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# Osteopontin inhibits expression of cytochrome *c* oxidase in RAW 264.7 murine macrophages☆

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**Abstract**

Osteopontin (OPN) functions as both a cell attachment protein and a cytokine that signals through two CAM molecules: $\alpha_v\beta_3$-integrin and CD44. OPN initiates a number of signal transduction pathways that control cell survival, proliferation, and migration. In this study, utilizing RAW 264.7 murine macrophages, we demonstrate that expression of the mitochondrial protein, CCOI, is significantly decreased in the setting of OPN stimulation. This effect is blocked by the CD44 competitive ligand, hylauronate; GRGDSP, a hexapeptide that blocks OPN–integrin binding, had no effect. CCOI mRNA and transcription were significantly decreased in the presence of OPN; CCOI mRNA half-life was unaltered by OPN. Additional mitochondrial run-on studies, which included genes from L-strand and H-strand, suggest that OPN terminates transcription of the distal H-strand. CCO enzyme activity was also significantly decreased by OPN. Our results indicate that OPN inhibits CCOI expression as the result of a novel CD44-dependent transcriptional regulatory mechanism of the mitochondrial H strand.


*Keywords:* Osteopontin; Mitochondria; Macrophage; Endotoxin; Cytochrome oxidase

OPN functions as both a cell attachment protein and a cytokine that signals through two CAM molecules: $\alpha_v\beta_3$-integrin and CD44. OPN initiates a number of signal transduction pathways that control cell survival, proliferation, and migration [1]. Initially discovered as an inducible, tumor-promoter gene, OPN is overexpressed in human tumors, is the major phosphoprotein secreted by malignant cells in patients with advanced metastatic cancer, and plays a key role in cell adhesion, survival, and migration/aggregation of neoplastic cells [2,3]. Using OPN-null mutant mice as a model system, Crawford et al. [4] recently demonstrated that OPN enhances the growth or survival of metastatic cells. However, the mechanism by which OPN may enhance tumor survival has not been well characterized. Lin et al. [5] have demonstrated that OPN produced as a result of growth factor signaling exerts an antiapoptotic activity on the cultured cells, and they suggest that malignant cells that have evolved to produce an increased level of OPN enjoy a growth advantage in vitro and in vivo.

We hypothesized that exposure of immune effector cells, such as macrophages, to tumor-expressed concentrations of OPN may also enhance malignant cell survival. To identify patterns of gene expression associated with OPN exposure, we utilized the technique of suppression subtractive hybridization (SSH) in RAW 264.7 murine macrophages [6]. A number of mitochondrial genes were found to be significantly decreased in the setting of OPN stimulation, most notably, cytochrome *c* oxidase subunit 1 (CCOI). In addition, pyruvate kinase and calmodulin were increased, while cytochrome *c* oxidase subunit 3 and cytochrome *b* were decreased in the initial screen. A mitochondrial heavy strand gene product, cytochrome *c* oxidase (CCO), is the terminal complex of the mitochondrial respiratory chain, responsible for 90% of cellular oxygen consumption, and essential for cellular energy production. CCOI is the most critical of the 13 cytochrome *c* oxidase

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subunits [7]. In this study, we demonstrate that OPN inhibits expression of the mitochondrial protein, CCOI, as the result of an CD44-dependent mitochondrial H strand transcriptional regulatory mechanism.

## Materials and methods

*Cell culture.* RAW 264.7 cells were grown as monolayer cultures in DMEM with the addition of 10% FBS. Medium was changed every 3–4 days and the cells were maintained at 37 °C in a humidified atmosphere containing 5% $CO_2$. In selected instances, the cells were treated for 12 h with 10 nM murine osteopontin (Sigma, St. Louis, MO) in the presence or absence of the competitive inhibitors of integrin binding, GRGDSP, or CD44 binding, hyaluronate (HA) [8]. Cell viability, as measured by trypan blue exclusion, was not significantly different among the various treatment groups following 1, 6, and 12 h of OPN (10 nM) incubation.

*Differential screening of the subtracted cDNA library.* SSH was performed as previously described [6]. To identify genes differentially expressed in OPN-stimulated cells, RNA from OPN-treated cells was used as 'tester' and RNA from untreated Control cells was used as 'driver.' Differentially expressed sequences in subtracted cDNA were amplified by PCR to amplify only cDNA with different adaptors at both ends. Further enrichment was performed by a second PCR amplification with nested primers. The differentially expressed sequences were inserted into a T/A vector, pT-Adv cloning vector (Clontech). After a blue/white visual assay, PCR was used to rapidly amplify cDNA inserts. PCR products were blotted on nylon membrane (Hybond $N^+$, Amersham). Following hybridization, positive colonies were sequenced with the ABI PRISM 377 Genetic Analyzer (PE Applied Biosystems, Foster City, CA). Resulting sequences were compared to the GenBank database. Northern blot analysis was then utilized with probes derived from the cDNA sequence.

*Isolation of mitochondria.* Mitochondria were isolated using the ApoAlert Cell Fractionation Kit (Clontech). Purity and normalization of mitochondrial yield were confirmed by immunoblot analysis for the CCO IV mitochondrial protein marker.

*Cytochrome c oxidase activity.* Cytochrome *c* oxidase activity was determined using a spectrophotometric microtiter plate assay based upon the oxidation of cytochrome *c* by CCO and the subsequent re-reduction by 3,3′-diaminobenzidine-tetrachloride (DAB), resulting in an oxidized DAB polymer which is detectable at 450 nm [9]. The rate of formation is directly proportional to CCO activity. Results were normalized to succinate dehydrogenase activity and total cell protein.

*RNA preparation and Northern blot analysis.* Total RNA was isolated from RAW 264.7 macrophages using Trizol reagent (Gibco-BRL, Rockville, MD). The RNA samples (10 μg/lane) were fractionated by electrophoresis on a 1% agarose formaldehyde gel and transferred to Hybond-C nylon membrane (Amersham Pharmacia). [$^{32}$P]dATP-labeled probes were constructed based upon the murine CCOI (GenBank NF 001569; nt +616 to +1216) and the NADH:ubiquinone oxidoreductase subunit 6 (ND6; GenBank NF 001569; nt +13,550 to +14,000) mitochondrial cDNA sequence. Hybridization was performed at 42 °C for 18 h in ULTRAhyb hybridization buffer (Ambion, Austin, TX). Following hybridization, filters were washed twice and subjected to autoradiography. cDNA probes were prepared by random primer labeling, followed by purification using a Sephadex G-50 mini-column (BioMax, Odenton, MD).

*Immunoprecipitation and immunoblot analysis.* Cell culture medium was removed and plates were rinsed with PBS at room temperature. All the following steps were performed using ice-cold buffers. RIPA buffer (0.6 ml: 1× PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml PMSF, and 60 μg/ml aprotinin) was added to a 65 mm cell culture plate. Plates were scraped and the cells lysed. Ten microliters of 10 mg/ml PMSF stock was added followed by incubation for 30–60 min on ice. Whole cell lysate was precleared by adding 0.25 μg of normal mouse control IgG together with protein A–Agarose conjugate and incubation at 4 °C for 30 min. The beads were pelleted and the supernatant incubated with primary antibody (monoclonal mouse CCOI antibody). Resuspended protein A–Agarose was added and the tubes incubated at 4 °C on a rocker platform overnight. The pellet was collected by centrifugation at 1000*g* for 5 min at 4 °C and the supernatant discarded. The pellet was washed with RIPA buffer multiple times and resuspended in electrophoresis sample buffer. Protein concentration was determined by absorbance at 650 nm using protein assay reagent (Bio-Rad). Cell lysates (50 μg/lane) were separated by SDS–12% PAGE and the products were electrotransferred to PVDF membrane (Amersham Pharmacia). The membrane was blocked with 5% skimmed milk PBS-0.05% Tween for 1 h at room temperature. After being washed three times, blocked membranes were incubated with primary mouse CCOI monoclonal antibody (Molecular Probes, Eugene, OR) for 1 h at room temperature, washed three times in PBS-0.05% Tween, and incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. After an additional three times washing, bound peroxidase activity was detected by the ECL detection system (Amersham Pharmacia, Piscataway, NJ).

*Mitochondria run-on assays.* Macrophage mitochondria were prepared in lysis buffer (10 mM Tris–Cl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% Nonident P-40) and pelletted at 500*g*. The nuclei ($2 \times 10^7$) were resuspended in 100 μl glycerol buffer, then 150 μCi of [α-$^{32}$P]UTP (800 Ci/mmol) in 100 μl of 10 mM Tris–Cl, pH 8.0, 5 mM DTT, 5 mM $MgCl_2$, 300 mM KCl, and 1 mM (each) ATP, CTP, and GTP for 30 min at 30 °C was added. Labeled RNA was treated with 10 U RNase-free DNase I (GIBCO) for 5 min at 30 °C and extracted with phenol:chloroform (24:1) and chloroform alone. Before ethanol precipitation, 10 μg yeast tRNA was added and labeled RNA was treated with 0.2 M NaOH for 10 min on ice. The solution was neutralized by the addition of Hepes (acid free) to a final concentration of 0.24 M. After ethanol precipitation, the RNA pellet was resuspended in 10 mM *N*-tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid, pH 7.4, 0.2% SDS, and 10 mM EDTA. CCOI cDNA was spotted onto nylon membranes with a slot blot apparatus (Bio-Rad). NADH:ubiquinone oxidoreductase subunit 6 from the mitochondrial L-strand and pT-Adv vector served as positive and negative controls, respectively. In selected instances, 16s rRNA and cytochrome *c* oxidase subunit II cDNA (CCOII; GenBank NF 001569) were also utilized. Hybridization was performed at 42 °C for 48 h with $5 \times 10^6$ cpm of labeled RNA in hybridization buffer (50% formamide, 4× SSC, 0.1% SDS, 5× Denhardt's solution, 0.1 M sodium phosphate, pH 7.2, and 100 μg/ml salmon sperm DNA). After hybridization, the membranes were washed twice at room temperature in 2× SSC and 0.1% SDS, and three times at 56 °C in 0.1× SSC and 0.1% SDS. The membranes were exposed to X-ray film with an intensifying screen.

*Data analysis.* Data are expressed as means ± standard deviation of three or four assays. Statistical analysis was performed using the Student's *t* test. *P* values less than 0.05 were considered significant. All blots are representatives of four experiments.

## Results

### *CCOI Northern blot analysis*

Previous work has demonstrated elevated OPN serum levels of 8–15 nM in patients with metastatic colon, breast, and prostate cancer [10]. Therefore, in an attempt to mirror the in vivo clinical state, RAW 264.7 cells were incubated in the presence of 10 nM OPN for a period of 12 h. In subsequent SSH experiments, RAW

264.7 expression of CCOI mRNA was determined to be down-regulated in the presence of 10 nM OPN. Steady state mRNA levels of cellular CCOI were then determined by Northern blot analysis (Fig. 1). β-Actin and ND6 mRNA were used as measures of constitutive nuclear and L-strand mitochondrial gene expression. Of note, both β-actin and ND6 mRNA were unaltered by treatment conditions. OPN treatment decreased CCOI mRNA by over 10-fold in comparison to Control cells. The hexapeptide, GRGDSP, blocks binding of RGD-containing proteins, such as OPN, to cell surface integrins. OPN–integrin binding is thought to be an RGD-dependent process, while OPN–CD44 binding is an RGD-independent process which can be inhibited by the competitive CD44 ligand, hyaluronate (HA). In the presence of OPN and HA (50 nM), CCOI mRNA expression is unchanged in comparison to unstimulated Controls. Addition of OPN (50 nM) with OPN decreased CCOI mRNA expression in a fashion similar to that noted with OPN alone. These data indicate that OPN binds to its CD44 receptor to decrease steady state CCOI mRNA levels.

### *CCOI protein expression*

Cellular CCOI protein expression was determined using immunoblot analysis (Fig. 2). CCOIV protein, encoded by the nuclear genome, was used as a marker to normalize for levels of total mitochondria. Under all treatment conditions, CCOIV expression was unaltered, indicating that total mitochondrial mass and subunits of CCO encoded by the nuclear genome did not change with the various experimental conditions. OPN treatment decreased normalized CCOI protein by over 5-fold in comparison to Control cells. These results indicate that OPN inhibits CCOI protein expression.

RAW 264.7 cells were stimulated for 1 or 12 h with OPN. The cells were washed and media containing HA (50 nM) were added to inhibit OPN–CD44 binding. CCOI protein expression was then measured after an additional 6 h (Fig. 3). In both groups, CCO protein was restored to levels noted in unstimulated Control cells. These data suggest that OPN inhibits CCO protein expression in a reversible fashion.

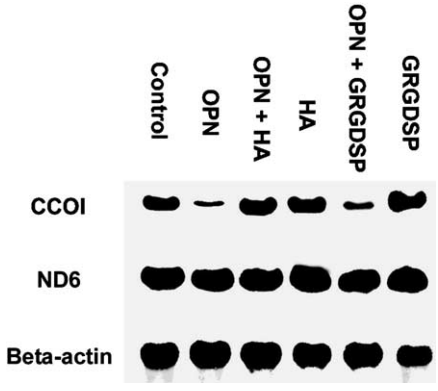


Fig. 1. Northern blot analysis of CCOI steady state mRNA expression. Steady state mRNA levels of cellular CCOI were determined by Northern blot analysis. β-Actin and NADH:ubiquinone oxidoreductase subunit 6 mRNA were used as measures of constitutive nuclear and L-strand mitochondrial gene expression. Northern blot analysis was performed as described in Materials and methods section. A [$^{32}$P]dATP-labeled 600 bp probe was constructed based upon the murine CCOI mitochondrial cDNA sequence (GenBank AF259518; nt +616 to +1216). OPN, osteopontin (10 nM); HA, hyaluronate (50 nM); CCOI, cytochrome *c* oxidase subunit 1; ND6, NADH:ubiquinone oxidoreductase subunit 6; GRGDSP, and hexapeptide inhibitor of OPN–integrin binding.

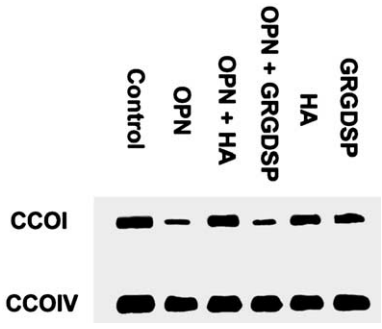


Fig. 2. Immunoblot analysis of CCOI and CCOIV protein expression. CCOI and CCOIV protein expression were determined as measures of mitochondrial and nuclear encoded CCO protein subunits, respectively. In selected instances, cells were stimulated with OPN (10 nM) for 12 h. Mitochondria were isolated using the ApoAlert Cell Fractionation Kit (Clontech). Purity and normalization of mitochondrial yield were confirmed by immunoblot analysis for the CCOIV mitochondrial protein marker. Immunoprecipitation and immunoblot analysis were performed as described in Materials and methods section. OPN, osteopontin (10 nM); HA, hyaluronate (50 nM); CCOI, cytochrome *c* oxidase subunit 1; CCOIV, cytochrome *c* oxidase subunit 4; and GRGDSP, hexapeptide inhibitor of OPN–integrin binding.

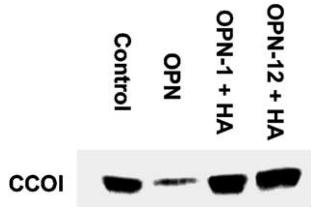


Fig. 3. Reversibility of OPN-mediated inhibition of CCOI protein expression. CCOI protein expression was determined in Control and OPN (10 nM × 12 h) stimulated cells. In selected instances, the cells were washed following 1 h (OPN-1 and HA) and 12 h (OPN-12 and HA) of incubation in the presence of OPN and new media containing GRGDSP were added. CCOI protein expression was then determined after 6 h. Mitochondria were isolated using the ApoAlert Cell Fractionation Kit (Clontech). Immunoprecipitation and immunoblot analysis were performed as described in Materials and methods section. OPN, osteopontin (10 nM); CCOI, cytochrome *c* oxidase subunit 1; and HA, inhibitor of OPN–CD44 binding.

*Cellular cytochrome c oxidase activity*

Cellular CCOI enzyme activities were normalized to succinate dehydrogenase activity and total cell protein and determined after 1, 6, and 12 h of OPN treatment (Table 1). One hour following OPN stimulation, CCO activity was decreased by ~50% in comparison to unstimulated Control cells. Addition of HA with OPN resulted in restoration of CCO activity. HA alone did not alter CCO activity. Following 6 and 12 h of stimulation, there was a serial decline in CCO activity in OPN-treated cells. At 12 h of stimulation, OPN inhibited CCO activity was 20% of that noted in Controls. Cell viability, as measured by trypan blue exclusion, was not significantly different among the various treatment groups following 1, 6, and 12 h of incubation. In subsequent studies, 1 and 12 h treatment groups of LPS-stimulated cells were washed, media containing GRGDSP were added to inhibit OPN binding, and CCO activity measured after an additional 6 h. In both groups, CCO activity ($1.8 \pm 0.3$/mg protein) was restored to levels noted in unstimulated Control cells. These data suggest that OPN inhibits CCO enzyme activity in a reversible fashion.

*CCOI mRNA half-life*

CCOI mRNA half-life was determined using actinomycin (100 μM) and performing the assays at 0 °C to inhibit mitochondrial RNA polymerase. (Data not shown.) These conditions were selected because mitochondrial RNA polymerase is resistant to normal inhibitors of nuclear RNA polymerase [11]. One hour following stimulation, actinomycin D was added. Again, densitometric measures of mRNA were normalized to 16s rRNA expression. In Control and OPN cells, CCOI mRNA half-life was found to be $2.9 \pm 0.2$ and $3.1 \pm 0.3$ h, respectively, indicating that OPN does not accelerate CCOI mRNA degradation.

*Mitochondrial CCOI transcription*

In most instances, the rate of transcription determines relative differences in mRNA expression. To determine the effect of OPN-mediated NO synthesis on mitochondrial gene transcription, mitochondrial run-on assays were performed (Fig. 4). The mitochondrial genome is a closed circular double-stranded DNA molecule of ~16.6 kb that is highly conserved among mammals [7]. Transcription is polycistronic. The H-strand encodes two rRNAs, 14 tRNAs, and 12 polypeptides, including CCOI. The L strand codes for eight tRNAs and a single polypeptide, ND6 [7]. Mitochondrial L-strand, as represented by ND6, transcription was used as a positive control, while pT-Adv vector served as a negative control. In the setting of OPN treatment, CCOI transcription was significantly decreased. GRGDSP did not alter the OPN-mediated change in CCOI transcription. In OPN + HA treated cells, the extent of CCOI transcription was not significantly different from that of Controls. As noted previously, mitochondrial gene transcription is polycistronic. Therefore, we performed corroborating experiments using 16s rRNA and CCOII, which are also encoded on the H strand. In a fashion similar to that seen for CCOI, gene transcription for CCOII was also significantly diminished in the presence of OPN. However, 16s rRNA was unaltered. These results indicate that the OPN acts through the CD44 receptor to decrease transcription of CCOI and CCOII. L strand transcription is unaltered. Two potential initiation sites in the H-strand promoter control transcription of the H strand. The genes encoded by the H-strand are classified into two categories: the promoter-proximal region encoding the $tRNA^{Phe}$,

Table 1
Cellular cytochrome *c* oxidase activity

| | 1 h | 6 h | 12 h |
|---|---|---|---|
| Control | $1.9 \pm 0.3$ | $1.7 \pm 0.3$ | $2.0 \pm 0.2$ |
| OPN (10 nM) | $0.8 \pm 0.2^*$ | $0.5 \pm 0.2^*$ | $0.3 \pm 0.1^*$ |
| OPN + GRGDSP (50 nM) | $0.9 \pm 0.1^*$ | $0.5 \pm 0.3^*$ | $0.1 \pm 0.1^*$ |
| OPN + HA (50 nM) | $1.8 \pm 0.2$ | $1.7 \pm 0.2$ | $2.1 \pm 0.1$ |

Data, normalized to succinate dehydrogenase activity and total cell protein, are expressed as mg protein$^{-1}$. Values are presented as means ± SEM of four experiments. OPN, osteopontin; HA, hyaluronate; and GRGDSP, hexapeptide inhibitor of OPN–integrin binding.
$^*$ $p < 0.05$ vs Control and OPN + HA.

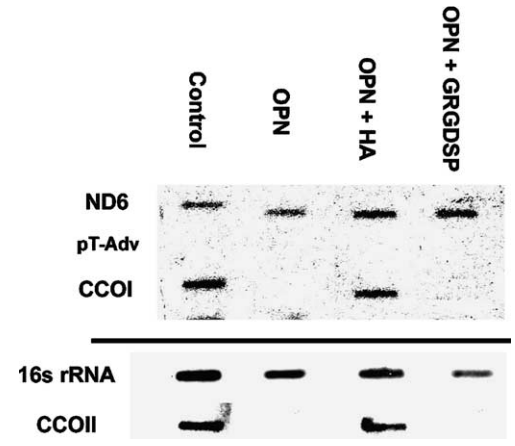


Fig. 4. Mitochondrial run-on analysis of CCOI transcription. To determine the effect of LPS-mediated NO synthesis on mitochondrial gene transcription, mitochondrial run-on assays were performed. ND6 was chosen as a surrogate for L-strand transcription and used as a positive control, while pT-Adv vector served as a negative control. Run-on analysis was performed as described in Materials and methods section. OPN, osteopontin (10 nM); HA, hyaluronate (50 nM); CCOI, cytochrome *c* oxidase subunit 1; CCOII, cytochrome *c* oxidase subunit 2; 16s rRNA, 16s ribosomal RNA; ND6, NADH:ubiquinone oxidoreductase subunit 6; and GRGDSP, hexapeptide inhibitor of OPN–integrin binding.

12S rRNA, and the 16S rRNA genes and the promoter-distal region which encodes the majority of mRNAs (including CCOI and CCOII), tRNAs, and the 0.8 kb D-loop region RNA of unknown function. Given the polycistronic nature of mitochondrial transcription, these data suggest that OPN inhibits transcription of the promoter-distal region of the H strand.

## Discussion

In this study, utilizing OPN stimulation of RAW 264.7 murine macrophages, we have demonstrated that OPN significantly decreases both CCO activity and CCOI protein expression by decreasing CCOI transcription. In addition, we demonstrate that cytochrome *c* oxidase activity is rapidly decreased in the presence of OPN (~60% decrement after one hour). In the setting of unaltered CCOI mRNA half-life of ~3 h and the slow turnover of mammalian cytochrome *c* oxidase proteins, we must also invoke an OPN-mediated alteration in turnover or activity of a component protein of the CCO complex.

A mitochondrial heavy strand gene product, cytochrome *c* oxidase (CCO), is the terminal complex of the mitochondrial respiratory chain, responsible for 90% of cellular oxygen consumption, and essential for cellular energy production. The primary three subunits, CCOI, II, and III, are encoded by mitochondrial DNA and perform the catalytic functions of the holoenzyme. CCOI binds three of the enzyme's redox centers (heme a, heme $a_3$, and $Cu_B$) and is the most highly conserved member of the entire CCO complex. As such, it is considered to be the most critical of the 13 cytochrome *c* oxidase subunits [7,8,12–14]. In theory, inhibition of CCOI protein expression by OPN should dramatically decrease CCO holoenzyme function and inhibit mitochondrial respiration.

The mitochondrial genome is a closed circular double-stranded DNA molecule of ~16.6 kb that is highly conserved among mammals [7]. The H-strand encodes two rRNAs, 14 tRNAs and 12 polypeptides, including CCOI. The L strand codes for eight tRNAs and a single polypeptide, ND6. All of the 13 polypeptide products are constituents of enzyme complexes of the oxidative phosphorylation system. Both the H- and L-strands of the mtDNA in vertebrate cells are transcribed symmetrically, and nearly completely as polycistronic precursor RNAs starting from strand-specific promoters, HSP, and LSP. Transcription and replication depend upon trans-acting nuclear encoded factors. Two potential initiation sites in the HSP control transcription of the H strand. The genes encoded by the H-strand are classified into two categories: the promoter-proximal region encoding the $tRNA^{Phe}$, 12s rRNA, and the 16s rRNA genes and the promoter-distal region which encodes the majority of mRNAs, tRNAs, and the 0.8 kb D-loop region RNA of unknown function. The majority of the mt RNA species is processed from larger polycistronic precursors. The promoter-proximal and -distal regions of the H-strand are expressed at distinctly different rates: the promoter-proximal region is transcribed at 40- to 80-fold higher rate than the promoter-distal region [15]. Our run-on data suggest that transcription of the promoter-distal regions is inhibited by OPN.

Mechanisms have been described which cause transcriptional termination of the mt distal H strand. Investigators have identified a tridecamer DNA sequence that supports transcription termination at the end of the 16S rRNA gene. The putative tridecamer terminator sequence has been mapped to the 5′ end of the $tRNA^{Leu}$ gene, which occurs immediately downstream of the 16S rRNA gene [16,17]. A 36-kDa protein, which binds to the tridecamer sequence motif, has been purified and characterized by cDNA cloning [18]. This protein, termed the mt transcription termination factor (mTERF) binds to the promoter-proximal terminator sequence (mt-TERM) and promotes transcription termination. Additional studies by Fernandez-Silva et al. [19] suggest that the binding of sea urchin mt displacement (D)-loop binding protein (mtDBP) to the 133 bp region between $tRNA^{Thr}$ and $tRNA^{Pro}$ leads to transcription termination. Sea urchin mtDBP exhibits bipolar transcription termination factor. Finally, Camasamudram has also described a novel sequence-specific termination of the mouse mt H-strand distal transcripts. They characterized the corresponding binding proteins (D-TERM) and found that they bind in a sequence-specific manner to terminator motif localized in the D-loop of mouse mt DNA. However, in contrast to the findings of Fernandez-Silva, these D-TERM proteins exhibit polarity [15].

In a manner reminiscent of these D-TERM proteins, OPN appears to inhibit transcription of the mt distal H strand possibly by early termination, while preserving L-strand transcription. This effect is mediated through binding to CD44 and is reversible. In contrast to our previous findings with nitric oxide mediated inhibition of CCOI expression, there is no change in CCOI mRNA half-life. Our results suggest that tumor cell elaboration of high concentration OPN may promote tumor survival by inhibition of macrophage respiratory function. This represents a novel mechanism that has not been previously described [11,20,21].

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