we found that human LEC lines A549, HS-24, BEAS-2b, as well as cultures of PBEC express WT1 mRNA (Fig. 3A). Because cell type-specific expression of exon 5 of WT1 has been reported (39), we studied exon 5-positive and -negative isoforms of WT1 in LEC. Primers binding to exons 4 and 6 were designed to detect differential expression of exon 5. The isoform lacking exon 5 is 51 bp shorter (see Materials and Methods). All LEC described above coexpressed WT1 isoforms with and without exon 5. The two RT-PCR bands of 412 and 361 bp (Fig. 3A) were
cloned and the sequence identity was confirmed to be 100% homologous to WT1 splice variants with and without exon 5.

To study WT1 protein expression, A549, HS-24, and BEAS-2b cell lysates were analyzed by Western blot using the mAb Ab-1 (Fig. 3B). A single band at 54 kDa was detected in all three cell lines. WT1 was immunoprecipitated from A549 cell lysate with the mAb F-6, separated by 2-D electrophoresis, and analyzed by Western blot using Ab-1. A single spot was detected at the expected molecular mass of 54 kDa (Fig. 3Ci). Three additional spots were also detected and confirmed to be IgG H chain by mass spectrometry sequence analysis (Fig. 3Cii).

To determine whether WT1 is also expressed in normal LEC, immunohistochemical analysis of sections of human lung biopsies were examined. Immunoreactivity for WT1 was associated with the epithelial cells using two different Abs (Fig. 4). WT1 displayed both nuclear and perinuclear subcellular localization (Fig. 4C).

WT1 binds to the MMP-9 promoter in vitro

Given our observation that there is a WT1-binding site on the MMP-9 promoter, we designed a DNA-PMP construct with the MMP-9 promoter sequence containing the putative WT1 site (H11002 to H11002) to isolate WT1 (see Materials and Methods). Following incubation of the DNA-PMP with A549 cell lysate, the WT1-DNA-PMP complex was precipitated using a magnet and resolved by 2-D electrophoresis. Western blot analysis using the mAb Ab-1 detected a spot of 54 kDa (Fig. 3Ciii). A total of four

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**FIGURE 4.** WT1 is expressed in the nuclei of human lung epithelial cells. A, Immunohistochemistry analysis was performed in normal tissue from adult human lung biopsies. The tissue was stained with the anti-WT1 polyclonal Ab N20. B, N20 was preincubated with its peptide blocker N20-P before staining tissue samples. C, The anti-WT1 mAb F-6 and its IgG1 isotype control (D) were also used to study WT1 expression and its distribution. Arrow points to a nucleus displaying perinuclear WT1 localization. The majority of nuclei showed positive granular staining to WT1 (arrowhead).

**FIGURE 5.** TNF-induced translocation of WT1 from the nucleus to the cytosol is NOS dependent. A, Confocal microscopy of A549 cells was performed using the anti-WT1 Ab Ab-1 (red). Nuclei were stained with DAPI (green). Colocalization was observed by merging the green and the red channels (yellow). The isotype control (iso) is shown for the sham treatment. Bars, 10 μm (n = 5). B, A549 cells were treated as described in the text followed by enrichment of nuclear fraction and Western blot analysis (n = 3). The nuclear-specific loading control histone H1 was used to correct for loading to perform the densitometric analysis (C). The cytosolic marker α-tubulin (α-tub) was measured to monitor contamination of the nuclear fraction. Statistical significance: p < 0.05 (*).
WT1 subcellular localization is regulated by NO

We next examined whether up-regulation of MMP-9 expression in response to proinflammatory stimuli such as TNF could be associated with altered WT1 expression. RT-PCR using cDNA extracted from A549, HS-24, and BEAS-2b stimulated with TNF (10 ng/ml) revealed that WT1 mRNA levels did not change at time points from 10 min to 24 h (data not shown). Similar results were obtained using a more potent proinflammatory stimulus consisting of PMA, IFN-γ, and LPS (see Materials and Methods) that we previously showed induces expression of iNOS, cyclooxygenase-2, and MMP-9 (18) (data not shown). Furthermore, WT1 protein levels did not change under similar conditions (data not shown).

Because there was no apparent change in relative abundance of WT1, we assessed the possibility that intracellular localization of WT1 may change upon stimulation. A549 cells were treated with TNF and analyzed using confocal microscopy using an mAb (Fig. 5). In sham-treated cells (sham), the nuclear stain DAPI colocalized with WT1 signal (merge). After 10 min of TNF (10 ng/ml) treatment, the WT1 signal (red) was detected only in the perinuclear region (Fig. 5A). Because NO and PKA can regulate MMP-9 expression and WT1 has been shown to be regulated by targets downstream of NO, such as PKA, we examined whether the apparent translocation of WT1 following TNF treatment was NO dependent. Indeed, preincubating the cells with the iNOS inhibitor 1400W before TNF stimulation blocked the alteration in the apparent location of WT1 at 10 min (Fig. 5A), as well as at 1, 3, and 6 h (data not shown).

Additionally, evidence confirming loss of WT1 from the nuclear milieu is provided by Western blot (Fig. 5B). As indicated by the confocal data (Fig. 5A), nuclear WT1 signal was significantly lower in TNF-treated cells and when cells were pretreated with the NO synthase inhibitor 1400W, WT1 remained in the nucleus (Fig. 5A). The nuclear and cytosolic markers histone H1 and α-tubulin were used as controls. Together, these results provide strong evidence that the levels of nuclear detectable WT1 drop in response to TNF in a NO-dependent mechanism.

WT1 depletion increases MMP-9 expression

WT1 is a transcription factor that can act as a gene repressor (30–32). As such, we reasoned that under conditions where WT1 is shuttled to the cytosol, it cannot fulfill its repressor functions resulting in up-regulation of MMP-9. To test this postulate, A549 cells were depleted of WT1 by RNA interference and treated with TNF (Fig. 6A). WT1 knockdown (39%) (Fig. 6A, left) did not result in a significant increase of MMP-9 expression (Fig. 6A, right). Although we thought that siRNA would increase MMP-9 expression, there was no significant increase when compared with TNF stimulation alone. Thus, it is likely that the TNF-dependent shuttling of WT1 to the cytosol is sufficient to derepress MMP-9 and WT1 knockdown does not result in a measurable increase of MMP-9 expression. To further test this hypothesis, TNF-induced MMP-9 expression was examined in the presence or absence of 1400W. Under these conditions, 1400W reduced the TNF-driven MMP-9 expression and WT1 knockdown reversed this blockade (Fig. 6, B and C). In the absence of 1400W, siRNA treatment alone did not increase MMP-9 enzyme activity. These data indicate that

FIGURE 6. WT1 functions as a MMP-9 repressor. A, Effect of WT1 knockdown in the presence of TNF. WT1 levels were monitored by Western blot. The loading control α-tubulin (α-tub) was also tested. MMP-9 expression was measured by zymography to study the effect of WT1 knockdown in the presence of TNF. Sham-treated cells were incubated with lipofectamine (sham). B, Effect of 1400W on the TNF-induced MMP-9 up-regulation and the recovery of MMP-9 expression by WT1 knockdown. Controls treated with TNF or TNF plus 1400W but no siRNA were included (lanes 2 and 3). The siRNA control sequence was also included (siRNA cont) in lanes 5 and 7. The loading control α-tubulin (α-tub) was measured (n = 5). MMP-9 protein expression was studied using gelatin zymography. C, Upper graph, Densitometric analysis of B was conducted and used to estimate the WT1/α-tubulin ratio and plotted as a percent of control of WT1 expression (n = 4). C, Lower graph, Densitometric analysis for zymograms was performed and the ratio MMP-9/MMP-2 plotted as a percent of control of MMP-9 activity (n = 5). D, F, and G, ChIP analysis was performed for MMP-9, IL-13, and Oct-1, respectively (n = 3). Input DNA was used as the positive control. Immunoprecipitation was performed using an anti-histone-3 (αH3) or anti-WT1 (αWT1) and their respective isotype controls (H3 iso) and (WT1 iso). PCR primers were used to detect MMP-9 (D), IL-13 (F), and Oct-1 (G) coprecipitated promoter sequences. E, Densitometric analysis of D was conducted and the WT1 bound relative to H3 bound in sham and TNF treatment groups was plotted as in vivo DNA-bound WT1. G, Oct-1 binding was measured as a constitutive promoter control. Statistical significance: *p < 0.05; **p < 0.01; ***p < 0.001.
WT1 is a MMP-9 repressor and TNF-induced NO functions to derepress the MMP-9 gene.

**WT1 binds to the MMP-9 promoter in vivo and binding is regulated by TNF**

To confirm in vivo binding of WT1 to the MMP-9 promoter, we performed ChiP analysis of A549 cells. Immunoprecipitation was performed with Abs against histone H3 (αH3), WT1 F-16 (αWT1), and the appropriate isotype controls. Primers designed to amplify the MMP-9 promoter generated a product from sonicated DNA before immunoprecipitation (input) as well as sham- or TNF-treated cells immunoprecipitated with αH3. However, the product generated with DNA immunoprecipitated with αWT1 following TFN treatment is reduced compared with sham treatment, when corrected against αH3 (n = 3, Fig. 6, D and E). No bands were amplified with primers for the IL-13 promoter which lacks a WT1-binding site. To confirm the specificity of TNF treatment on loss of WT1 binding, we performed ChiP for Oct-1 on the constitutive promoter of the gene thioredoxin reductase 1 (40). Fig. 6G shows that Oct-1 binding to the thioredoxin promoter region, unlike WT1 binding to the MMP-9 promoter, was observed in both sham- and TNF-treated cells. These findings support the hypothesis where WT1 functions as a transcriptional repressor of MMP-9.

**Discussion**

NO is an important second messenger produced at high concentrations in conditions such as asthma and COPD. We have identified WT1 as a novel target by which NO exerts regulation of MMP-9 gene expression.

Human LEC express MMP-9 in resting conditions and this expression was driven by TNF. We showed that TNF-induced up-regulation of MMP-9 was NO dependent in A549. Although NO production could not be detected after 10 min of TNF stimulation using the DAF assay (Fig. 1A), intracellular NOS blockade using the selective iNOS inhibitor 1400 W reduced MMP-9 mRNA expression but not expression of iNOS (Fig. 1C). Furthermore, 1400 W also blocked TNF-induced translocation of WT1 (Fig. 5). These data indicate that within 10 min of TNF activation NOS activity induces WT1 translocation to the cytosol, an event that promotes the formation of MMP-9, which accumulates in the medium to measurable levels after 12 h (Fig. 1B).

Downstream of NO, sGC was shown to mediate this pathway through activation of PKA (Fig. 2). In this study, we have extended our observations in rat vascular smooth muscle cells (18) into a new human cell compartment, the lung epithelium. In addition, we have identified PKA as an important target of NO in the regulation of MMP-9 mRNA expression and enzymatic activity. Recently, PKA has been shown to activate MMP-9 in human ovarian epithelium (41) and we have confirmed this findings in human LEC. WT1 is regulated by PKA through phosphorylation of its serine residues (26, 34, 42) in agreement with our findings.

WT1 expression was not restricted to carcinoma cell lines, such as A549, HS-24, and Calu-3, but was also present in primary cell cultures (PBEC) and in BEAS-2B, a virus immortalized human LEC line. Additional evidence supporting the expression of WT1 in human LEC was obtained from immunohistochemical analysis of normal adult lung (Fig. 4). In addition to demonstrating the expression of WT1 in human tissue, these data indicate that WT1 may change its subcellular microlocalization in vivo (Fig. 4C).

A number of different WT1 splice variants have been described in the literature. Exons 1, 5, and 10 are either spliced or modified before a mature mRNA is generated (39). By designing our RTPCR primers to recognize exons 4 and 6, we were able to amplify PCR products with and without exon 5. Different cell types may alternatively express WT1 isoforms with or without exon 5, whereas other cell types may coexpress both isoforms (39). It has been reported that exon 5 might play a role in repressor functions of WT1 (43). Our results indicate that the isoform variant containing exon 5 is more abundant at the mRNA level (data not shown), however, both isoform mRNA types are coexpressed in all LEC tested. In our studies, we were able to identify only one WT1 isoform at the protein level. The 54-kDa isoform is generated from the full-size transcript encoding all 10 exons, which was the most abundant isoform detected by PCR. We were able to detect only one band at 54 kDa using Western blot and only one spot using 2-D electrophoresis. Thus, the 54-kDa WT1 isoform seems to be the major WT1 variant in LEC and the isoform that undergoes changes in cellular microlocalization.

Stimulation of A549, HS-24, and BEAS-2B with 10 ng/ml TNF for 10 min to 24 h did not change WT1 mRNA expression levels or protein levels (data not shown). However, this treatment induced a change in localization of WT1 from the nucleus to the perinuclear area (Fig. 5). Interestingly, this response to TNF was blocked with the NOS inhibitor 1400 W, suggesting that NO mediates TNF effects in this pathway.

To test whether NO regulates the MMP-9 gene through WT1, siRNA was used to knockdown WT1 (Fig. 6). We found that WT1 levels were significantly reduced with the siRNA treatment and that WT1 knockdown resulted in MMP-9 up-regulation in a reaction driven by TNF but blocked with 1400 W (Fig. 6B), indicating that NO controlled the MMP-9 gene expression through regulation of WT1 microlocalization. Additionally, in the absence of 1400 W, WT1 knockdown did not increase MMP-9 expression indicating that under these conditions WT1 shuttling and not WT1 protein levels is the mechanism involved (Fig. 6, B and C). Phosphorylation of WT1 results in loss of DNA affinity (42) and this may also explain why reducing WT1 levels does not increase MMP-9 expression. Thus, we have shown with carefully optimized, semi-quantitative RT-PCR, in combination with Western blot analysis and with MMP-9 enzyme activity, that MMP-9 is regulated by WT1. The evidence we provide clearly supports the model in which WT1 acts as a MMP-9 gene repressor, regulated by PKA.

A powerful technique that studies in vivo binding of transcription factors to their specific DNA-binding sites, ChiP, was used. We found that TNF treatment significantly reduces the amount of WT1 that binds to the MMP-9 promoter (Fig. 6, D and E). To confirm the specificity of WT1 binding to the MMP-9 promoter, we used primers for the IL-13 promoter that is known to lack WT1-binding sites (Fig. 6F). The binding of the constitutive transcription factor Oct-1 was not affected in these conditions. Collectively, these data indicate that WT1 is a repressor of the MMP-9 gene in human LEC and in conditions of high NO, WT1 translocates to the perinuclear region thus derepressing the MMP-9 gene.

Our findings add the MMP-9 gene to a growing number of growth related genes that have been reported to be repressed by WT1, including platelet-derived growth factor A-chain, insulin-like growth factor II, and early growth response 1 (30–32).

In contrast to our results, using rat embryo fibroblasts and a transient transfection approach, Himelstein et al. (44) found that deletion of the WT1-binding site in the MMP-9 promoter inhibited MMP-9 promoter activity. In this study, the authors created a promoter sequence lacking the WT1-binding site. The WT1-binding site is 42 nt long and it is located near the TATA box at position -131 bp. It is possible that deletion of 42 bases in this region would result in a frame shift that would interfere with the functionality of the promoter, thus explaining contrasting results found by the authors. Additionally, the approach that they used with transient transfection has a number of limitations e.g., uncontrolled
plasmid copy number and no genomic integration. By contrast, in human LEC BEAS-2B, Wu et al. (45) found that the same sequence had inhibitory properties in the cytosolic phospholipase A2 gene, raising the possibility that there is differential expression of WT1 in different cell types. Interestingly, we have found that human lymphocytes express WT1 and that NO reduces WT1 levels rather than changing WT1 subcellular localization. These findings support the view that NO can regulate WT1 at multiple levels (e.g., expression and localization) and that these mechanisms are cell specific (unpublished observations).

Using a novel homologous recombination technique, Yan et al. (46) reported that the MMP-9 gene was potentially controlled by a gene repressor and that this phenomenon was not apparent through transient transfection, explaining contradictory results found by Himelstein et al. (44). Our findings confirm this proposal and provide new insight into the regulation of the MMP-9 gene.

The WT1 gene is located at 11p13. At position 11p15, the mucin family of genes MUC2, MUC5AC, MUC5B, and MUC6 are found. These glycoproteins are the major macromolecular component of mucus and are also known to be regulated by PKA (47). Mucin gene expression varies with differentiation, inflammation, and carcinogenesis (48), processes known to affect transcription factor WT1 expression and subcellular localization. Recent observations have shown increased expression of MUC2, MUC5AC, and MUC5B in association with secretory cell hyperplasia and metaplasia in the airways and fostered our interest in the processes controlling mucin secretion. Genomic proximity and detection of binding sites of WT1 in the promoter of mucins (detected by TESS, see Methods) make these genes attractive targets to assess for WT1 derepression.

Most of the literature regarding the transcription factor WT1 has focused on its role in cancer as WT1 is overexpressed in numerous solid tumors. It would be interesting to determine the role of NO in relation to WT1 function and oncogenesis. Our data provide new directions for research in cancer, inflammation, and allergy and the role of NO in the expression of several genes.

Acknowledgments

We extend our thanks to members of the Pulmonary Research Group and Lynelle Watt for their support.

Disclosures

The authors have no financial conflict of interest.

References


