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# Identification and Characterization of Unique Tumoricidal Genes in Rat Umbilical Cord Matrix Stem Cells

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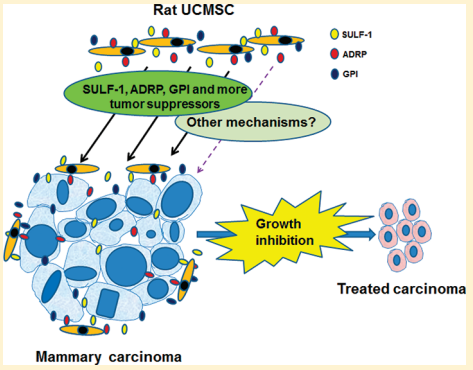
Supporting Information

**ABSTRACT:** Rat umbilical cord matrix stem cells (UCMSC) have been shown to exhibit a remarkable ability to control rat mammary adenocarcinoma (Mat B III) cell proliferation both *in vivo* and *in vitro*. To study the underlying mechanisms and genes involved in Mat B III growth attenuation, total RNA was extracted from the naive rat UCMSC alone and those cocultured with Mat B III in Transwell culture dishes. Gene expression profiles of naive rat UCMSC alone and those cocultured with Mat B III cells were investigated by microarray analysis using an Illumina RatRef-12 Expression BeadChip. The comparison of gene expression profiles between untreated and cocultured rat UCMSC identified five upregulated candidate genes (follistatin (FST), sulfatase1 (SULF-1), glucose phosphate isomerase (GPI), HtrA serine peptidase (HTRA1), and adipocyte differentiation-related protein (ADRP)) and two downregulated candidate genes (transforming growth factor, beta-induced, 68 kDa (TGF$\beta$I) and podoplanin (PDPN)) based upon the following screening criteria: (1) expression of the candidate genes should show at least a 1.5-fold change in rat UCMSC cocultured with Mat B III cells; (2) candidate genes encode secretory proteins; and (3) they encode cell growth-related proteins. Following confirmation of gene expression by real-time PCR, ADRP, SULF-1 and GPI were selected for further analysis. Addition of specific neutralizing antibodies against these three gene products or addition of gene-specific siRNA's individually in cocultures of 1:20 rat UCMSC:Mat B III cells significantly increased cell proliferation, implying that these gene products are produced under the cocultured condition and functionally attenuate cell growth. Immunoprecipitation followed by Western blot analysis demonstrated that these proteins are indeed secreted into the culture medium. Individual overexpression of these three genes in rat UCMSC significantly enhanced UCMSC-dependent inhibition of cell proliferation in coculture. These results suggest that ADRP, SULF-1 and GPI act as tumor suppressor genes, and these genes might be involved in rat UCMSC-dependent growth attenuation of rat mammary tumors.




**KEYWORDS:** rat umbilical cord matrix stem cells, rat mammary tumor cells, Mat B III, microarray, real-time PCR, thymidine uptake, tumor suppressor genes, ADRP, GPI, SULF-1

## INTRODUCTION

Umbilical cord matrix stem cells (UCMSC) represent a promising source of therapeutics for various diseases including cancer. The tumor-tropism of UCMSC has been reported[1−3] and was utilized for targeted delivery of therapeutic genes for cancer therapies in mouse preclinical studies.[4,5] This type of stem cell-based cancer-targeted delivery of therapeutic genes, drugs, or nanoparticles has been successfully tested with UCMSC as well as with various mesenchymal stem cells (MSC) from bone marrow, neuronal tissues or adipose tissues.[6−10] The benefit of using UCMSC is the ease of preparing relatively large quantities of stem cells without feeder cells,[2,11] the absence of ethical concerns with the origin of the stem cells, and the lack of tumorigenicity of the cells.[3]

Recently, Khakoo et al. showed that bone marrow-derived MSC have intrinsic antitumor effects on Kaposi sarcoma in a nude mouse model.[12] Through *in vitro* and *in vivo* studies they proved that MSC cause antitumor effects through direct contact with the Kaposi sarcoma cells. On the contrary, several studies have reported that bone marrow MSC support tumor growth both directly and indirectly.[13−15] Since tumor cells appear to recruit circulating bone marrow MSC and create the appropriate tumor microenvironment, supporting tumor growth may be a reasonable function for bone marrow MSC. However, Ganta

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et al. have shown that naive rat UCMSC have an antiproliferative effect on rat Mat B III mammary adenocarcinoma cells *in vitro* and have demonstrated that rat UCMSC treatment completely abolishes Mat B III grafts *in vivo* with no recurrence during a lengthy survival study.[2] This powerful antitumor effect has been confirmed in interspecies transplantation in pancreatic[14] and lung carcinoma-bearing mice.[16] The rat UCMSC antitumor effect does not appear to be cell contact-dependent since conditioned medium from rat UCMSC,[2,16] as well as rat UCMSC separated from cancer cells by Transwell inserts, significantly attenuated cancer cell growth. In addition, rat UCMSC-dependent attenuation of cell proliferation may be more pronounced by exposure to tumor cells such as Mat B III cells. These findings suggest that rat UCMSC produce specific factors with an antiproliferative effect and expression of these factors may be increased in the presence of Mat B III cells. However, the existence and identity of rat UCMSC-dependent antiproliferative factors has yet to be clarified.

To clarify the antiproliferative factors produced by rat UCMSC, the following hypotheses were formulated: (1) rat UCMSC-dependent antitumor factors are produced by specific genes; (2) these factors should be secretory gene products and are cell growth regulation-related proteins; and (3) the proteins' expression profiles may be modified when rat UCMSC are cocultured with mammary tumor cells. Testing these hypotheses will clarify the underlying mechanisms and potential genes involved in rat UCMSC-dependent tumor growth attenuation. Accordingly, gene expression profiles of naive rat UCMSC alone and those cocultured with Mat B III cells were investigated by microarray analysis using a rat genome-wide gene expression bead chip. The microarray analysis initially suggested 16 candidate genes. The differential expression of 7 genes was confirmed by quantitative real-time PCR (qRT-PCR). Further analysis revealed that at least three genes have a tumor suppressor function and are associated with rat UCMSC-dependent antitumor activity.

## MATERIALS AND METHODS

**Cell Culture.** Rat UCMSC were harvested from E19 pregnant Fisher 344 rats according to the method previously described.[2] The rat UCMSC were maintained in low-serum defined medium containing the following mixture per 100 mL: 57 mL of low-glucose DMEM (Invitrogen, Carlsbad, CA), 37 mL of MCDB 201 (Sigma-Aldrich, St. Louis, MO), 2 mL of fetal bovine serum (FBS; Equitech Bio, Inc., Kerrville, TX), 1 mL of 100× insulin-transferrin-selenium-X (Invitrogen); 1 mL of 0.15 g/mL AlbuMax1 (Invitrogen), 1 mL of 100× Pen/Strep (Invitrogen), 10 nmol/L dexamethasone (Sigma-Aldrich), 100 $\mu$mol/L ascorbic acid 2-phosphate (Sigma-Aldrich), 10 ng/mL epidermal growth factor (R&D Systems, Minneapolis, MN), and 10 ng/mL platelet derived growth factor-BB (R&D Systems). The Mat B III rat mammary adenocarcinoma cell line (ATCC, Manassas, VA) was maintained in McCoy's 5A modified medium (Invitrogen) supplemented with 10% FBS and 1% of 100× Pen/Strep (Invitrogen). Primary rat uterus fibroblasts from Fisher 344 rat pups were prepared following an established method.[17] Rat uterus fibroblasts were cultured in DMEM/Ham's F-12 medium (1:1) (Invitrogen) supplemented with 10% FBS and 1% of 100× Pen/Strep (Invitrogen). All cells were cultured at 37 °C in a humidified atmosphere containing 5% $CO_2$.

**Antibodies.** Rabbit and goat polyclonal antibodies against adipose differentiation-related protein (ADRP), sulfatase-1 (SULF-1), glucose phosphate isomerase (GPI), decorin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Horseradish peroxidase-conjugated anti-rabbit, anti-goat IgG antibodies were obtained from GE Healthcare Bioscience Corp (Piscataway, NJ) and Santa Cruz Biotechnology, Inc.

**Indirect Coculture of Rat UCMSC with Mat B III Cells and RNA Isolation.** Indirect coculture of rat UCMSC ($1 \times 10^5$ cells per dish) with Mat B III cells ($1.5 \times 10^6$ cells per dish) were carried out using a Transwell cell culture system (Corning Life Sciences, Lowell, MA), which allows free diffusion of substances without contact between tumor cells and stem cells. Mat B III cells were cultured in a Transwell insert with a porous membrane (10 cm in diameter, pore size 0.4 $\mu$m), and rat UCMSC were maintained in the bottom of the culture dish using defined medium. Total RNA was isolated from the rat UCMSC cocultured with or without Mat B III cells using TRIzol (Invitrogen) according to the manufacturer's protocols. The concentration and quality of the samples were measured by the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA) and Agilent 2100 bioanalyzer (Agilent Technologies, Inc. Santa Clara, CA), respectively.

**Microarray Analysis Procedures.** Microarray experiments including RNA quality evaluation, hybridization, and initial data analysis were carried out at the National Institute on Aging, National Institutes of Health (Baltimore, MD). For each sample, biotinylated cRNA was prepared using an Illumina Total Prep RNA Amplification Kit (Applied Biosystems, Foster City, CA, USA). Briefly, 5 $\mu$g of total RNA was converted to double stranded cDNA using T7-oligo (dT) primers, followed by an *in vitro* transcription (IVT) reaction to amplify biotinylated cRNA as described in the manufacturer's instructions (Illumina Inc., San Diego, CA). The biotinylated cRNA was hybridized to a RatRef-12 Expression BeadChip platform that contains 22,519 probes (Illumina Inc.). The hybridization, washing, and scanning were performed according to the manufacturer's instructions. The chips were scanned using a BeadScan 2.3.0.10 (Illumina Inc.) at a multiplier setting of 2. The microarray images were registered and extracted automatically during the scan using the manufacturer's default settings.

**Microarray Data Analysis.** The resulting microarray data set was analyzed with DIANE 6.0, a spreadsheet-based microarray analysis program. An overview of DIANE can be found online at http://www.grc.nia.nih.gov/branches/rrb/dna/diane_software.pdf. Raw intensity data for each experiment was normalized by $z$ transformation. Intensity data were first log10-transformed and used for the calculation of $z$ scores.[18] $z$ scores were calculated by subtracting the average gene intensity from the raw intensity data for each gene and dividing that result by the SD of all the measured intensities. Gene expression differences between any two experiments were calculated by taking the difference between the observed gene $z$ scores. The significance of calculated $z$ differences can be directly inferred from measurements of the SD of the overall $z$ difference distribution. Assuming a normal distribution profile, $z$ differences are assigned significance according to their relation to the calculated SD of all the $z$ differences in any one comparison. To facilitate comparison of $z$ difference between several different experiments, $z$ differences were divided by the appropriate SD to give the $z$ ratios. Further hierarchical cluster analysis was performed using open source software Cluster 3.0 and Java Treeview.

**Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR).** Microarray results were validated by qRT-PCR using the

**Table 1. List of the Primers Used for qRT-PCR**

| gene symbol | forward primer (5′−3′) | reverse primer (5′−3′) |
|---|---|---|
| ADRP | CATTCAAGACCAGGCCAAAC | AGGAGGTAACATTGCGGAAC |
| SULF-1 | AAACAGTGCAACCCAAGACC | TTGCCAGTTGGTGTCTGAAG |
| GPI | TGCCAAAGAGTGGTTTCTCC | CTTCACTTTGTCCGTGTTCG |
| PDPN | AAAGCCCAAGTTGAGGAACC | TCCATCGTCCAGAAAGAAGC |
| FST | TGCTGCTACTCTGCCAATTC | TGCAACACTCTTCCTTGCTC |
| HTRA1 | TTATCGCTGATGTGGTGGAG | AATGAATCCTGACCCACTCG |
| TGF$\beta$I | GCGGCTAAAGTCTCTCCAAGGT | TGACACTCACCACATTGTTTTTCA |
| GAPDH | AGACAGCCGCATCTTCTTGT | CTTGCCGTGGGTAGAGTCAT |

same RNA samples as those used for the microarray. qRT-PCR was carried out using the iScript One-Step RT-PCR Kit with SYBR Green (Bio-Rad, Hercules, CA), and the reactions were conducted on the real-time PCR detection system iCycler (Bio-Rad, Hercules, CA). The PCR was performed as follows: 45 cycles with initial incubation at 50 °C for 10 min, 95 °C for 5 min, and final extension at 72 °C for 4 min. Each cycle consisted of denaturation for 10 s at 95 °C, annealing for 20 s at 58 °C, and extension for 50 s at 72 °C. The results were quantified as $C_t$ values, where $C_t$ is defined as the threshold cycle of PCR at which the amplified product is first detected and signifies relative gene expression (the ratio of target/control). qRT-PCR results were analyzed by the comparative $C_t$ method.[19] The primers used in this study are indicated in Table 1.

**[$^3$H]Thymidine Uptake Assay.** In the [$^3$H]thymidine incorporation assay, rat UCMSC ($2.5\times 10^3$ cells per well) were plated in 24 well culture plates and were cultured in a $CO_2$ incubator overnight. Mat B III cells ($5 \times 10^4$ cells per well) were added to the culture plates on the next day. Cells were cultured for an additional 24 h and pulsed for the last 4 h of the culture time with 1.0 $\mu$Ci [$^3$H]thymidine per well. Mat B III cells in suspension culture were collected into a 1.5 mL centrifuge tube, and the free [$^3$H]thymidine in the medium was washed away with PBS. The [$^3$H]thymidine taken by Mat B III cells and rat UCMSC was solubilized by 0.2 M NaOH separately, combined and counted by the Packard liquid scintillation counter Tri-Carb 2100TR (Perkin-Elmer Life Science, Boston, MA, USA). For proteins neutralizing experiment, neutralizing antibodies (1−4 $\mu$g per well) were added to the culture medium 2 h prior to beginning coculture with Mat B III cells.

**Live Cell Counting.** Rat UCMSC ($1.25 \times 10^3$ cells per well) were plated in 24 well culture plates and were cultured in a $CO_2$ incubator overnight. Neutralizing antibodies (1 $\mu$g per well) were added to the culture medium 2 h prior to beginning coculture with Mat B III cells ($2.5 \times 10^4$ cells per well). Cells were cocultured an additional 48 h and live cells were counted after trypan blue staining (0.2% trypan blue for 5 min).

**Immunoprecipitation and Western Blotting.** Rat UCMSC ($2.5 \times 10^5$ cells per dish) were seeded in 10 cm culture dishes and were cultured in a $CO_2$ incubator overnight. Mat B III cells ($4 \times 10^6$ cells per dish) were added to the rat UCMSC and further incubated for 24 h. Mat B III cells alone ($4 \times 10^6$ cells per dish) were also separately cultured for 24 h. One mL of the culture medium was collected from each culture dish and precleared with 30 $\mu$L of protein A agarose resin slurry (50%, Thermo Fisher Scientific, Rockford, IL) for 2 h at 4 °C. Supernatant from each sample was separately collected into new tubes after centrifugation at 13,000 rpm for 5 min at 4 °C. Three $\mu$g/mL of respective gene-specific antibody against ADRP, GPI or SULF-1 was added and incubated overnight at 4 °C with gentle rocking. At the end of the incubation, 70 $\mu$L of protein A agarose resin slurry was added and incubated for an additional 3−4 h at 4 °C. The agarose resin−antibody−protein complexes were washed three times with ice-cold 1× Tris buffered saline (TBS), followed by centrifugation at 6,500 rpm for 5 min. Protein−antibody complexes were dissociated from the protein A agarose resin by adding 30 $\mu$L of 2× sample loading buffer and boiling for 5 min. Protein samples were separated by 8 or 12% SDS−PAGE, electroblotted onto nitrocellulose membrane (GE Healthcare Bioscience Corp) and blocked with 4% nonfat dry milk in 0.1% Tween 20 in phosphate buffered saline (PBST) for 1 h at room temperature. The membranes were washed and incubated with specific antibodies against SULF-1, ADRP, or GPI (1:200, Santa Cruz Biotechnology) with 0.1% nonfat dry milk in PBST for 1 h at room temperature and then incubated with secondary antibody (1:2000, GE Healthcare Biosciences Corp.). The protein expression signal was detected with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL).

**Table 2. $z$ Ratio of Differentially Expressed Genes in Rat UCMSC Cocultured with Mat B III Cells[a]**

| gene | $z$ ratio |
|---|---|
| ADRP | 1.581055553 |
| HTRA1 | 1.905298131 |
| GPI | 2.077145532 |
| P4HA1 | 2.275806017 |
| COL1A2 | 2.432693795 |
| SULF1 | 2.738457286 |
| FST | 4.78920589 |
| SERPINE2 | 5.333449106 |
| PTGS2 | 6.077754681 |
| LOXL1 | −1.561672755 |
| BGN | −1.793571385 |
| LTBP4 | −1.83925686 |
| PAM | −2.334943964 |
| PDPN | −2.469104398 |
| PDIA5 | −3.113440321 |
| TGF$\beta$I | −3.790700019 |

[a] Total RNA was extracted from rat UCMSC 24 h after culture with or without coculturing with Mat B III rat mammary carcinoma cells. Coculture was carried out in Transwell culture dishes so that the two types of cells were not mixed with each other. Microarray analysis was performed as described in Materials and Methods to identify genes differentially expressed in rat UCMSC cocultured with Mat B III cells relative to rat UCMSC not exposed to coculture.

**Overexpression of Candidate Genes.** Rat UCMSC ($2.5 \times 10^3$ cells per well) were seeded in 24 well plates and were cultured in a $CO_2$ incubator overnight. The next day, cells were transfected with human ADRP, human GPI, or human SULF-1 plasmid DNA (Open Biosystem, Huntsville, AL) using FuGENE HD transfection reagent (Roche Applied Science) according to manufacturer's instructions. Since these human genes are highly homologous with rat genes (homology: ADRP, 77%; GPI, 86%; SULF1, 60%), human genes were used in this study. After transfection, cells were incubated for 48 h. Successful gene transfection was confirmed by qRT-PCR. Mat B III cells ($5 \times 10^4$ cells per well) were added to the transfected rat UCMSC in the 24 well plates and further incubated for 24 h. At the end of the incubation, the [$^3$H]thymidine uptake assay was performed to monitor cell proliferation as described above. [$^3$H]Thymidine uptake by Mat B III cells cocultured with GFP transfected rat UCMSC was used as negative control. As a control study, these three genes were also transfected rat uterus fibroblasts and treated identically as rat UCMSC.

**Transient Gene Knockdown of Candidate Genes in Rat UCMSC.** Knockdown of candidate genes in rat UCMSC was carried out using small interfering RNA (siRNA). For gene knockdown, rat UCMSC ($2.5 \times 10^3$ cells per well) were seeded in a 24 well plate, cultured in a $CO_2$ incubator. After 20 h cells were transfected with respective siRNA for SULF-1, ADRP, GPI genes or scramble RNA (Sigma-Aldrich) as a universal negative control using N-TER transfection reagent (Sigma-Aldrich) following the manufacturer's instructions. After 6 h of transfection with the siRNA, transfected rat UCSMC were cocultured with Mat B III cells ($5 \times 10^4$ cells per well). DNA synthesis was measured by [$^3$H]thymidine uptake assay after 24 and 48 h of coculture. Efficiency of knockdown was confirmed by qRT-PCR.

**Statistical Analysis.** All data are reported as mean ± SE. Statistical significance was assessed by one-way ANOVA. Group comparisons were deemed significant for 2-tailed $P$ values below 0.05.

## RESULTS

**Screening of Unique Tumoricidal Genes by Microarray Analysis.** To screen genes associated with rat UCMSC-dependent growth attenuation of rat mammary tumor cells, genome-wide microarray analyses were carried out using an Illumina RatRef-12 Expression BeadChip, which contains 22,519 distinctive rat oligonucleotide probes. Although this microarray chip does not contain all rat genes, it encompasses the largest number of rat genes available at the time of the analysis. In this study, total RNA was extracted from the rat UCMSC either cocultured with or without Mat B III cells in Transwell culture dishes; the two cell types were separated by a porous membrane. The quality of the extracted RNA was proven to be homogeneous and high (Supplemental Figure 1 in the Supporting Information). For unique gene screening, the following criteria were set to identify candidate genes: (1) candidate gene expression should show at least a 1.5-fold change in rat UCMSC cocultured with Mat B III cells; (2) they encode secretory proteins; and (3) they encode cell growth regulation-related proteins. Data analysis by $z$ normalization of the hybridization signals identified sixteen genes which were differentially expressed in the two culture conditions, i.e., their expression levels were at least 1.5-fold different (Figure 1). Nine of these genes were upregulated in rat UCMSC, and seven of these genes were downregulated when

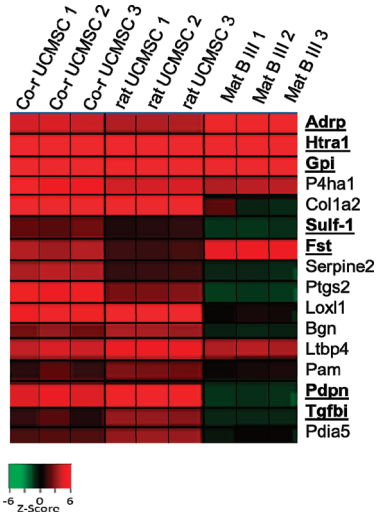


**Figure 1.** Gene clustering of secretory protein encoding genes. Expression levels of mRNA ($z$ score) of rat UCMSC alone, Mat B III cells alone, and rat UCMSC cocultured with Mat B III cells (co-rUCMSC). Underlined genes were further characterized.

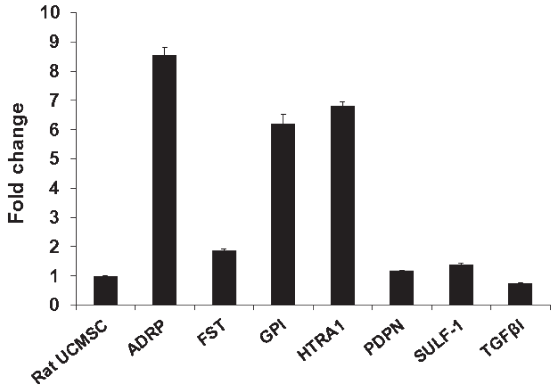


**Figure 2.** Fold changes in expression of genes differentially expressed in rat UCMSC cocultured with Mat B III cells relative to rat UCMSC not cocultured was measured by qRT-PCR analysis of candidate genes selected initially by microarray analysis. Fold changes in expression was confirmed by qRT-PCR analysis.

cocultured with the Mat B III cells (Table 2). Among these genes, a total of five upregulated genes (HTRA1, SULF-1, GPI, ADRP, and FST) and two downregulated genes (TGF$\beta$I and PDPN) were further characterized as candidate genes associated with growth regulation of tumor cells, i.e., these genes encode secretory proteins and are associated with cell growth regulation.

The mRNA expression of these seven genes in rat UCMSC was verified by qRT-PCR using the total RNA extracted for microarray analysis (Figure 2). Of the five upregulated genes selected from microarray analysis, all five genes showed concordant results between microarray analysis and qRT-PCR. However, although PDPN and TGF$\beta$I showed significantly decreased expression in microarray analysis (Table 2), their expression was determined by qRT-PCR to be identical to the unstimulated rat UCMSC levels. The mRNA expression of seven genes was reevaluated by qRT-PCR using separately prepared samples. Their expression patterns were similar to qRT-PCR results described above (data not shown). Accordingly, overall results

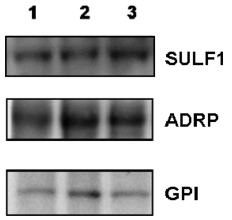


**Figure 3.** Detection of SULF-1, ADRP, and GPI in the culture medium by Western blot analysis. Rat UCMSC and Mat B III cells were either cultured individually or cocultured together in defined medium. Three gene products were immunoprecipitated using specific goat polyclonal antibodies against SULF-1, ADRP, or GPI. Each immunoprecipitate was subjected to SDS−PAGE and then immunoblotted using rabbit polyclonal antibodies against SULF-1, ADRP and GPI. Lane 1, immunoprecipitated protein from the medium from rat UCMSC cultured alone; lane 2, immunoprecipitated proteins from the medium from Mat B III cells cultured alone; lane 3, immunoprecipitated proteins from the medium of rat UCMSC and Mat B III cells cocultured at a ratio of 1 to 16.

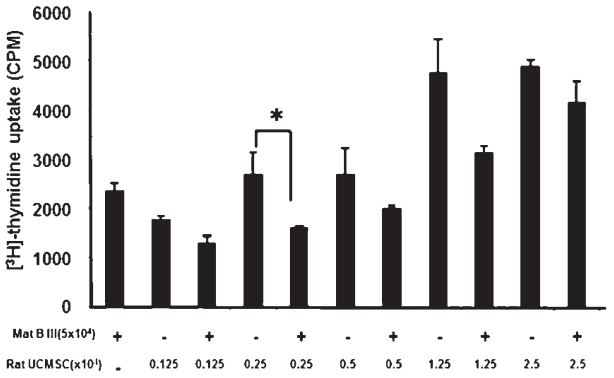


**Figure 4.** Optimization of the cell ratio of rat UCMSC to Mat B III cells for [$^3$H]thymidine uptake assay. In order to evaluate the most appropriate growth inhibition of Mat B III cells by rat UCMSC, both cells were cocultured at various ratios as indicated in the figure. [$^3$H]Thymidine uptake was determined at 24 h after coculturing of both cell types. Each value represents mean ± SE of triplicate determinations. *, $P < 0.05$, compared with rat UCMSC alone or Mat B III alone.

suggest that the data obtained from qRT-PCR correlate well with the microarray results. Results from both analyses suggest that the sensitivity of the two methods appears to be different in each gene. However, the qRT-PCR method may be more sensitive than the microarray. It is important to note that the fold change obtained by the qRT-PCR assays was bigger than the $z$ ratios in the array data. This is because the $z$ ratios are $z$ differences divided by standard deviation and not fold change ratios. Out of the seven genes, five genes (HTRA1, SULF-1, ADRP, FST, and TGF$\beta$I) have been characterized as tumor suppressor genes, one gene (GPI) has been identified as a metastasis support gene, and one gene (PDPN) has been identified as a tumor promoter gene in published studies.[20,21] Based on the preliminary screening of their growth attenuation ability (ADRP > SULF-1 > GPI > FST > HTRA1) measured by [$^3$H]thymidine uptake assay in the presence of specific neutralizing antibodies, the extent of the differential expression, and individual characterization of these genes, SULF-1, GPI, and ADRP were further studied to determine their roles in the rat UCMSC-dependent tumor growth attenuation.

**Detection of Candidate Gene Products in Culture Medium by Western Blot Analysis.** To confirm that ADRP, GPI, and SULF-1 products are indeed secreted proteins, the presence of these proteins in the culture medium was analyzed by Western blot analysis. Individual proteins were collected from the culture medium by immunoprecipitation as described in Materials and Methods. All three of the gene products were detected in the culture media from rat UCMSC alone, Mat B III cells alone, and the coculture of rat UCMSC and Mat B III cells (Figure 3). Although protein levels were not quantitatively determined due to the technical difficulty of normalization with loading standard, rat UCMSC-dependent production of these proteins seems higher than that by Mat B III cells since only a small number of rat UCMSC (1:16 = rat UCMSC:Mat B III cells) produced protein bands similar to those by Mat B III cells alone. These results support our hypothesis that these proteins are produced by rat UCMSC, secreted from the cells, and could potentially play a role in the control of tumor cell proliferation.

**Neutralizing Antibodies Specific to Selected Candidate Genes Increased Cell Proliferation.** In the initial experimental optimization study, it was found that a small number of rat UCMSC ($2.5 \times 10^3$ cells) significantly inhibited [$^3$H]thymidine uptake by Mat B III cells ($5 \times 10^4$ cells) when they were cocultured together (rat UCMSC:Mat B III = 1:20, Figure 4). Accordingly, the lowest rat UCMSC cell number exhibiting a growth attenuation effect (1:20 ratio) was used for all of the following direct coculture experiments. As shown in Figure 5, when SULF-1, GPI, and ADRP proteins were inhibited by their neutralizing antibodies, rat UCMSC-dependent inhibition of [$^3$H]thymidine uptake was decreased, suggesting that all three genes play a role in the Mat B III cell growth attenuation by rat UCMSC. As a control experiment, rat UCMSC were cocultured with Mat B III cells in the presence of an anti-decorin antibody (a gene not differentially expressed in rat UCMSC cocultured with Mat B III cells). However, the anti-decorin antibody did not alter the [$^3$H]thymidine uptake (Supplemental Figure 2 in the Supporting Information). As an additional control experiment, rat UCMSC were replaced with rat uterus fibroblasts. However, rat uterus fibroblasts did not show any growth attenuation of Mat B III cells (data not shown). These results were further confirmed by live cell counting. As shown in Figure 6, total live cell numbers were significantly increased when the SULF-1, GPI, or ADRP proteins were inhibited by their neutralizing antibodies. These results clearly suggest that rat UCMSC-dependent attenuation of mammary tumor cell growth is rat UCMSC-specific, and the three selected genes play a role in rat UCMSC-dependent growth attenuation.

**Knockdown of Candidate Genes Increased the Cell Proliferation.** To confirm the results obtained from neutralizing antibody-based attenuation of gene function, three selected genes were knocked down by specific siRNA. Specific siRNA-dependent attenuation of mRNA expression for the three genes was approximately 80% for ADRP, 70% for GPI, and 77% for SULF-1 when 20 nM siRNA was transfected. Gene knockdown for ADRP, GPI, and SULF-1 increased rat UCMSC-dependent cell proliferation approximately 67.1%, 40.5% and 49.9%, respectively (Figure.7). These results confirmed that the three selected genes play a role in rat UCMSC-dependent growth attenuation.

**Overexpression of Candidate Genes Inhibited Cell Growth.** To confirm that ADRP, GPI, and SULF-1 are indeed functional tumor suppressors, the effect of overexpression of these genes in rat UCMSC was analyzed using a [$^3$H]thymidine uptake assay.

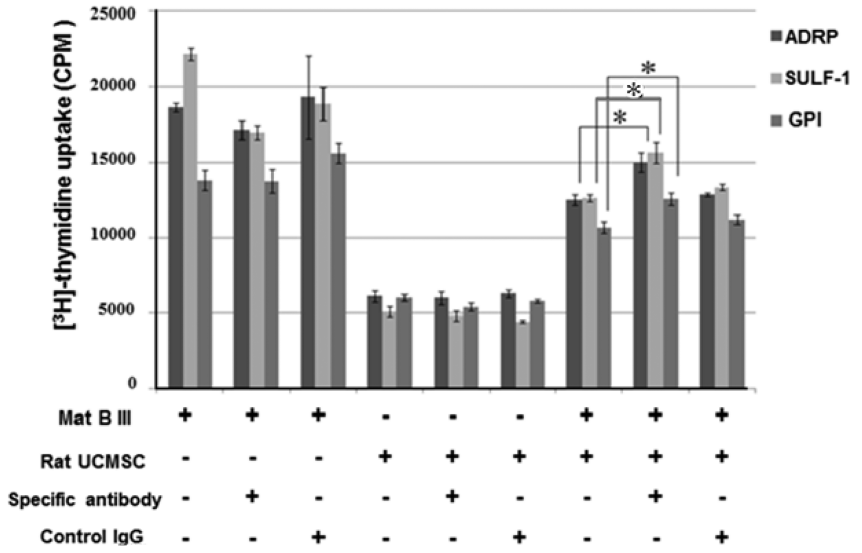


**Figure 5.** The effect of neutralizing antibodies against ADRP (ADFP), SULF-1 and GPI on cell proliferation of Mat B III cells cocultured with rat UCMSC. [$^3$H]Thymidine uptake was determined at 24 h after coculturing rat UCMSC with Mat B III cells in the presence or absence of anti-ADRP (left column), -SULF-1 (middle column), and -GPI (right column) antibodies (4 $\mu$g/well). Each value represents mean ± SE of triplicate determinations. *, $P$ value <0.05, compared with thymidine uptake by Mat B III cells cocultured with rat UCMSC in the absence of antibody.

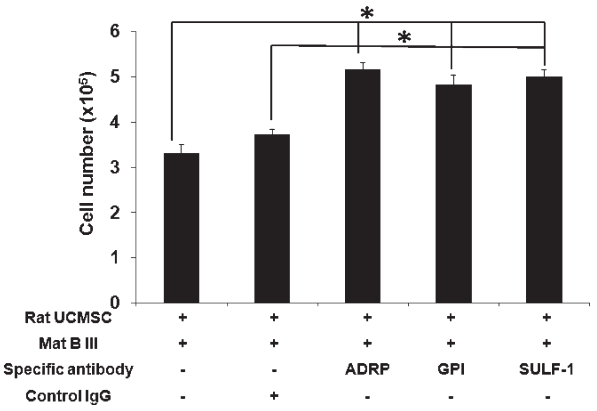


**Figure 6.** The effect of neutralizing antibodies against ADRP (ADFP), SULF-1 and GPI on live cell count of Mat B III cells cocultured with rat UCMSC. Total live cell numbers were counted after 48 h of coculture in the presence or absence of anti-ADRP, -SULF-1, and -GPI antibodies (1 $\mu$g/well). The experiment was performed twice with triplicate determinations. Each value represents mean ± SE of samples. *, $P < 0.05$, compared with Mat B III cells cocultured with rat UCMSC in the absence of antibodies and in the presence of control IgG.

The qRT-PCR confirmed that expression of ADRP, GPI, and SULF-1 was respectively 3.0 ± 0.7, 2.0 ± 0.5, and 2.5 ± 0.4 (mean ± SE, triplicate determinations) fold higher than that of nontransfected rat UCMSC at 48 h after the transfection. While [$^3$H]thymidine uptake in rat UCMSC overexpressing these genes was not altered, [$^3$H]thymidine uptake significantly decreased to approximately 70%, 71% and 66%, respectively, in Mat B III cocultured with rat UCMSC transfected with ADRP, GPI, or SULF-1 48 h prior to the coculture as compared with GFP transfected cells (Figure 8). This enhancement of rat UCMSC-dependent attenuation of the [$^3$H]thymidine uptake in Mat B III rat mammary carcinoma cells is attributable to the gene products of overexpressed genes. Therefore, it is suggested that these genes, which are differentially upregulated in rat UCSMC cocultured with Mat B III (Figure 2), are at least partially responsible for growth attenuation of the Mat B III cells.

## DISCUSSION

Increasing evidence suggests that adult stem cells can be effective therapeutic tools for various diseases including cancer.[1,2,13,22,23] Indeed, multiple adult stem cells engineered to express therapeutic genes have been reported to be very effective in attenuating various cancers.[3,4,22] A few papers have also reported that naive adult stem cells have an intrinsic ability to attenuate growth of several types of cancer cells, such as Kaposi's sarcoma and glioma.[12,24,25] We reported earlier that unengineered rat UCMSC attenuate rat mammary carcinoma,[2] mouse pancreatic carcinoma,[16] and lung carcinoma[26] in immunocompetent animals. A drawback of cancer cytotherapy using engineered stem cells could be unexpected gene expression of the transfected gene or, if viral vectors are used, mutation of the vector genes into a virulent form or insertion into inappropriate genomic regions. Thus, if naive stem cells can be used for cancer cytotherapy, the safety of cytotherapy will be increased significantly. Accordingly, the aim of the present study was to determine the molecular mechanism of the intrinsic tumoricidal activity in rat UCMSC. In the present study, we identified potential genes involved in the intrinsic tumoricidal ability of rat UCMSC against rat malignant breast carcinoma cells *in vitro*. The study provides evidence that multiple tumor suppressor genes are involved in rat UCMSC-dependent tumoricidal activity. This evidence can be utilized for the future development of a safe cancer-targeted cytotherapy for breast carcinoma.

Microarray analysis provides the means to perform genome-wide parallel analysis of genes in a single assay, resulting in a semiquantitative assessment of changes in mRNA expression. Although the Illumina RatRef-12 Expression BeadChip used for this study does not cover entire the entire rat genome, this chip covers over 22,500 rat genes. From our microarray analysis, we screened only 16 significant genes that are differentially expressed in rat UCMSC cocultured with Mat B III rat mammary carcinoma

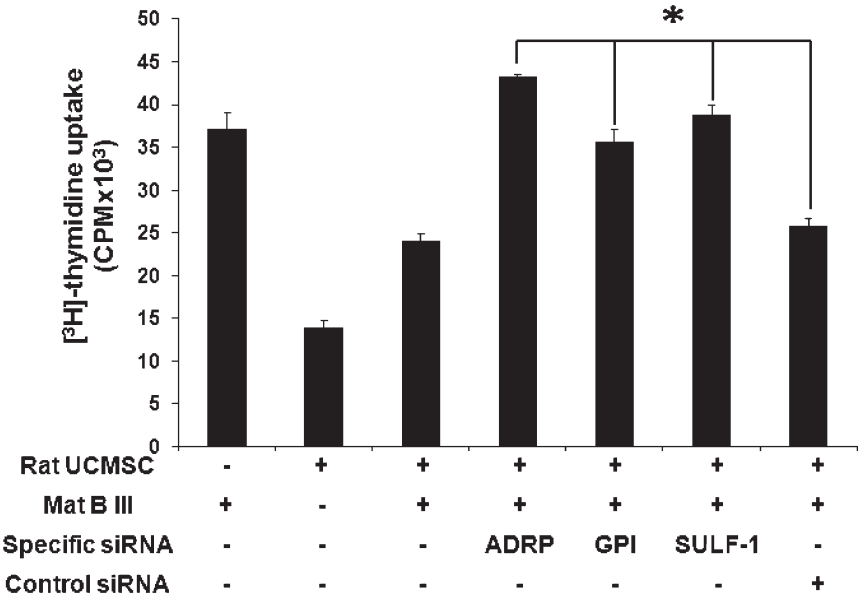


**Figure 7.** The effect of gene-specific siRNA against ADRP (ADFP), SULF-1 and GPI on cell proliferation of Mat B III cells cocultured with rat UCMSC. [$^3$H]Thymidine uptake was determined at 24 h after coculturing gene-specific siRNA transfected rat UCMSC with Mat B III cells. The experiment was performed twice with triplicate determinations. Each value represents mean ± SE of samples. *, $P < 0.05$, compared with Mat B III cells cocultured with rat UCMSC transfected with control scramble RNA.

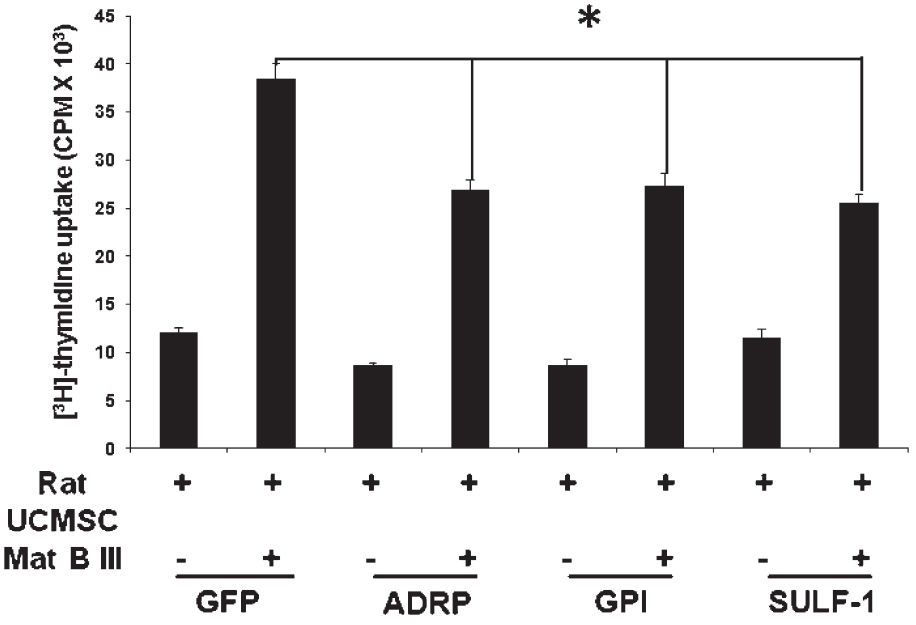


**Figure 8.** The effect of ADRP (ADFP), GPI, and SULF-1 overexpression of rat UCMSC on cell proliferation in cocultured Mat B III cells. ADRP (ADFP), GPI, and SULF-1 were overexpressed in rat UCMSC using the transfection reagent FuGENE HD and cultured for 48 h. Mat B III cells were cocultured with rat UCMSC transfected with individual genes. [$^3$H]Thymidine uptake was determined after 24 h coculture of Mat B III cells and gene-overexpressing rat UCMSC. Each value represents mean ± SE of triplicate determinations. *, $P$ value <0.05, compared with [$^3$H]thymidine uptake by Mat B III cells cocultured with GFP-transfected rat UCMSC.

cells (Figure 1). Among these 16 genes, seven genes were further screened based on the criteria that they should produce secretory proteins and their gene products should be involved in tumor growth regulation. Differential expression of these seven genes was further confirmed by qRT-PCR using the same RNA samples (Figure 2). A remarkable finding in this screening was that five genes (ADRP, FST, HTRA1, SULF-1 and TGF$\beta$I) are known tumor suppressor genes,[27−33] and the first four out of these five genes were determined to be overexpressed by both microarray and qRT-PCR. Although the GPI gene has been identified as a metastasis promoter gene,[21] it was shown to be significantly upregulated in rat UCMSC when they were cocultured with Mat B III cells. In contrast, expression of the TGF$\beta$I and PDPN genes was downregulated in microarray analysis but

slightly upregulated in qRT-PCR. Upregulation of multiple tumor suppressor genes in rat UCMSC is conceivable and reasonable, since we have previously found that rat UCMSC significantly attenuate tumor growth *in vivo* and *in vitro*.[2,16] Therefore, screening of the intrinsic tumoricidal genes in rat UCMSC by genome wide microarray and confirming their expression by qRT-PCR analysis appears to have produced useful information.

Among the seven genes screened, although qRT-PCR analysis indicated that ADRP, GPI, and HTRA1 exhibited the highest upregulation when rat UCMSC were cocultured with mammary carcinoma cells, SULF-1 draws our attention since SULF-1 upregulation attenuates breast cancer cells by decreasing heparin binding growth factor signaling.[30] Although the GPI gene is known to be associated with metastasis,[21] upregulation in rat UCMSC was very high when they were cocultured with Mat B III cells. Accordingly, ADRP, GPI and, SULF-1 were further characterized by their potential function. For this characterization purpose, the [$^3$H]thymidine uptake assay was utilized, since this assay is a sensitive evaluation of cell proliferation.[34,35] The proportion of the cells in coculture was determined by varying their ratio from 1:40 to 1:2 (Figure 4). As shown in Figure 4, a 1:20 ratio of rat UCMSC:Mat B III showed the best attenuation effect. Although a lower ratio of rat UCMSC (1:2−1:4) exhibited Mat B III cell growth attenuation effect, its effect was less pronounced. This result may suggest that rat UCMSC may possess both growth attenuation and promotion effects and the growth attenuation effect is more visible in a higher ratio of two cells while the growth promotion effect is more pronounced at a lower ratio in coculture. Accordingly, a 1:20 ratio was used for most of the following experiments.

As described in Results, individual neutralizing antibodies against the three gene products significantly increased not only [$^3$H]thymidine uptake (Figure 5) but also direct cell counts (Figure 6) by Mat B III cells cocultured with rat UCMSC. These results were further confirmed using gene-specific siRNA-dependent knockdown of mRNA. As shown in Figure 7, individual gene-specific siRNA transfection significantly increased [$^3$H]-thymidine uptake. These results suggest that these three gene proteins are secretory and attenuate cell proliferation of Mat B III cells. Negative control experiments confirmed that the effect of these neutralizing antibodies was specific to these three tumor suppressor proteins, since a neutralizing antibody against the tumor suppressor protein decorin (nondifferentially expressed) and control IgG did not show any effect on [$^3$H]thymidine uptake by Mat B III cells. In addition, overexpression of these selected genes in rat UCMSC significantly enhanced their ability to attenuate growth suppression of Mat B III cells in coculture (Figure 8). Furthermore, coculture with rat uterus fibroblasts did not affect Mat B III cell proliferation (data not shown). Product secretion from the three identified genes was confirmed by immunoprecipitation of the proteins in the culture medium and resultant Western blot analysis (Figure 3). These results suggest that the tumoricidal effect of rat UCMSC is specific and, at least in part, carried out by upregulation of multiple tumor suppressor genes. However, these gene-dependent tumor suppressor functions are only partially responsible for the tumoricidal action of rat UCMSC, since the present study identified at least 16 genes are involved in this rat UCMSC-dependent tumor growth attenuation. In addition, it is noteworthy to point out that these gene products were also produced in Mat B III tumor cells (Figure 3). Therefore, it is possible that tumor cell-dependent production of these tumor suppressor proteins also plays a role in cell growth control in an autocrine and paracrine manner. A surprising discovery in the present study is that GPI, which has been considered to be associated with tumor metastasis, acted as a cell growth suppressor in this experiment. Although it is unclear whether GPI acts as a tumor suppressor *in vivo*, this possibility might be of interest as a future study. Larmonier et al. have reported that nitric oxide plays a role in bone marrow mesenchymal stem cell-induced growth alteration of cancer cells.[25] However, rat UCMSC-dependent growth attenuation *in vitro* may not be due to nitric oxide since nitric oxide is very unstable in culture media.[36]

In summary, the present study demonstrates that rat UCMSC significantly attenuate growth of Mat B III rat mammary carcinoma cells in culture. Rat UCMSC-dependent tumor cell growth attenuation is associated with upregulation of multiple tumor suppressor genes in rat UCMSC. These tumor suppressor proteins were shown to be secretory regulators of tumor cell proliferation. These results clearly suggest that naive UCMSC are a potential cytotherapeutic tool for breast cancer therapy.

## ■ ASSOCIATED CONTENT

**Supporting Information.** Figures depicting (1) quality analysis of total RNA obtained from rat UCMSC alone and cocultured with Mat B III and (2) the effect of neutralizing antibody against decorin on cell proliferation of Mat B III cells with or without coculture with rat UCMSC. This material is available free of charge via the Internet at http://pubs.acs.org.

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## ■ ABBREVIATIONS USED

ADRP, adipose differentiation-related protein, adipophilin, ADFP, perilipin; BGN, biglycan; COL1A2, collagen, type I, alpha 2; FST, follistatin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPI, glucose phosphate isomerase; HTRA-1, HtrA serine peptidase 1; LOXL1, lysyl oxidase-like 1; LTBP4, latent transforming growth factor beta binding protein 4; Mat B III, Fisher 344 rat derived mammary adenocarcinoma cell line;

MSC, mesenchymal stem cells; PAM, peptidylglycine alpha-amidating monooxygenase; PDIA5, protein disulfide isomerase family A, member 5; PDPN, podoplanin; PTGS2, prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and; cyclooxygenase)P4HA1, prolyl 4-hydroxylase, alpha polypeptide 1; qRT-PCR, quantitative real-time polymerase chain reaction; SERPINE2, serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 2; siRNA, small interfering ribonucleic acid; SULF-1, sulfatase-1; TGF$\beta$I, transforming growth factor beta-induced; UCMSC, umbilical cord matrix stem cells

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