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# *SEL1L* Affects Human Pancreatic Cancer Cell Cycle and Invasiveness through Modulation of *PTEN* and Genes Related to Cell–Matrix Interactions

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**Abstract**

**Previously, it was reported that SEL1L is able to decrease the aggressive behavior of human pancreatic tumor cells both *in vitro* and *in vivo*. To gain insights into the involvement of SEL1L in tumor invasion, we performed gene expression analysis on the pancreatic cancer cell line Suit-2 subjected to two complementary strategies: upregulation and downregulation of *SEL1L* expression by stable transfection of the entire cDNA under an inducible promoter and by RNA-mediated interference. SuperArray and real-time analysis revealed that *SEL1L* modulates the expression of the matrix metalloproteinase inhibitors *TIMP1* ($P < .04–.03$) and *TIMP2* ($P < .03–.05$), and the *PTEN* gene ($P < .03–.05$). Gene expression modulations correlate with the decrease in invasive ability ($P < .05$) and in accumulation of *SEL1L*-expressing cells in G1. Taken together, our data indicate that *SEL1L* alters the expression of mediators involved in the remodeling of the extracellular matrix by creating a microenvironment that is unfavorable to invasive growth and by affecting cell cycle progression through promotion of G1 accumulation.**

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**Keywords:** *SEL1L*, cell cycle, cancer invasion, extracellular matrix–related genes, siRNA.

## Introduction

Most studies on *SEL1L*, the human ortholog of the *Caenorhabditis elegans sel-1* gene [1], have focused on its role in cancer development and have provided significant *in vitro* and *in vivo* evidence to link its increased expression to a decrease in tumor aggressiveness.

Previously, it was reported that ectopic and inducible expression of *SEL1L* reduces the aggressive behavior of breast cancer cells, possibly involving cell–matrix interactions. Moreover, its downregulation has been significantly correlated to poorer outcome in cancer patients [2]. A similar situation was also found in pancreatic cancer, where inducible expression of *SEL1L* in stably transfected cells caused both a decrease in clonogenity and anchorage-independent growth and a delayed tumor growth when inoculated in immunodeficient mice [3]. To investigate the involvement of *SEL1L* in human breast cancer biology, we used proteomic approach and global expression screening (Affymetrix platform) and found that *SEL1L* ectopic expression changed the levels of proteins and transcripts that operate in different signaling pathways and in cytoskeletal reorganization; several tumor-associated proteins were also modulated [4].

SEL1L activation has been reported in the early stages of esophageal, prostate, and non–small cell lung cancers [5,6] (Ferrero et al., unpublished).

The function of SEL1L may be associated with degradation or trafficking of several proteins, as revealed in yeast, plant, and nematode studies. In *Saccharomyces cerevisiae*, the HRD3 protein is required for the degradation of malfolded endoplasmic reticulum (ER)–resident proteins [7]. In *C. elegans*, sel-1, along with ABU-1, is a component of cell survival pathway that is induced when unfolded proteins accumulate in the ER [8]. The SEL1L protein of *Arabidopsis thaliana* is a membrane-anchored glycoprotein that increases under ER stress [9]. It has also been reported that the human *SEL1L* gene is induced in response to ER stress and contributes, along with HRD1, to the protection of cells by degrading unfolded proteins accumulated in the ER [10]. ER stress has been implicated in the pathogenesis of a variety of human diseases, including neural degenerative diseases, diabetes, viral pathogenesis, and cancer [11].

To understand the role of *SEL1L* in human pancreatic cancer, we used the cell line Suit-2 containing the entire *SEL1L* cDNA [3] and two complementary technical strategies: cDNA macroarray (GEArray Q Series Human Tumor Metastasis Gene Array; DBA, Segrate, Italy) and RNA-mediated interference of *SEL1L de novo* transcription.

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Here we demonstrate that *SEL1L* triggers an antitumor response by modulating the expression of *TIMP1*, *TIMP2*, and *PTEN* genes. All these gene products have widely been studied in several aspects of cancer development and progression [12,13]. Here we also show that gene expression modulations observed in *SEL1L*-expressing cells correlate with their decreased invasive ability and with alteration of cell cycle progression.

## Materials and Methods

### *Cell Culture and* In Vitro *Induction of Exogenous* SEL1L *Transcript*

The human pancreatic cancer cell line Suit-2 stably transfected with the entire *SEL1L*-coding region driven by a dexamethasone (DEX)–inducible promoter as well as control mock-transfected cells were grown in RPMI 1640 (Microbiological Associates, Walkersville, MD) supplemented with 10% fetal bovine serum (Hyclone, Euroclone, Devon, UK), penicillin/streptomycin (100 IU/ml), and G418 sulfate antibiotic (200 μg/ml) in a humidified chamber (95% air and 5% $CO_2$) at 37°C. Cells were regularly seeded to maintain exponential growth.

For experimental purposes, the cells were treated for 7 to 14 days with 1 μM DEX, after which they were harvested and analyzed by reverse transcription–polymerase chain reaction (RT-PCR), Northern blot analysis, and Western blot analysis.

### *Northern Blot Analysis*

Total RNA (20 μg) from cultured cells was isolated using the Total Quick RNA Kit (Talent, Trieste, Italy) and fractionated in denatured agarose gels using well-standardized procedures. Gel-purified, double-stranded DNA probes were labeled with [$^{32}$P]dCTP using a random primer labeling kit (Promega, Madison, WI). Hybridization was performed overnight at 42°C. RNA loading was normalized by hybridizing the stripped blots with $^{32}$P-labeled *HPRT* DNA probe. Northern blots were imaged and quantified using the Quanty one program (Bio-Rad Laboratories S.r.l., Segrate, Italy). The experiments were repeated twice using independent RNA preparations. The oligonucleotide primer sequences used to amplify the DNA probes are as follows:

| | |
|---|---|
| *MMP1* | 5′-cctccactgctgctgctgct-3′ |
| | 5′-gggagagtccaagagaatgg-3′ (770 bp) |
| *MMP7* | 5′-gaatgttaaactcccgcgtc-3′ |
| | 5′-catccgtccagcgttcatc-3′ (390 bp) |
| *TIMP1* | 5′-ccctggcttctggcatcctg-3′ |
| | 5′-ggacctgtggaagtatccgc-3′ (280 bp) |
| *TIMP2* | 5′-gtagtgatcagggccaaagg-3′ |
| | 5′-ctggtacctgtggttcaggc-3′ (320 bp) |
| *PTEN* | 5′-cgaactggtgtaatgatatg-3′ |
| | 5′-catgaacttgtcttcccgtc-3′ (330 bp) |
| *HPRT* | 5′-aattatggacaggactgaacgtc-3′ |
| | 5′-cgtggggtccttttcaccagcaag-3′ (388 bp) |

### *RT-PCR Analysis*

Total RNA (1 μg) treated with RNase-free DNaseI (Clontech, Palo Alto, CA) was used in each RT reaction containing 5 μM $MgCl_2$, 1× reaction buffer [50 mM Tris–HCl (pH 8.8), 8 mM $MgCl_2$, 30 mM KCl, and 1 mM dithiothreitol], 1 μM dNTPs, 5 U of RNase inhibitor (RNAsin), 0.8 μg of oligo-$p(dT)_{15}$ primer, 1.6 μg of random primer, and 15 U of Avian Myeloblastosis Virus Reverse Transcriptase (Amersham, Piscataway, NJ). The reaction mixture was incubated for 10 minutes at 25°C, and for 60 minutes at 42°C. The enzyme was denatured at 99°C for 5 minutes and chilled on ice. PCR amplifications were performed with a Perkin-Elmer (Foster City, CA) thermal cycler using 2 μl of RT product per reaction. PCR conditions used to specifically detect *SEL1L* induction and downmodulation were as follows: 94°C for 3 minutes, followed by 20 to 26 cycles at 94°C for 1 minute, annealing at 60°C for 1 minute, extension at 72°C for 1 minute, and a final extension at 72°C for 5 minutes.

PCR conditions used to detect constitutive *HPRT*, *Activin receptor II*, and *TIMP1–TIMP2–MMP1–MMP7–PTEN–Activin A* expressions were as follows: 3 minutes at 94°C, followed by 23 to 26 cycles at 94°C for 1 minute, annealing at 55°C to 63°C for 1 minute, extension at 72°C for 1 minute, followed by a final extension at 72°C of 5 minutes.

All the PCR products were electrophoresed on 1.5% agarose gels and stained with ethidium bromide.

Sets of primers used to amplify *HPRT* and *MMP7* genes are listed above, whereas the *SEL1L* primer set is as follows:

| | |
|---|---|
| *SEL1L* | 5′-CCTCAGAGTAATGAGACAGCTCTCC-3′ |
| | 5′-GCCACTGGCATGCATCTGAGC-3′ (314 bp) |

### *cDNA Expression Array*

The commercially available SuperArray (GEArray Q Series Human Tumor Metastasis Gene Array; DBA) was used to compare the gene expression profiles of the pancreatic cancer cell line stably transfected with *SEL1L* cDNA and pDEX.1 constructs. The arrays were handled following the manufacturer's protocol. Briefly, total RNA (3 μg) from each cell line was reverse-transcribed into $^{32}$P-labeled cDNA using MMLV reverse transcriptase. Probes were purified and used to hybridize the filters overnight at 60°C. Blots were then washed twice with 2× SSC/1% SDS and with 0.1× SSC/0.5% SDS at 60°C. Damp membranes were wrapped immediately and exposed to X-ray film. The GEArray Analyzer version 1.2 software was used to interpret the results. The SuperArray contains 96 functionally well-characterized genes involved in several tumor metastatic processes and grouped according to their functions and structural features into seven categories, including growth factors and receptors, cell–cell and cell–matrix interaction molecules, metastasis-associated proteases, protease inhibitors, signal transduction molecules, oncogenes, and metastasis suppressors. Controls are represented by four potentially normalized

features (glyceraldehyde-3-phosphate dehydrogenase, peptidylprolyl isomerase A, ribosomal protein L13a, and β-actin), plasmid pUC18, and blank. The experiment was performed more than three times using different preparations of RNA. Student's *t* test was used to determine statistical significance.

*Western Blot Analysis*

Cells were lysed using a buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 10% glycerol, 0.5% NP40, 10 μg/ml aprotinin, and 1 μM phenylmethylsulfonyl fluoride. Protein concentration was determined by the Bradford assay; 40 μg of each sample was resolved in 10% SDS polyacrylamide gel, and Western blot analysis was performed as previously described [14]. Monoclonal antibody MSEL1 was used at 10 μg/ml.

*siRNA Transfection*

Two different chemically synthesized siRNA targeting *SEL1L* (NM_005065) (siRNA-SEL1L-1 and siRNA-SEL1L-2) and one scrambled siRNA (siRNA control) were purchased from Ambion, Huntingdon, UK (predesigned siRNA-annealed standard purity). The siRNA were introduced into Suit-2 cells using two different transfection agents (siPORT amine and siPORT lipid; Ambion), according to the manufacturer's instructions. Briefly, the cells were seeded into six-well plates at a density of $1 \times 10^5$ cells/well. The transfection agents (3 μl of siPORT lipid and 8 μl of siPORT amine) and the siRNA complex were added to the cells and incubated for 24, 48, and 72 hours. The final concentration of siRNA was 60 mM. Each assay was performed in duplicate in at least five independent experiments.

*Real-Time PCR*

Real-time PCR was used to quantify the expression of target genes in siRNA-Suit-2 cells, using the iCycler iQ real-time detection system (Bio-Rad) and SYBR Green. Real-time PCR amplifications were performed using 20 ng of cDNA, 2× Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, S. Giuliano M.se, Italy), 50× Rox Dye, and 100 nM forward and reverse gene-specific primers. The cycling program was as follows: 5 minutes at 95°C, followed by 40 cycles of 15 seconds at 94°C, 30 seconds at 60°C, and 50 seconds at 72°C. To assess PCR specificity, melting curves from 55°C to 95°C in 0.5°C steps of 10 seconds each were generated. A relative standard curve was generated by amplifying serial dilutions of cDNA obtained from siRNA-control-Suit-2. The standards and cDNA samples were amplified in triplicate in the same reaction plate in at least three independent experiments. The amount of product in each sample was quantified using the threshold cycle ($C_t$) method normalized to the ribosomal protein S14 gene. PCR efficiencies of the reactions were between 85% and 95%. Fold increase or fold decrease in the expression of the target gene relative to ribosomal protein S14 in each sample was calculated by the formula: $2^{-\Delta\Delta C_t}$, where $\Delta C_t = C_t$ target gene $- C_t$ S14 and $\Delta\Delta C_t = \Delta C_t$ test sample $- \Delta C_t$ control. Student's *t* test was used to determine statistical significance.

The sets of primers used to amplify the target genes are as follows:

| | |
|---|---|
| Ribosomal | 5′-ggcagaccgagatgaatcctca-3′ |
| *protein S14* | 5′-caggtccaggggtcttggtcc-3′ (123 bp) |
| *SEL1L* | 5′-ctcgctaacaggaggctcagta-3′ |
| | 5′-catggcatgtgaattgccag-3′ (251 bp) |
| *TIMP1* | 5′-ccctggcttctggcatcctg-3′ |
| | 5′-ggacctgtggaagtatccgc-3′(280 bp) |
| *TIMP2* | 5′-aagcggtcagtgagaagga-3′ |
| | 5′-tctcaggccctttgaacatc-3′ (108 bp) |
| *PTEN* | 5′-cgacgggaagacaagttcat-3′ |
| | 5′-aggtttcctctggtcctggt-3′ (163 bp) |
| *MMP1* | 5′-ggtctctgagggtcaagcag-3′ |
| | 5′-tggtccacctttcatgttca-3′ (161 bp) |
| *MMP7* | 5′-tgctcacttcgatgaggatg-3′ |
| | 5′-tggggatctccatttccata-3′ (159 bp) |
| *Activin A* | 5′-ggagggcagaaatgaatgaa-3′ |
| | 5′-ccttggaaatctcgaagtgc-3′ (102 bp) |
| *Activin receptor II* | 5′-acacagcccacttcaaatcc-3′ |
| | 5′-aggagggtaggccatcttgt-3′ (144 bp) |

In Vitro *Invasion Assay*

Cell invasion assay was performed using the Chemicon International system. The kit contains 24 inserts with a polycarbonate membrane (8 μm pore size) coated with a thin layer of ECMatrix (Chemicon International, Temecula, CA), a reconstituted basement membrane matrix of proteins derived from the Engelbreth-Holm-Swarm mouse tumor. Briefly, cells untreated and treated with 1 μM DEX for 1 week were trypsin-detached and placed in 300 μl of serum-free medium containing 5% BSA in the presence or absence of DEX (1 μM) over the inner chamber of the insert at a density of $1.25 \times 10^5$, whereas 500 μl of serum-free medium containing 20 ng/ml epidermal growth factor (chemoattractant) was placed in the outer chamber of the insert. The plates were incubated for 48 hours at 37°C. Noninvading cells that remained on the upper surface of the insert were removed, whereas invasive cells that migrated to the lower surface of the membrane were stained and counted under a fluorescence plate reader at 485 nm/535 nm. Each assay was carried out in triplicate in at least three independent experiments. Student's *t* test was used to determine statistical significance.

*Colony Formation on Soft Agar*

Cells untreated and treated with 1 μM DEX for 1 week were trypsin-detached and seeded in duplicate in six-well plates at a density of $15 \times 10^3$ cells/well in a semisolid medium containing 0.3% Bacto-Agar (Difco, Detroit, MI) over a 0.8% agarose layer containing DEX, where indicated. Fresh medium was added weekly. Colony formation was scored after 14 days of growth. Each assay was carried out in duplicate in at least three independent experiments.

*Cell Cycle Analysis*

Cells untreated and treated with 1 μM DEX for 1 week were trypsin-detached, washed with PBS, and incubated for 15 minutes with 0.37% NP40 and 20 μg/ml RNAse A (Sigma,

St. Louis, MO) in 1% sodium citrate (pH 7.4). Samples were then stained for 15 minutes with 1 μg/ml DAPI and analyzed on a FACSVantage instrument (Becton Dickinson, Milan, Italy) using an ultraviolet excitation laser beam.

## Results

### *Identification of Genes Modulated by* SEL1L *Induction in Pancreatic Cancer Cells*

Previously, it was reported that the inducible expression of *SEL1L* in the human pancreatic tumor cell line Suit-2 strongly inhibited its anchorage independence and delayed tumor growth in nude mice [3]. To gain insights into molecular mechanisms underlying tumor regression, we compared the gene expression profile of induced Suit-2-SEL1L to uninduced parental cells and induced/uninduced vector-transfected clones (Suit-2-pDEX.1) using filter macroarrays. The SuperArray (GEArray Q Series Human Tumor Metastasis Gene Array; DBA) used in our study consists of 96 functionally well-characterized genes involved in several tumor metastatic processes, four normalized housekeeping genes, and plasmid sequences. Macroarray analysis was performed more than three times using harvested cells that were treated for 7 days with DEX, as previously reported [3].

Among the seven gene categories comprising the array, ''cell–cell and cell–matrix interactions'' and ''metastasis supressors'' displayed significant modulation. Two *MMPs* (*MMP1* and MMP7) of a total of 23 proteases associated with metastasis were significantly downmodulated, whereas two protease inhibitor genes *TIMP1* and *TIMP2* out of eight were upregulated, in addition to the metastasis-suppressor gene *PTEN* (Table 1).

To validate macroarray results, Northern blot analysis was performed using selected gene fragments as probes. A clear and constant decrease of *MMP1* and *MMP7* gene expression and an increase of *TIMP1* and *TIMP2* transcription were observed in induced Suit-2-SEL1L cells, confirming filter array results (Figure 1). A 3.5–3.1-fold and a 4.2–3.7-fold decrease in the expression of *MMP1* and MMP7, respectively, as well as a 1.6–1.53 and 1.3–1.25-fold increase in the expression of *TIMP1* and *TIMP2*, respectively (Table 1 and Figure 1), were observed when the Suit-2-SEL1L cells were treated with DEX for 7 days. A further effect was seen when the Suit-2-SEL1L cells were treated with DEX (*MMP1* and *MMP7* gene expression decreased by 2.2–2.0 and 2.7–2.35-fold, respectively, whereas *TIMP1* and *TIMP2* increased by 1.8–1.63 and 1.7–1.8-fold, respectively) for 14 days (Table 1 and Figure 1).

A clear increase in the tumor-suppressor gene *PTEN*, a key element in controlling cell growth and survival, was observed in induced Suit-2-SEL1L cells with respect to controls (1.85–1.7 to 13–11-fold for 7 and 14 days of DEX treatment, respectively) (Table 1 and Figure 1).

Table 1 indicates fold changes in gene expression using the GEArray Analyzer software and the Quanty one program for Northern expression analysis. These values are significant, and the respective *P* values are reported in Table 1.

### *RNA-Mediated Interference of* SEL1L *mRNA in Suit-2 Cells*

To determine if inhibition of *SEL1L* mRNA would specifically affect genes encoding for cell–matrix proteins as well as *PTEN*, the Suit-2 cells were transiently transfected with two target-specific SEL1L-siRNA (siRNA-SEL1L-1 and siRNA-SEL1L-2, designed based on the second exon of *SEL1L* cDNA). The specificity of RNAi was assessed by simultaneously transfecting the Suit-2 cells with a scrambled siRNA (siRNA-control). Two transfection agents (siPORT lipid and siPORT amine; Ambion) were used, and each transfection was carried out for more than three times per transfection reagent.

Table 2 shows the fold decrease in *SEL1L* expression as evaluated by quantitative RT-PCR (qRT-PCR). *SEL1L* transcription was inhibited from 3- to 2.5-fold over control after 48 hours of siRNA-SEL1L-1 treatment. Gene silencing was already visible 24 hours after transfection, reached an optimum after 48 hours, and lasted for at least another 24 hours (data not shown). *SEL1L* mRNA silencing was stronger with siRNA-SEL1L-1 than siRNA-SEL1L-2, and no effects were observed on the ribosomal protein S14 gene, which was used as an internal control. Comparable results were obtained with the two transfection agents used.

**Table 1.** Summary of Differentially Expressed Genes Identified by Macroarray and Northern Blot Analysis in Suit-2-SEL1L *versus* Suit-2-pDEX.1 Cells.

| Gene | GenBank ID | Function | Fold Changes*,† | | |
|---|---|---|---|---|---|
| | | | Macroarray* | Northern Blot Analysis† | |
| | | | 7 days‡ | 7 days‡ | 14 days§ |
| *Genes downregulated* | | | | | |
| *MMP1* | X05231 | Metastasis-associated proteases | 2 ± 0.2 ($P < .05$) | 3.5–3.1 | 2.2–2 |
| *MMP7* | X07819 | Metastasis-associated proteases | 1.8 ± 0.1 ($P < .05$) | 4.2–3.7 | 2.7–2.35 |
| *Genes upregulated* | | | | | |
| *TIMP1* | NM_003254 | Protease inhibitors | 2 ± 0.18 ($P < .04$) | 1.6–1.53 | 1.8–1.63 |
| *TIMP2* | NM_003255 | Protease inhibitors | 1.3 ± 0.04 ($P < .03$) | 1.3–1.25 | 1.7–1.8 |
| *PTEN* | U96180 | Metastasis suppressor | 3.7 ± 0.4 ($P < .03$) | 1.85–1.7 | 13–11 |

*The reported ratio indicates the observed average fold increase or fold decrease of gene expression in the induced Suit-2-SEL1L cells *versus* the uninduced parental cells and the induced/uninduced mock control (± SD).
†The reported ratio indicates the observed two-fold increase or decrease of gene expression in the induced Suit-2-SEL1L cells *versus* the uninduced parental cells and the induced/uninduced mock control.
‡Cells were treated with DEX for 7 days.
§Cells were treated with DEX for 14 days.

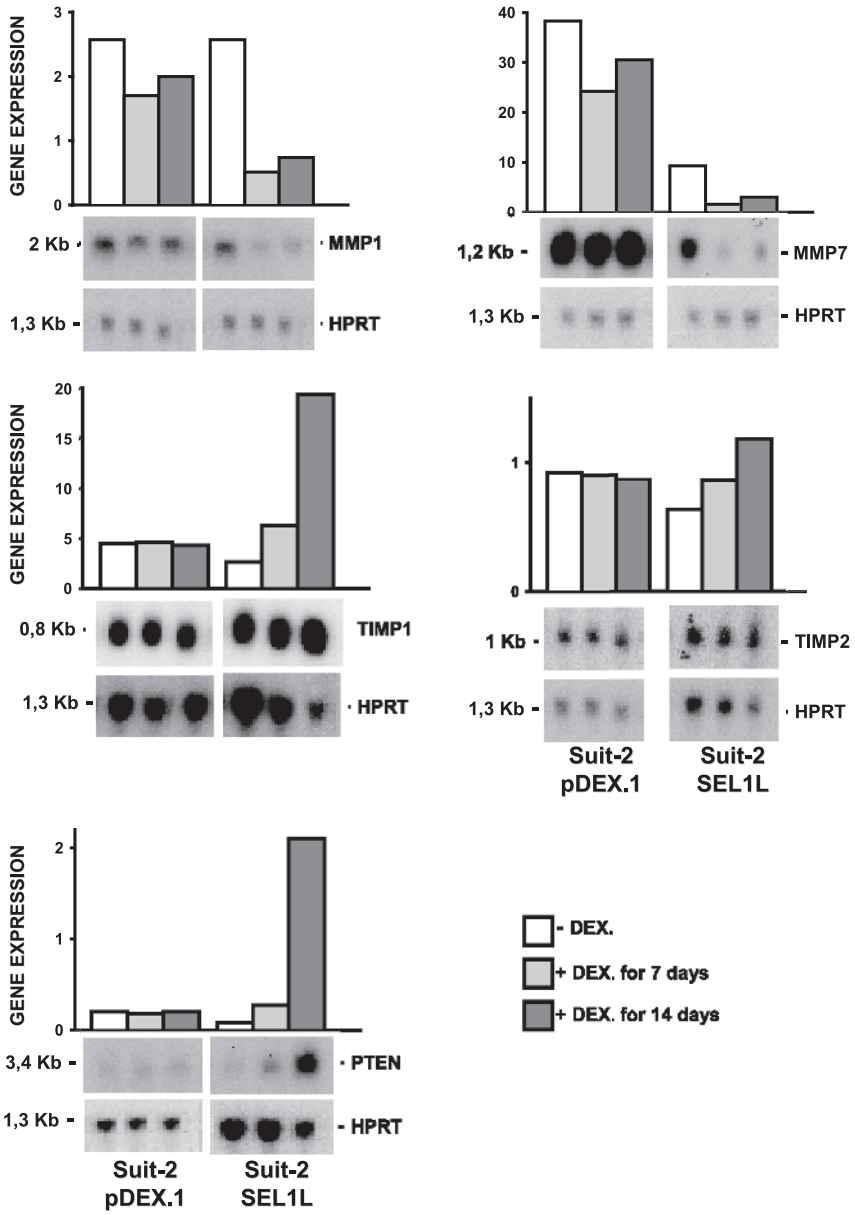


**Figure 1.** *Northern blot hybridization of RNA extracted from untreated and DEX-treated (for 7 and 14 days, respectively) Suit-2-SEL1L and Suit-2-pDEX.1 cells using MMP1, MMP7, TIMP1, TIMP2, and PTEN coding DNA fragments as probes. HPRT expression was used as endogenous control. Histograms show normalized values of gene expression, as determined by imaging through a Quanty one program, and correspond to the representative blot of two experiments using independent RNA preparations.*

Figure 2*A* shows RT-PCR experiments. As a consequence of *SEL1L* interference, Suit-2 cells underwent significant morphologic changes in that the cells grew in a disorganized manner, increased in cell volume, lost cell–cell contact, and acquired a spindle-type shape with numerous extrusions (Figure 2*B*). Cell growth rate, however, was unaffected, confirming previous results.

SEL1L *Directly Modulates the Transcription of* TIMP1*,* TIMP2*,* PTEN*,* Activin A*, and* Activin Receptor II

We looked at the modulation of *MMP1*, *MMP7*, *TIMP1*, *TIMP2*, and *PTEN* transcription in the Suit-2 cells treated with siRNA-SEL1L-1 and siRNA-control by qRT-PCR. Although *TIMP1*, *TIMP2*, and *PTEN* significantly decreased their expression (2.4- to 2-fold, 2- to 1.5-fold, and 2- to 1.5-fold, respectively) after *SEL1L* downregulation, the two *MMPs* (*MMP1* and *MMP7*) were unaffected. However, a decrease in *Activin A* and *Activin receptor II* expression was observed (2- to 1.5-fold and 2- to 1.5-fold, respectively). *Activin A*—but not *Activin receptor II*—modulation was in agreement with data previously obtained in response to *SEL1L* induction [3,15]. Relative *P* values are listed in Table 2.

*Effect of Exogenous* SEL1L *Expression on Pancreatic Cancer Cell Invasion* In Vitro

Because genes associated with the extracellular matrix are of paramount importance in tumor invasion and metastasis,

**Table 2.** *SEL1L Fold Decrease* after siRNA-SEL1L-1 in Suit-2 Cells and a Summary of Differentially Expressed Genes after *SEL1L* Depletion.

| Gene | Fold Changes* |
|---|---|
| *SEL1L* | 3–2.5 ± 0.25 ($P < .02$) |
| *TIMP1* | 2.4–2 ± 0.2 ($P < .03$) |
| *TIMP2* | 2–1.5 ± 0.25 ($P < .05$) |
| *PTEN* | 2–1.5 ± 025 ($P < .05$) |
| *Activin A* | 2–1.5 ± 025 ($P < .05$) |
| *Activin receptor II* | 2–1.5 ± 025 ($P < .06$) |

The values correspond to the results obtained using the two transfection reagents (± SEM).
*The reported ratio indicates the observed fold decrease of gene expression in the siRNA-SEL1L-1-Suit-2 cells *versus* the siRNA-control-Suit-2 cells 48 hours posttransfection.

we examined the effects of *SEL1L* on genes related to cell–matrix interactions in pancreatic cancer cell invasion through the use of an extracellular matrix–coated filter (ECMatrix; Chemicon International). Both Suit-2-SEL1L and mock cells were pretreated with DEX for 7 days prior to performing invasion assays. The *SEL1L*-expressing cells displayed a lower potential to penetrate Matrigel-coated filters compared to the uninduced and induced control cells; only a 27% decrease in the ability to invade the Matrigel was consistently observed on *SEL1L* induction, and this was significant at the $P < .05$ level (Figure 3*A*). RT-PCR analysis and Western blot analysis were performed on RNA and proteins obtained from untreated and DEX-treated Suit-2-SEL1L cells to ascertain the induction of *SEL1L* and the decrease of *MMP7* expression (Figure 3*B*).

### *Effect of Exogenous* SEL1L *Expression on Anchorage-Independent Growth of Pancreatic Cancer Cells*

We further investigated the anti-invasive effects of *SEL1L* on anchorage-independent growth. The ability to grow on soft agar to detect the capacity of tumor cells to survive and metastasize *in vivo* is an acceptable method; therefore, anchorage-independent growth strongly correlates with tumorigenesis and invasiveness. As previously described, soft agar cloning efficiency was markedly impaired in the induced Suit-2-SEL1L cells compared to uninduced parental cells and mock control [3]. Furthermore, the majority of the induced *SEL1L*-expressing cells failed to locally penetrate and degrade the surrounding semisolid medium, exhibiting a drastically altered cellular morphology (Figure 3*C*).

### *Effect of Exogenous* SEL1L *Expression on Cell Cycle Distribution in Pancreatic Cancer Cells*

The effects of *SEL1L* on cell cycle distribution were analyzed using DEX-treated and untreated Suit-2-SEL1L and mock cells. The *SEL1L*-expressing cells showed an

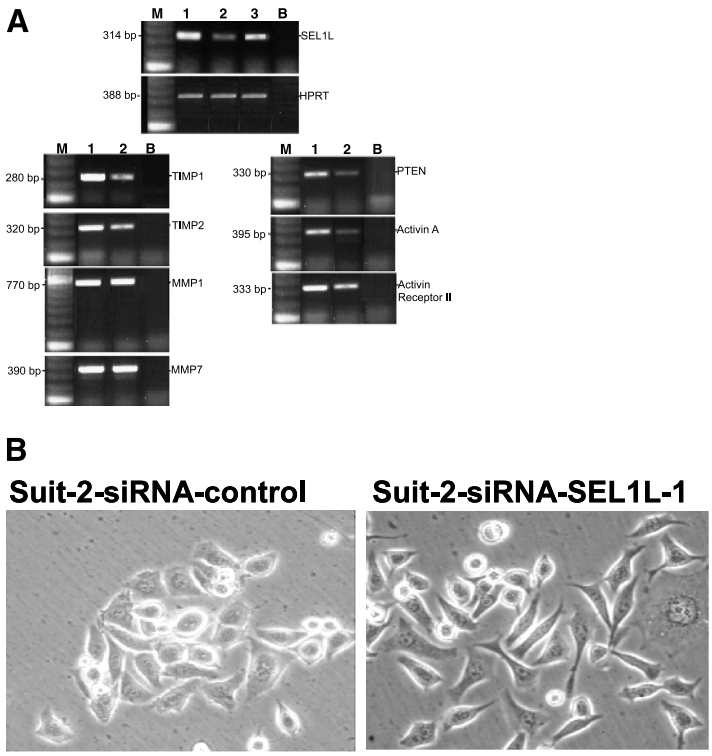


**Figure 2.** *Effects of* SEL1L *downmodulation on target gene expression (A) and on morphologic changes (B) in Suit-2 cells. (A) RT-PCR performed on RNA obtained from Suit-2 cells treated for 48 hours with siRNA-control (lane 1), siRNA-SEL1L-1 (lane 2), and siRNA-SEL1L-2 (lane 3). The figure shows the downmodulation of* SEL1L *expression and the concomitant decreased expression levels of TIMP1, TIMP2, PTEN, Activin A, and Activin receptor II. M, marker, 100 bp; B, blank. HPRT housekeeping gene is used as endogenous control. (B) The figure shows morphologic changes after treating Suit-2 cells for 48 hours with siRNA-SEL1L-1.*

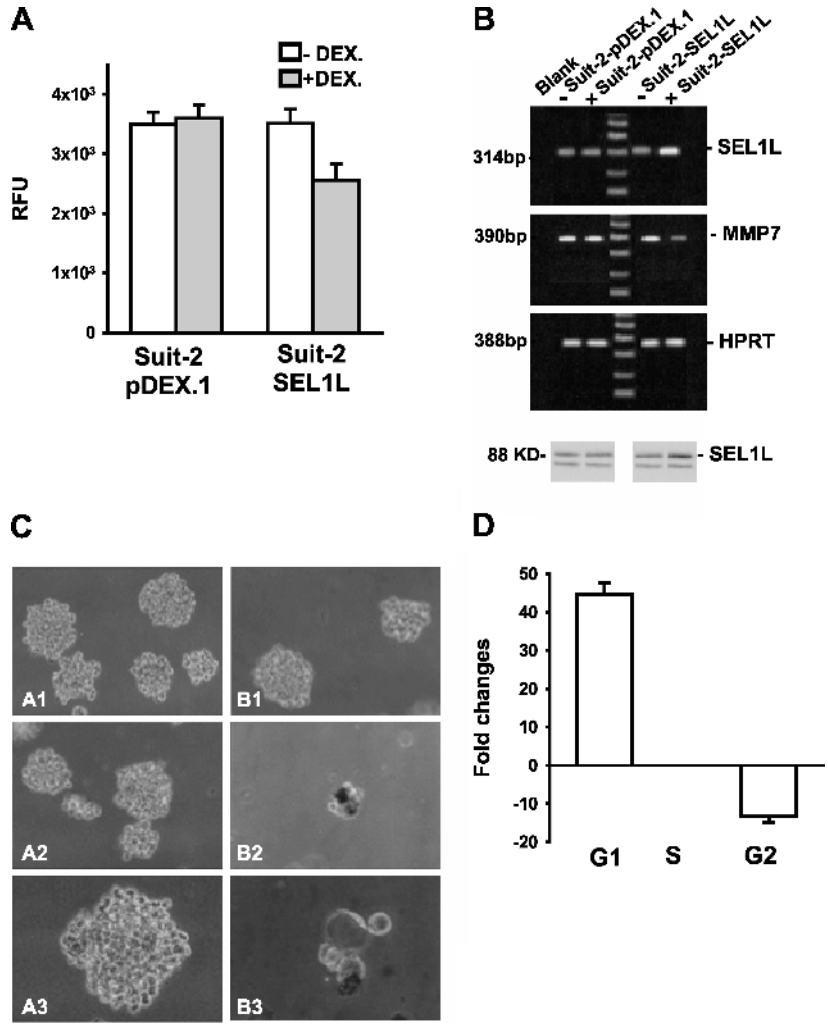


**Figure 3.** *Effects of exogenous* SEL1L *expression on cell invasion (A), anchorage-independent clonogenity (C), and cell cycle (D) of Suit-2 cells. (A) The invasion assay was performed on Suit-2-SEL1L and Suit-2-pDEX.1 cells untreated and DEX-treated for 7 days. Invasion was assessed in Matrigel-coated transwell chambers, as described in Materials and Methods section. Briefly, cells were pretreated with 1* $\mu$*M DEX for 7 days, or grown uninduced and seeded onto the Matrigel-coated 8-*$\mu$*M pore membrane of the upper compartment of each chamber at a density of 1.25* $\times$ $10^5$ *cells/well. As a chemoattractant, we used epidermal growth factor. The chambers were incubated for 48 hours at 37°C. The filters were then removed and stained. The graph shows the number of cells that migrated into the lower chamber. Induced Suit-2-SEL1L cells show 27% less invasion than uninduced parental cells or uninduced/induced mock control. The data are the average values from three separate experiments performed in triplicate. Student's t test, P < .05; bars, ±SE. (B) RT-PCR performed on RNA obtained from Suit-2-pDEX.1 and Suit-2-SEL1L cells untreated (−) and DEX-treated (+) for 7 days. The figure shows the induction of* SEL1L *expression and the concomitant decreased expression levels of* MMP7 *after DEX treatment. B, blank; M, marker, 100 bp. The* HPRT *housekeeping gene is used as endogenous control. Western blot analysis performed on proteins obtained from Suit-2-pDEX.1 and Suit-2-SEL1L cells untreated (−) and DEX-treated (+) for 7 days. The figure shows the induction of* SEL1L *protein after DEX treatment. (C) Anchorage-independent clonogenity in soft agar was performed on Suit-2-SEL1L and Suit-2-pDEX.1 cells untreated and DEX-treated for 7 days. Cells were pretreated with 1* $\mu$*M DEX for 7 days, or grown uninduced and seeded in 0.6% soft agar in duplicate at a density of 1.5* $\times$ $10^4$ *cells/well. Colonies were scored after 14 days. (A1) Uninduced Suit-2-pDEX.1 (original magnification,* $\times$*10). (A2) Induced Suit-2-pDEX.1 (original magnification,* $\times$*10). (A3) Induced Suit-2-pDEX.1 (original magnification,* $\times$*20). (B1) Uninduced Suit-2-SEL1L (original magnification,* $\times$*10). (A2) Induced Suit-2-SEL1L (original magnification,* $\times$*10). (A3) Induced Suit-2-SEL1L (original magnification,* $\times$*20). (D) Cell cycle analysis was performed on Suit-2-SEL1L and Suit-2-pDEX.1 cells untreated and DEX-treated for 7 days. The graph represents the fold increase or fold decrease of specific cell cycle phases observed in the induced Suit-2-SEL1L cells* versus *the uninduced parental and uninduced/induced control mock. Bars, ±SE.*

increase in G1 phase (44.7%) and a decrease in G2 phase (13.29%) compared to controls (Figure 3*D*), whereas S phase was unaffected.

## Discussion

This study provides evidence that *SEL1L* affects the aggressive ability of the pancreatic cancer cell line Suit-2 by reorganizing the expression of genes involved in cell–matrix interactions and by promoting cell cycle accumulation in G1 phase. Although G1 phase accumulation may result from the enhancement of *PTEN* transcription, the decreased invasive ability (as revealed by invasion and clonogenity assays) may be due to the sudden modulation of *TIMP1* and *TIMP2* gene expression and subsequent effects on *MMPs* (*MMP1* and *MMP7*). In fact, using two opposing experimental approaches on the same biologic system (Suit-2)—with the first consisting of upmodulation of *SEL1L* expression by stably transfecting the entire cDNA downstream of an inducible promoter, and the second consisting of the knockdown of *SEL1L* expression by RNAi—we demonstrated that *SEL1L* upmodulation/downmodulation led to upmodulation/

downmodulation of *PTEN*, *TIMP1*, and *TIMP2* genes. As for the pattern of MMP modulation, contrasting results have been obtained. Although increasing levels of *SEL1L* mRNA caused the downmodulation of *MMP1* and *MMP7* expression, interfering *SEL1L* transcription did not affect the levels of these two genes. This could be explained in two ways: 1) *MMP* modulation in the first case may be due to a general perturbation of the cells as a result of prolonged effects of DEX treatment (7–14 days) or may be attributable to inhibitor effects of *TIMP* genes rather than direct *SEL1L* regulation; and 2) in SEL1L-RNAi experiments, the entire process is relatively short, thus preventing the identification of *MMP* modulations.

MMPs and their inhibitors play key roles in extracellular remodeling and are known to be involved in development as well as several pathological conditions; these proteins have been associated with invasion and metastasis in cancer [12,16]. MMP activity is tightly regulated both at the transcriptional level and after secretion by virtue of proenzyme activators and endogenous proteinase inhibitors, including TIMP1, TIMP2, TIMP3, and TIMP4. Diseases associated with high levels of MMPs over the TIMPs enhance proteolysis of the extracellular matrix [17,18]. Inhibitory activities of TIMPs suggest that the net balance between MMPs and TIMPs is a major determinant of the proteolytic potential of tumors. This concept has been supported by several studies showing that overproduction of TIMPs reduces experimental metastasis [19], whereas low levels of these inhibitors correlate with tumorigenesis [20]. Hence, their modulations in the biologic systems under analysis may explain the invasion assay results presented in this paper.

It is also worth mentioning that the array panel used contained 12 *MMP* and 3 *TIMP* genes, but only two of the *MMPs* and *TIMPs* were actually modulated, indicating the cell-specific action of the protease family of proteins. This is in line with previous reports indicating that the increased expression of *MMP7* and the reduced expression of *TIMP2* are responsible for the aggressive phenotype of pancreatic carcinoma [21,22].

It is still unclear how *SEL1L* modulates *PTEN* and extracellular matrix–related genes, but it has been reported that both *MMP1* and *TIMP1* are *Smad* gene targets [23,24] and that *SEL1L* induces the expression of both *Activin A* and *Smad4*, which are members of the tumor growth factor (TGF) β pathway [3,15]. The *MMP1* proximal promoter contains a TGF-β–inhibitory element as well as an AP1 site—two elements that are essential for Smad interaction; the Smad-responsive region is contained in the *TIMP1* promoter. It can be hypothesized that *SEL1L* may elicit its effects through transcriptional regulation mediated by TGF-β signaling. In this report, we confirm, through the use of siRNA transfection, that *SEL1L* regulates the expression of *Activin A* gene. The expression of *Activin receptor II* was also downmodulated in response to *SEL1L* depletion, which was in contrast with our previous results but may be explained by considering the different points at which expression analysis was performed on the two model systems; thus, the modulation may be attributable to an earlier response, rather than a later response. In addition, as a consequence of *SEL1L* interference, Suit-2 cells underwent drastic morphologic changes in that cells grew in a disorganized fashion, showed an increase in cell volume and a significant loss of cell–cell contact, and acquired a spindle-type cell morphology with the presence of numerous extrusions. Cell growth rate was unaffected, confirming our previous results [3].

*PTEN* RNA levels show a remarkable increase after several days of ectopic *SEL1L* induction and cell cycle arrest in G1 phase. *PTEN* is a phosphoinositide (PI) 3 phosphatase that can inhibit cellular proliferation survival, growth, and motility by inactivating PI3-kinase–dependent signaling [25,26]. It promotes cell cycle arrest in G1 phase by limiting the activity of PI3-K/Akt effector [27]. Therefore, SEL1L may elicit its effects on cell cycle through the upmodulation of *PTEN* and Smad4. Because high levels of *PTEN* mRNA were observed after 14 days of DEX treatment, it may thus reflect the generic perturbation of several pathways. It has been reported that the human and plant *SEL1L* gene belongs to the so-called unfolded protein response genes, which are induced in response to the accumulation of unfolded and misfolded proteins in the ER (ER stress) [9,10]. During ER stress, PI3-K/Akt signaling is acutely activated and governs cell survival by directly counteracting ER stress–induced cells [28]. The UPR is activated in solid tumors by hypoxia. Based on this information and on the present results, which indicate SEL1L's ability to modulate PTEN expression, we then hypothesize that SEL1L may attenuate ER stress and elicit its antitumor response by affecting the PI3-K/Akt signals. Besides the known activity of *PTEN* to control cell proliferation, it was proven to efficiently inhibit invasiveness [29,30]. Although *PTEN* upregulation was moderate (but still within the range of the increase of *TIMP1* and *TIMP2* transcripts) after DEX treatment of Suit-2-SEL1 cells for 7 days, it might still contribute to reverting the invasive phenotype of this cell line. Interestingly, *TIMP1* proved to inhibit endothelial cell migration and to promote *PTEN* upregulation [31].

Presently, we do not have proteomic data on our experimental system (Suit-2 cells). Most likely, these proteins are present in the cells at a certain level; thus, it remains to be determined whether or not they will change levels on *SEL1L* induction.

Presently, we do not know the precise stage during which SEL1L exerts its anti-invasive activity. Metastasis requires the interaction of malignant cells with three distinct microenvironments: the primary organ, the circulation, and the target organ where a metastatic lesion may develop. Cancer cell invasion requires several steps: 1) invasion, which includes loss of cell–cell adhesion, detachment from tumor mass, and degradation of basement membranes, which enables neoplastic cells to migrate; 2) intravasation, wherein tumor cells penetrate through the endothelium of the blood or lymphatic vessels to enter the systemic circulation; and 3) extravasation, wherein the small number of cells that survive the voyage through the circulatory system extravasates through the capillary endothelium at the target organ parenchyma, where they proceed to proliferate in the new supportive microenvironment as micrometastases. Any agent that can inhibit the early steps of the invasive process may

be a powerful tool in the prevention of metastasis [32]. It would then be extremely interesting to dissect the specific stage during which SEL1L interferences with cancer progression; however, the lack of model systems that allow the investigation of the sequential stage of carcinogenesis renders this analysis very difficult.

By modulating genes related to cell–matrix interactions, it can be hypothesized that SEL1L may be involved in the early steps of the metastatic process because MMPs appear to be important in the early stage of cancer progression (local invasion and micrometastasis) and may no longer be required once metastases have been established [33].

Adenocarcinoma of the pancreas is an invasive neoplasm that is usually lethal. The dismal prognosis of pancreatic carcinoma is due to: 1) late-stage diagnosis; 2) its high invasive and metastatic potential; and 3) intense desmoplastic stromal reaction, resulting in low rates of curative resections and high frequencies of relapse [13,34,35]. Molecular and cellular processes underlying epithelial–stromal interactions are of great importance to understanding the biology of the disease and to developing effective strategies for diagnosis and treatment. Disturbing the balance between MMPs and their natural tissue inhibitors is an important target for pancreatic tumor therapy as well as for other cancers. Future direction should focus on a new conceptual basis for the design of strategies that use SEL1L as a molecule against these enzymes, thus preventing matrix breakdown.

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