IDENTIFICATION AND CHARACTERIZATION OF UNIQUE TUMORICIDAL GENES IN RAT UMBILICAL CORD MATRIX STEM CELLS

by

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B.Sc., Andhra University, India, 2007

A THESIS

Submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Department of Anatomy & Physiology
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KANSAS STATE UNIVERSITY
Manhattan, Kansas

2010

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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 naïve rat UCMSC alone and those co-cultured with Mat B III in Transwell culture dishes. Gene expression profiles of naive rat UCMSC alone and those co-cultured 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 co-cultured rat UCMSC identified five up-regulated candidate genes (follistatin (FST), sulfatase1 (SULF-1), glucose phosphate isomerase (GPI), HtrA serine peptidase (HTRA1), and adipocyte differentiation-related protein (ADRP)) and two down-regulated candidate genes (transforming growth factor, beta-induced, 68kDa (TGFβI) 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 co-cultured 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 individually in co-cultures of 1:20 rat UCMSC:Mat B III cells significantly increased cell proliferation, implying that these gene products are produced under the co-cultured condition and functionally attenuate cell growth. Immunoprecipitation followed by Western blot analysis demonstrated that these proteins are indeed secreted into the culture medium. Individual over-expression of these three genes in rat UCMSC significantly enhanced UCMSC-dependent inhibition of cell proliferation in co-culture.

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.

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List of abbreviations used

UCMSC- Rat umbilical cord matrix stem cells

Mat B III- Fisher 344 rat derived mammary adenocarcinoma cell line

RUF- Rat uterus fibroblasts

ADRP- Adipose differentiation-related protein, adipophilin

ADFP- perilipin

SULF-1- Sulfatase-1

GPI- Glucose phosphate isomerase

PDDN- Podoplanin

FST- Follistatin

HTRA-1- HtrA serine peptidase 1

TGFβI- Transforming growth factor beta-induced

GAPDH- Glyceraldehyde-3-phosphate dehydrogenase

Acknowledgements

Thanks to my major advisor, Dr. Masaaki Tamura (Department of Anatomy & Physiology, Kansas State University) for providing me an opportunity to study this challenging and intriguing subject and supporting me throughout my Master course period. I would also like to acknowledge Dr. Naomi Ohta and Atsushi Kawabata (Department of Anatomy & Physiology, Kansas State University) for supporting my entire Master research by providing critical and constructive discussion. I am very thankful to all of the members of Dr. Tamura's laboratory for assisting me in conducting day-to-day experiments and proofreading my thesis. I would also like to express my gratitude towards Drs. Troyer and Ganta for serving my advisory committee role for my Master thesis. This work was supported in part by Kansas State University (KSU) Terry C. Johnson Center for Basic Cancer Research, KSU College of Veterinary Medicine Dean's fund, and NIH P20 RR017686.

CHAPTER 1 - Introduction

Breast cancer continues to be one of the most common cancers and a major cause of death among women worldwide[1]. Breast cancer occurs when mammary epithelial cells multiply uncontrollably, causing damage to the mammary gland function and interfering with the normal function of other organs by metastasis[2]. The most common types of breast cancer are ductal and lobular carcinoma[3]. Other less common types of breast cancer include inflammatory breast cancer characterized by general inflammation of the mammary epithelium, medullary carcinoma (an invasive breast carcinoma that forms a distinct boundary between tumor tissue and normal tissue), mucinous carcinoma (formed by the mucus-producing cancer cells), and tubular carcinoma (cancer that begins inside the milk duct and spreads beyond it)[3].

Current statistics of breast cancer

Breast cancer is second only to lung cancer as the leading cause of cancer deaths among women in the United States of America (USA) with higher incidence rates than any other cancer type[4] . Breast cancer will affect one of every eight American women in their lifetime. American Cancer Society estimation for breast cancer incidence in USA for 2010 is about 207,090 for females and 1,970 for males and estimated numbers of deaths are 39,840 for females and 390 for males.

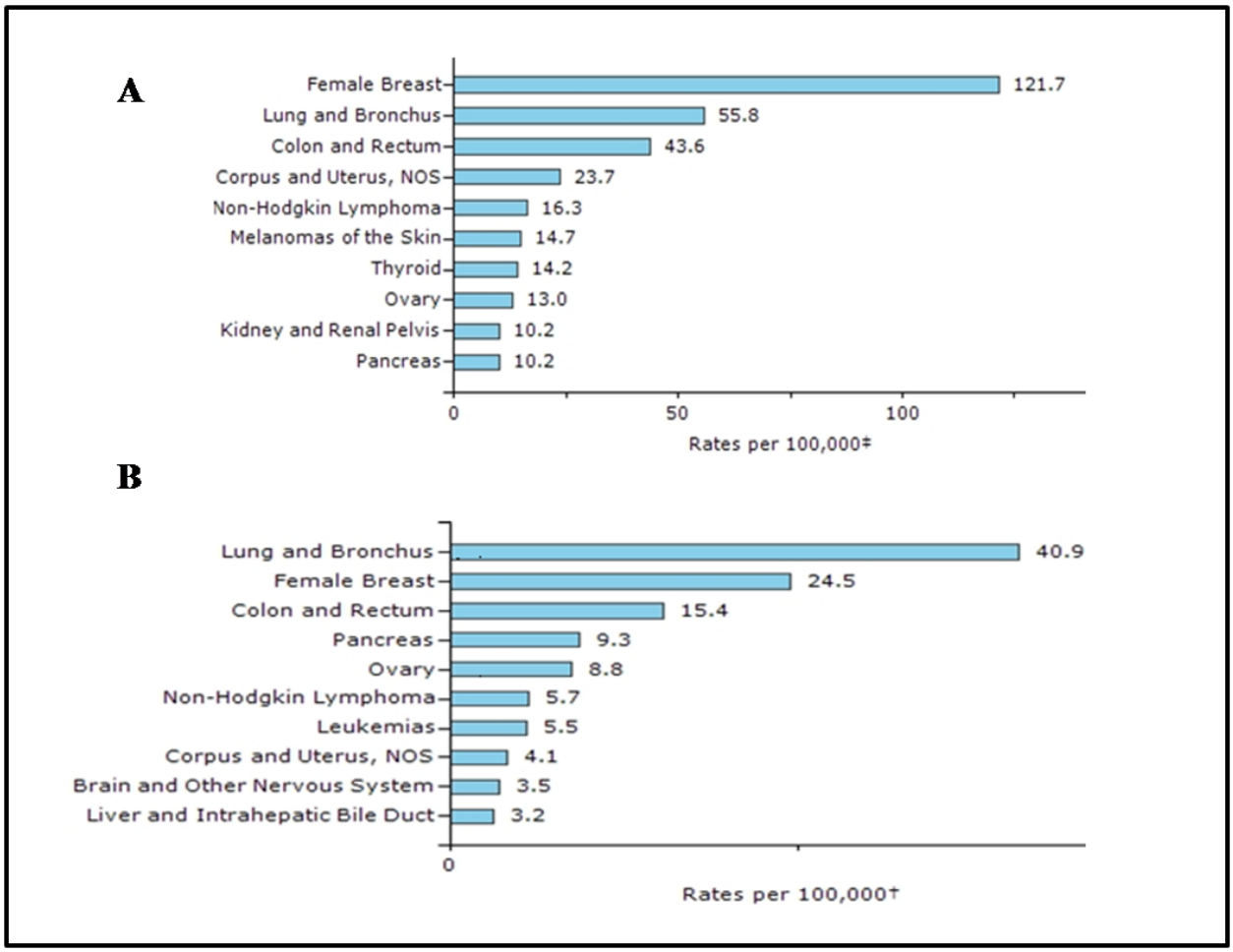


Figure 1.1. **The incidence and mortality of cancers in the USA[4]**.

A. Incidence of top 10 types of cancers in the United States. Breast cancer is the highest among the all cancers. B. The mortality rates of the top 10 cancers in the United States. Breast cancer ranked second among cancer mortality rates with a rate equaling those of colorectal cancer and pancreatic cancer combined.

Current breast cancer therapies

Currently, available clinical therapies for breast cancer include surgical resection, radiation therapy, hormone therapy, and chemotherapy[5]. Surgical resection is usually the most common and first line of therapy for breast cancer, Lumpectomy (breast-sparing surgery) and mastectomy are the two types of surgical therapies[6]. Radiation therapy uses ionizing (high-energy) radiation to kill the tumor cells and this procedure generally reduces the risk of breast cancer recurrence by approximately 70%[5, 7]. However, it often induces adverse side effects on normal tissues adjacent to the radiation site[5]. For both of the above therapies the tumor should be within clinical detection range and accessible to therapeutic procedures[5].

Chemotherapy is a major post-surgical treatment used to reduce the risk of cancer recurrence[5]. Chemotherapy decreases the tumor size by affecting rapidly dividing and highly metabolizing cells, which are the main characteristics of malignant cells[5]. Generally, chemotherapy involves the administration of drugs systemically through the bloodstream[5]. A large number of chemotherapeutics are clinically available. In many cases, a combination of two or more drugs will be used as a treatment regimen for breast cancer[5]. However, chemotherapy causes side effects like hair loss (alopecia), fatigue, anemia etc., by acting on dividing cells and cells, which have high metabolic activities[8].

Estrogen and progesterone promote breast cancer growth by affecting the mammary epithelial cells' multiplying ability[9]. Hormone therapy prevents breast cancer cells from utilizing these hormones but it is not effective against hormone-receptor-negative breast cancers (mostly used as adjuvant therapies with other therapies)[10, 11]. The drugs that will block the synthesis of estrogen like anastozole, letrozole (aromatase inhibitors), leuprolide acetate, goserelin acetate (LH-RH agonists), and estrogen receptor modulators like tamoxifen (estrogen

receptor downregulator) are in use as therapeutic agents in hormonal therapy[9]. Although these drugs have been used successfully, they exhibit serious side effects such as blood clots, strokes, uterine cancer, cataracts, thinning bones and increase in blood cholesterol level[8]. Trastuzumab and Lapatinib are the other two cancer-related protein-targeted drugs that have been shown to be effective for breast tumors expressing high levels of HER2 (Human epidermal growth factor receptor 2)[12].

Among these therapies, a breast cancer target therapy is effective in treating a particular subset of breast cancer and exhibits fewer side effects[13]. However, only a small portion of breast cancer patients receives this benefit since targeting cancer–specific abnormal protein is not always an effective method of treating breast cancer[13]. Accordingly, chemotherapy and/or radiation therapy along with surgical resection is still the most important treatment regimen[5]. Although these therapies listed above effectively treat breast cancer, once again, their adverse side effects are a big hampering issue. For the staggering therapy-related side effects and mortality rates of breast cancer are to be reduced, novel therapies which exclude the serious side effects of current treatments are needed. Several new therapies based on biological agents such as nanoparticle drug delivery, stem cell based anticancer therapy, photodynamic therapy, targeted antibody therapy, and cancer vaccines, which are currently in development, have been very effective in preclinical research, but they have not yet been shown to be effective in clinical trials. Accordingly, a novel powerful cancer therapy with fewer side effects is urgently needed.

Postnatal stem cells and cancer therapy

Postnatal stem cells or adult stem cells are undifferentiated cells found in tissues and organs, which have the ability to self-replicate [14]. These cells help in the maintenance of homeostasis in adult tissues by differentiating into different specialized cells [14, 15]. These cells have been identified in many organs and tissues like bone marrow (hematopoietic stem cells and bone marrow mesenchymal stem cells), skin, teeth, gut, liver, brain, blood, adipose tissue and umbilical cord blood and matrix. Among these stem cells, hematopoietic stem cells and bone marrow mesenchymal stem cells have been used in order to apply more intensive chemotherapeutic regimens [16]. However, these trials have yet to be successful.

Apart from supplemental therapeutic use, some of the adult stem cells have tumor tropic property, which can be applied for the purpose of anticancer therapy [17-20]. Hall *et al.,* showed that bone marrow-derived mesenchymal stem cells (MSCs) can migrate to the tumors and produce biological agents locally at tumor sites [21]. The tumor microenvironment preferentially promotes the engraftment of MSCs as compared with other tissues. MSCs engineered to over-express IFN-β inhibited the growth of malignant cells *in vivo* [20, 22]. Importantly, this effect requires the integration of MSCs into the tumors and could not be achieved by systemically delivered IFN-β or by IFN-β produced by MSCs at a site distant from the tumors. This may indicate that MSCs may serve as a platform for delivery of biological agents in tumors.

Recently, Dwain *et al.* and Aboody *et al.,* showed that intravascular injections of neural stem cells (NSCs) may lead to preferential migration towards central nervous system tumors [17, 23]. They have also demonstrated that, upon transplantation, genetically modified NSCs engineered to produce antitumor molecules have the ability to migrate towards tumors and reduce tumor mass directly or through a “bystander” effect. Modifying NSCs with either

endogenous promoters or viral vectors to express antitumor genes could be useful and practical for potential therapeutic use for brain tumors. NSCs modified to secrete HER2-targeting antibodies constitute a promising novel platform for targeted cancer immunotherapy [24]. Specifically, this NSC-mediated antibody delivery system has the potential to significantly improve clinical outcome for patients with HER2-overexpressing breast cancer [24].

Adipose-derived stem cells (ADSCs) are shown to possess the ability to home and deliver oncolytic myxoma virus (preferentially infects and lyse cancer cells) to gliomas xenografts [25]. In orthotopic xenograft studies, vMyxgfp transduced ADSCs injected intracranially distant from the tumor demonstrated that myxoma virus was delivered to tumor site by ADSCs and resulting in significant survival increase [25]. This result suggests that ADSCs are promising new carriers for oncolytic viruses, specifically myxoma virus, to brain tumors [19, 25]. Thus, adult stem cells hold a great promise for application in targeted anticancer therapy due to their unique tumor tropic characteristics.

Umbilical cord matrix stem cells

Umbilical cord matrix (Wharton's jelly) is a rich source of mesenchymal like stem cells. Unlike with embryonic stem cells, the destruction of an embryo is not involved in the collecting of umbilical cord matrix stem cells [26]. Studies have demonstrated that UCMSC can be differentiated into a variety of specialized tissues such as nerve, liver, heart, kidney, bone marrow, and cartilage cells [26-30]. The additional fact that umbilical cords would normally be discarded after a healthy birth makes such stem cells readily, easily and inexpensively available worldwide. This offers a further advantage since, unlike with embryonic stem cells, UCMSC are ethically and politically noncontroversial.

UCMSC have been shown to be effective in the treatment of stroke, spinal cord damage, myocardial infarction, diabetes, retinal disease, Ischemia, liver disease, parkinson's disease, etc [31-35]. Apart from these applications, UCMSC has tumor tropic properties [18, 36-40]. Although the exact signals that are recruiting UCMSC to the tumor area is still unknown, the same signaling process that is attracting the immune cells and MSCs to the neoplasia site may also be working for the tropism of UCMSC.

Because of the inflammatory state in invasive tumors, stem cells may migrate towards tumors by chemo-attraction mechanisms. Several groups have showed the tumor tropic properties of UCMSC from human [36, 38, 39, 41] and rat [18, 37]. Ganta *et al.* has shown that un-engineered rat UCMSC alone significantly attenuate growth of rat mammary carcinoma in F344 rats without any evidence of reoccurrence [18]. The tumor regression effect of the rat UCMSC is dose dependent and these cells did not show any significant acute side effects [18]. These results strongly suggest that un-engineered rat UCMSC is an ideal therapeutic tool for the treatment of breast tumors. Finding the underlying molecular mechanisms and potential genes involved in rat UCMSC-dependent tumor growth attenuation will help to develop human UCMSC-based therapy for human cancer patients.

Microarrays

DNA microarray is one of the most powerful technologies to screen up-regulated or down-regulated genes in various pathophysiological conditions. Defining gene expression profiles, i.e. comparing patterns of gene expression in different tissues, developmental stages, in normal and disease states, or in distinct *in vitro* conditions, is a big step toward understanding the roles of various genes. Using DNA microarrays, a single hybridization experiment can generate a

genome-wide gene expression profile at once[48]. While gene expression profiling is currently the dominant microarray application, microarrays is also increasingly being used in the research of pharmacogenomics and molecular diagnostics[49]. Therefore, the range of microarray technology applications is enormous. A DNA microarray uses an orderly arranged known DNA probes attached to a solid support[48]. These samples can be partial oligonucleotides of cDNAs. RNA is isolated from various tissues or cells, reverse transcribed into cDNA and futher into cRNA and labeled by biotin. Then it is hybridized on a microarray chips/plates, stained with streptavidin and visualized by multiple techniques.

Cancer is caused by repeating mutations of various genes such as tumor suppressor genes, oncogenes, genes related with cell cycle regulation, apoptosis, DNA repair, etc. Therefore, DNA microarray data might be one of the clues to uncover the basic biology, diagnosis, treatment of different cancers and shedding light on the uncountable questions about this problematic disease. Because our colleagues have shown that rat UCMSC have a potent ability to suppress the growth of Mat B III cells *in vitro* and *in vivo*[18]. DNA microarray would be the simplest and appropriate way to discover potential genes responsible for the tumoricidal function in rat UCMSC.

Objective of project and experimental design

Our colleagues, Ganta *et al*., showed that un-engineered rat UCMSC significantly regressed mammary carcinoma xenografts in F344 rats without reoccurrence for 100 days[18]. Ayuzawa *et al*. have discovered that naïve human UCMSC attenuate the growth of metastatic breast carcinoma xenografts in SCID mice [36]. Doi *et al*. have reported that a treatment with un-engineered rat UCMSC significantly attenuated the growth of peritoneal xenografts of pancreatic

tumors and increased survival time of pancreatic tumor-bearing mice [37]. There are now compelling evidences that human UCMSC can be used as cancer-targeted gene delivery vehicles. Naive rat and human UCMSC that are not engineered to secrete therapeutic proteins also exert a potent anticancer effect in preclinical models [18, 36, and 37].

Identifying underlying molecular mechanisms of tumor growth attenuation or intrinsic tumoricidal genes of rat UCMSC helps to understand how rat UCMSC are regressing the neoplasm and this mechanisms may be utilized to develop novel treatment procedures for breast cancer. The present study is designed to identify the intrinsic tumor growth attenuating mechanisms of un-engineered rat UCMSC. For this purpose, rat UCMSC gene expression profiles in the presence and absence of Mat B III rat mammary carcinoma cells were analyzed using microarray analysis. Functions of screened candidate genes for intrinsic tumor growth attenuation by rat UCMSC were analyzed using [^{3}H] thymidine uptake cell proliferation assay and the effect of neutralizing antibodies against selected gene products (proteins) on the cell proliferation. The present study clearly indicates that multiple tumor suppressor genes were up-regulated in the rat UCMSC co-cultured with Mat B III cells. These gene products were determined to be functional in attenuation of the growth of Mat B III mammary carcinoma cells.

CHAPTER 2 - Materials & Methods

Cell culture

The rat UCMSC were maintained in low-serum defined medium containing the following mixture per 100 mL: 57 mL low-glucose DMEM (Invitrogen, Carlsbad, CA), 37 mL MCBD 201 (Sigma, St Louis, MO), 2 mL fetal bovine serum (FBS; Atlanta Biologicals Inc.,Georgia), 1 mL of 100× insulin-transferrin-selenium-X (Invitrogen, Carlsbad, CA); 1 mL of 0.15 g/mL AlbuMax1 (Invitrogen, Carlsbad, CA), 1 mL of 100× Pen/Strep (Invitrogen,Carlsbad, CA), 10 nmol/L dexamethasone (Sigma, St Louis, MO), 100 μmol/L ascorbic acid 2-phosphate (Sigma, St Louis, MO), 10 ng/mL epidermal growth factor (R&D systems, Minneapolis, MN), and 10 ng/mL platelet derived growth factor-BB (R&D systems, Minneapolis, MN). Mat B III rat mammary adenocarcinoma line (ATCC, Manassas, VA), was maintained in McCoy's 5A modified medium (Invitrogen, Carlsbad, CA) supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA).Primary rat uterus fibroblasts (RUF) from Fisher 344 rat pups were prepared following an established method [50]. RUF were cultured in DMEM/Ham's F-12 medium (1:1) supplemented with 10% FBS, 100 units/ml pencillin, and 100 μg/mL streptomycin. 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 (SULF1), Glucose phosphate isomerase (GPI), Podoplanin (PDPN), 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. (Santa Cruz, CA).

Co-culture of rat UCMS cells with Mat B III cells and RNA isolation

Indirect co-culture of rat UCMSC (1×10^5 cells/dish) with Mat B III cells (1.5×10^6 cells/dish) were carried out by 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 porous membrane (10 cm in diameter; pore size: 0.4μm), and rat UCMSC were maintained in the bottom culture dish using defined medium. Total RNAs were isolated from the rat UCMSC co-cultured with or without Mat B III cells and from Mat B III cells in the Transwell insert 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 by Drs. Zhang, Becker, and Mr. Wood III at National Institute of Aging, National Institute of Health, (Baltimore, MD). For each sample, biotinylated cRNAs were prepared using an Illumina total prep RNA amplification kit (Applied Biosystems, Foster City, CA, USA). Briefly, 5ug of total RNA were converted to double stranded cDNA using T7-oligo (dT) primers and were followed by an *in vitro* transcription (IVT) reaction to amplify biotinylated cRNA as described in manufacturer's instruction (Illumina Inc., San Diego, CA, USA). The biotinylated cRNA was hybridized to Rat Ref-12 Expression BeadChip platform that contains 22,519 probes (Illumina Inc., San Diego, CA, USA). 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., San Diego, CA, USA) 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

Image Quant software (Molecular Dynamics; Sunnyvale, CA) was used to convert the hybridization signals on the image into raw intensity values, and the data thus generated were transferred into MS Excel spreadsheets, predesigned to associate the Image Quant data format to the correct gene identities. 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. *Z* scores were calculated by subtracting the average gene intensity from the raw intensity

data for each gene and dividing that result by the S.D. 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 S.D. of the overall *z* difference distribution. Assuming a normal distribution profile, *z* differences are assigned significance according to their relation to the calculated standard deviation 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 standard deviation to give the *z* ratios.

Detection of differentially expressed genes

The lists of significant differentially expressed genes were identified based upon the following screening criteria: 1) candidate gene expression should show at least 1.5 z-ratios, p-value < 0.05, false recovery rate < 0.3 in rat UCMSC co-cultured with Mat B III cells; 2) candidate genes encode secretory proteins; and 3) candidate genes encode growth regulation-related proteins. Further hierarchical cluster analysis was performed using open source softwares Cluster 3.0 and Java Treeview .

Quantitative real time-polymerase chain reaction (qRT-PCR)

Microarray results were validated by qRT-PCR using the same RNA samples as those used for the microarray. qRT-PCR was carried out using 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 94°C for 3 min and final extension at 72°C for 7 min. Each cycle

consisted of denaturation for 45 s at 94°C, annealing for 45 s at 58°C, and extension for 1 min 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). The primers used in this study are indicated in Table 2.1.

[^{3}H] Thymidine uptake assay

In all [^{3}H] thymidine incorporation experiments, rat UCMSC (0.25×10^4 per well) were plated in 24-well culture plates and were cultured in CO_2 incubator overnight. Neutralizing antibodies (4 μg per well) were added to the culture medium two hours prior to the co-culture with Mat B III cells (5×10^4 cells per well). Cells were cultured additional 24 h and pulsed for the last 4 h of the culture time with 1.0 μCi [^{3}H] thymidine per well. Mat B III cells in suspension culture were collected, and the free-[^{3}H] thymidine in the medium was washed away with PBS. The cell-incorporated [^{3}H] thymidine was solubilized by adding 0.2 M NaOH and counted by the Packard liquid scintillation counter Tri-Carb 2100TR (Perkin-Elmer Life Science, Boston, MA, USA).

Immunoprecipitation and Western blotting

Rat UCMSC (0.25×10^6 cells per well) were seeded in a 10 cm culture dish and were cultured in CO_2 incubator overnight. Mat B III cells (4×10^6 cells per well) were added to the rat UCMSC cells grown in 10 cm culture dish and further incubated for 24 h. Mat B III in suspension was removed and the culture medium was collected for further analysis.

One ml of supernatant was pre-cleared with 30 μl of protein-A agarose resin slurry (50%)(Thermo Fisher Scientific, Rockford, IL) for 2 h at 4°C. Supernatant was collected into

new tubes by centrifugation at 13,000 rpm for 5 min at 4°C. Three µg/mL of respective gene specific neutralizing antibody was added and incubated overnight at 4°C with gentle rocking. Seventy µl of protein-A agarose resin slurry was added and incubated for 3-4h at 4°C. The beads (resin-antibody-protein complexes) were washed three times with ice-cold 1xTris buffered saline (TBS) by centrifugation at 6,500 rpm for 5 min. Protein-antibody complexes were eluted by suspending the Protein A Resin in 30µl of 2X sample loading buffer and boiled for 5 minutes. The samples were stored at -80°C for future use.

Protein samples were separated by an 8 or 12 % SDS-PAGE gel, electroblotted onto nitrocellulose membrane (GE Healthcare Bioscience Corp, Piscataway, NJ) and blocked with 4% nonfat dry milk in 0.1% Tween20 in phosphate buffered saline (PBST) for 1hr at room temperature. The membranes were washed and incubated with specific antibodies against PDPN, ADRP and GPI (1:200, Santa Cruz Biotechnology) with 4% nonfat dry milk in PBST for 1hr at room temperature and then with the secondary antibody (1:2000, GE Healthcare, Piscataway, NJ). The protein expression signal was detected with Pierce ECL Western Blotting substrate (Pierce, Rockford, IL).

Statistical analysis

Statistical analysis was conducted using student's t-test in Microsoft Excel. The data were presented as mean ± SE. Experiments were performed at least three times with different batches of cells. Results were considered to be statistically significant at $p \leq 0.0$

Table 2.1

The 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
TGFBI	GCGGCTAAAGTCTCTCCAAGGT	TGACACTCACCACATTGTTTTTCA
GAPDH	AGACAGCCGCATCTTCTTGT	CTTGCCGTGGGTAGAGTCAT

CHAPTER 3 - Results

Screening of unique tumoricidal genes by cDNA microarray analysis

To screen genes associated with rat UCMSC-dependent growth attenuation of rat mammary tumors, [18] genome-wide cDNA microarray analyses was carried out using an Illumina RatRef-12 Expression BeadChip which contains 22,519 distinctive rat cDNA oligonucleotide probes. Although this microarray chip does not contain whole rat genes, it encompasses the largest number of rat genes at the time of the analysis. In this study, total RNA was extracted from the rat UCMSC either co-cultured with (defined as stimulated UCMSC) or without Mat B III rat mammary adenocarcinoma cells (defined as unstimulated UCMSC) in the Transwell culture dish (two cell types were divided with a porous membrane with 0.4μm pores). Total RNA was reverse transcribed to cDNA. Hybridization with biotinylated cRNA transcribed from the cDNA was carried out in triplicate and gene expression profiles of rat UCMSC in two conditions were compared. As shown in the Figure 3.1, RNA quality was high enough for further microarray analysis. For unique gene screening, the following criteria were set: 1) candidate gene expression should show at least a 1.5 fold change in rat UCMSC co-cultured with Mat B III cells; 2) candidate genes must encode secretory proteins; and 3) candidate genes should encode positive or negative cell growth-related proteins. Data analysis by *Z*-normalization of the hybridization signals identified sixteen differentially expressed genes in two culture conditions, i.e. their expressions were at least 1.5 fold different (Figure 3.2). Nine of these genes were up regulated in rat UCMSC when co-cultured with the Mat B III cells and seven of these genes were down regulated (Figure 3.3). Among these genes, a total of five up-regulated (HTRA1, SULF1, GPI, ADRP, and FST) and two down-regulated genes (TGFβI and PDPN) were further

characterized as candidate genes associated with growth regulation of tumor cells, i.e. these genes encoding secretory proteins and associated with cell growth regulation.

The mRNA expressions of these seven genes in rat UCMSC were verified by a qRT-PCR using the total RNA extracted for microarray analysis. Of the five up regulated genes selected from microarray analysis, all five genes showed concordant results between microarray analysis and qRT-PCR. However, although PDPN and TGFβI showed a significantly decreased expression in microarray analysis, their expressions were detected to be identical to the un-stimulated rat UCMSC levels (Figure 3.4). These results indicate 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 *z*-ratios in the array data. This is because the *z*-ratios are *z*-differences divided by standard deviation and not fold change ratios. As shown in Table 3.1 below , out of the seven genes, six genes, HTRA1, SULF1, GPI, ADRP, FST and TGFβI, have been characterized as tumor suppressor genes, whereas one PDPN gene has been identified as tumor promoter gene. Based on the extent of the differential expression and individual characterization of these genes, SULF1, GPI, and ADRP were further studied to determine their roles in the rat UCMSC-dependent tumor growth attenuation.

Effect of neutralizing antibodies specific to selected candidate genes on the DNA synthesis in mammary carcinoma cells

In the initial experimental optimization study, it was found that a small number of rat UCMS cells (0.25×10^4) most significantly inhibited [^{3}H] thymidine uptake by Mat B III cells (5×10^4) when they were co-cultured together (rat UCMSC:Mat B III = 1:20, Figure 3.5). Accordingly, this condition was utilized for the following experiment.

Decrease in the rat UCMSC-dependent inhibition of [^{3}H] thymidine uptake by individual neutralizing antibodies against SULF-1, GPI or ADRP suggested that all three genes play a role in the tumor growth attenuation by rat UCMSC (Figure 3.6). We further investigated the effect of all three neutralizing antibodies added together to the culture medium for rat UCMSC co-culturing with Mat BIII cells. Adding three antibodies together markedly increased [^{3}H] thymidine uptake in Mat B III cells (Figure 3.7). This result suggests that these neutralizing antibodies appear to attenuate tumor suppressor protein function individually. As a control experiment the rat UCMSC were co-cultured with Mat B III cells in the presence of anti-decorin antibody (an un-related gene to rat UCMSC-dependent growth inhibition) (Figure 3.8). This is consistent with the original hypothesis that rat mammary carcinoma cell stimulated genes in rat UCMSC are responsible for growth regulation of Mat B III.

As a control study, rat UCMSC were substituted with rat uterus fibroblasts. However, rat uterus fibroblasts did not show any growth attenuation of Mat B III cells (data not shown). This result clearly suggests that rat UCMSC-dependent attenuation of mammary tumor cell growth is an UCMSC-specific event.

Detection of candidate gene products in culture medium by Western blot analysis

To confirm all three gene products are indeed secreted out and play tumor suppressor roles in adjacent tumor cells, presence of all three proteins in the culture medium was analyzed by Western blot analysis. For this purpose, secreted proteins were collected from the culture medium by immunopreciptation as described in the Methods. As shown in the Figure 3.9, all three gene products were detected in the culture media conditioned with the rat UCMSC alone, Mat B III cells alone, and two cell types together. Although these protein levels were higher in the medium used for the co-culture, it was difficult to compare their expressions due to a technical difficulty of normalization with loading standard. However, these results support our hypothesis that tumor suppressor proteins are produced by rat UCMSC, secreted out from the cells and play role in the control of tumor growth.

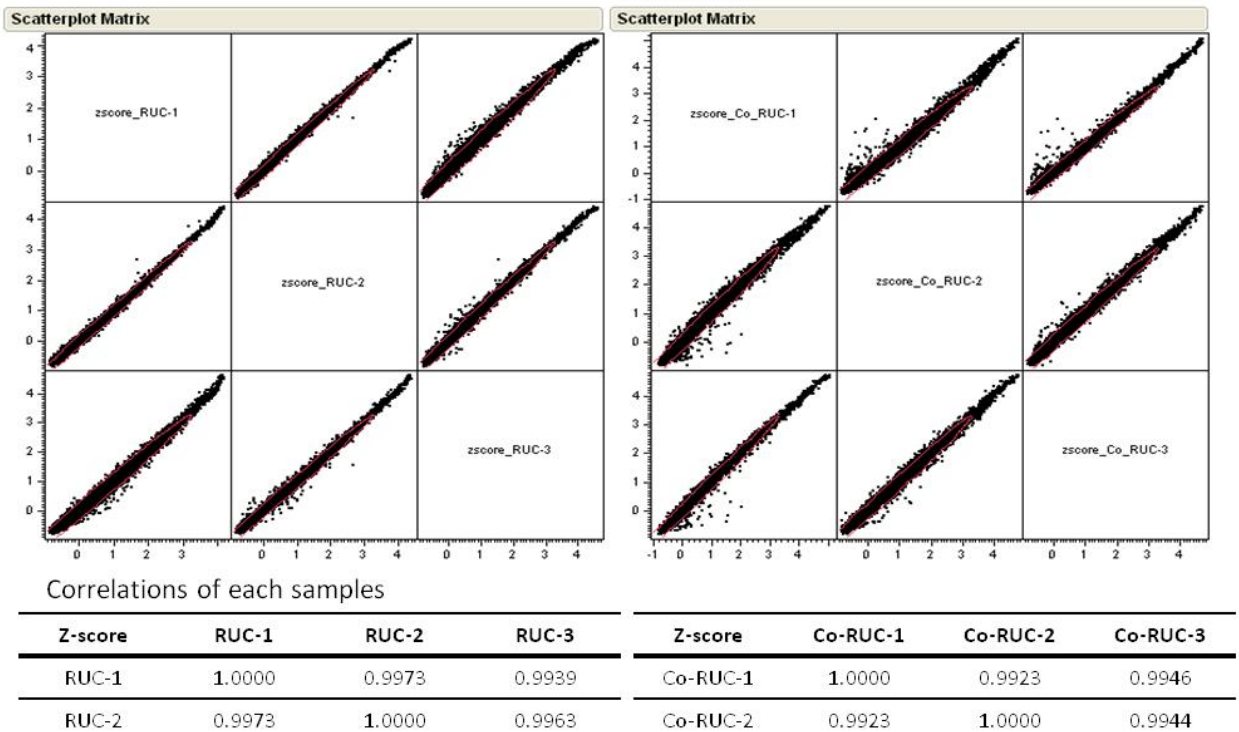
Table 3.1

Functions of differentially expressed genes.

GENE	FUNCTION
HtrA1	TUMOR SUPPRESSOR
SULF1	TUMOR SUPPRESSOR
GPI	TUMOR SUPPRESSOR
TGFBI	TUMOR SUPPRESSOR
PDPN	TUMOR PROMOTER
ADFP	TUMOR SUPPRESSOR
FST	TUMOR SUPPRESSOR

Figure 3.1

Quality analysis of total RNA obtained from rat UCMSC alone and rat UCMSC co-cultured with Mat BIII group. Totally three RNA samples were prepared for each group. As the z-score in the correlation table shows, both rat.UCMSC (RUC) and rat UCMSC co-cultured with Mat B III (co-RUC) groups have high quality of RNA.



Correlations of each samples

Z-score	RUC-1	RUC-2	RUC-3
RUC-1	1.0000	0.9973	0.9939
RUC-2	0.9973	1.0000	0.9963
RUC-3	0.9939	0.9963	1.0000

Z-score	Co-RUC-1	Co-RUC-2	Co-RUC-3
Co-RUC-1	1.0000	0.9923	0.9946
Co-RUC-2	0.9923	1.0000	0.9944
Co-RUC-3	0.9946	0.9944	1.0000

Figure 3.2

Gene clustering of secretory protein encoding genes. Expression levels of mRNA (z-score) of rat UCMSC alone, Mat B III alone and rat UCMSC co-cultured with Mat BIII (co-UCMSC). Highlighted genes were further characterized.

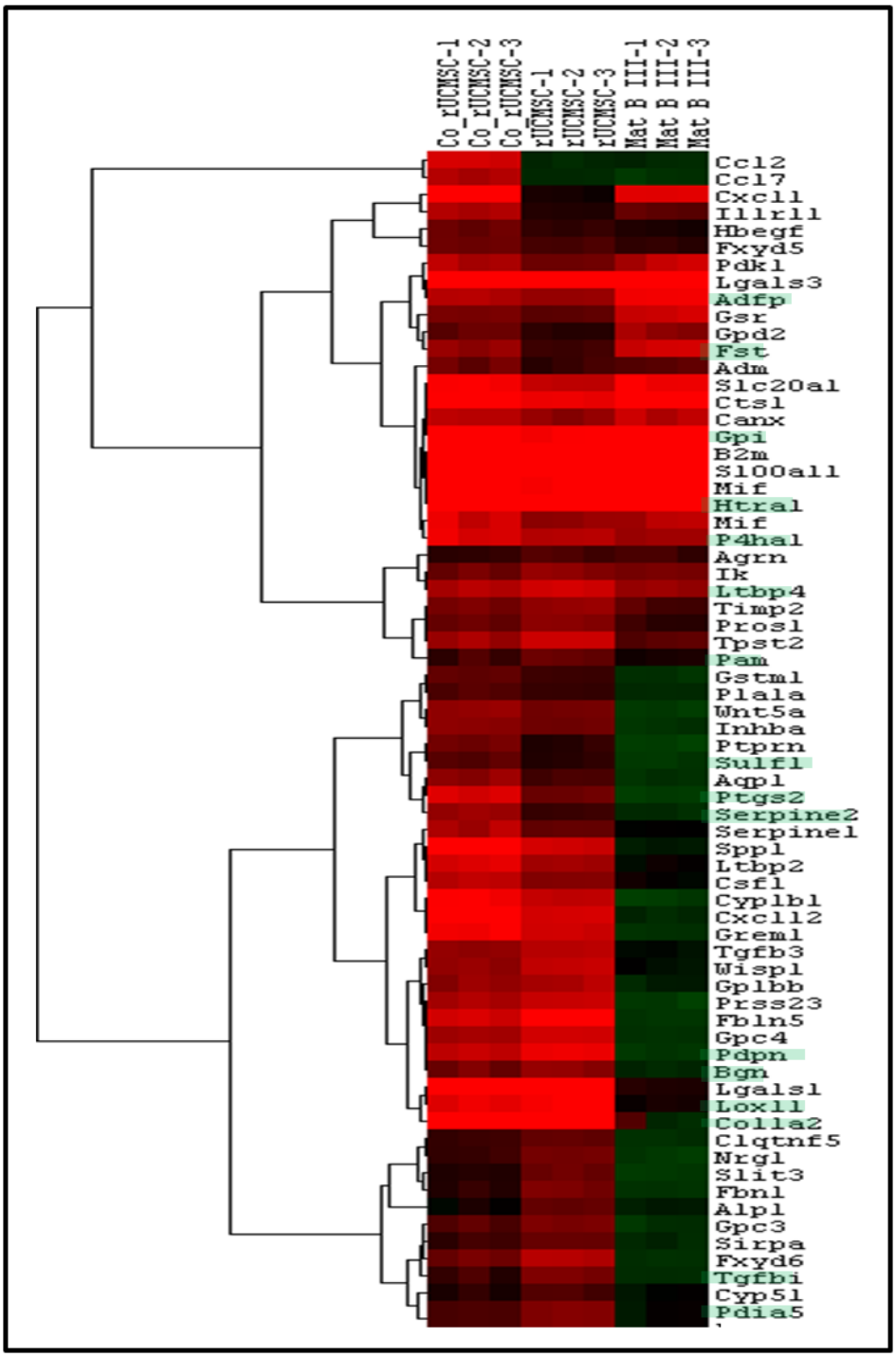


Figure 3.3

Z-ratio of differentially expressed genes in rat UCMSC co-cultured with Mat B III cells. Total RNA was extracted from rat UCMSC 24 hour after co-culturing with or without Mat B III rat mammary carcinoma cells. Co-culture was carried out in the Transwell culture dish so that two types of cells were not mixed each other. Microarray analysis was performed as described in the Methods to identify differentially expressed genes in rat UCMSC co-cultured with or without the rat mammary adenocarcinoma cells.

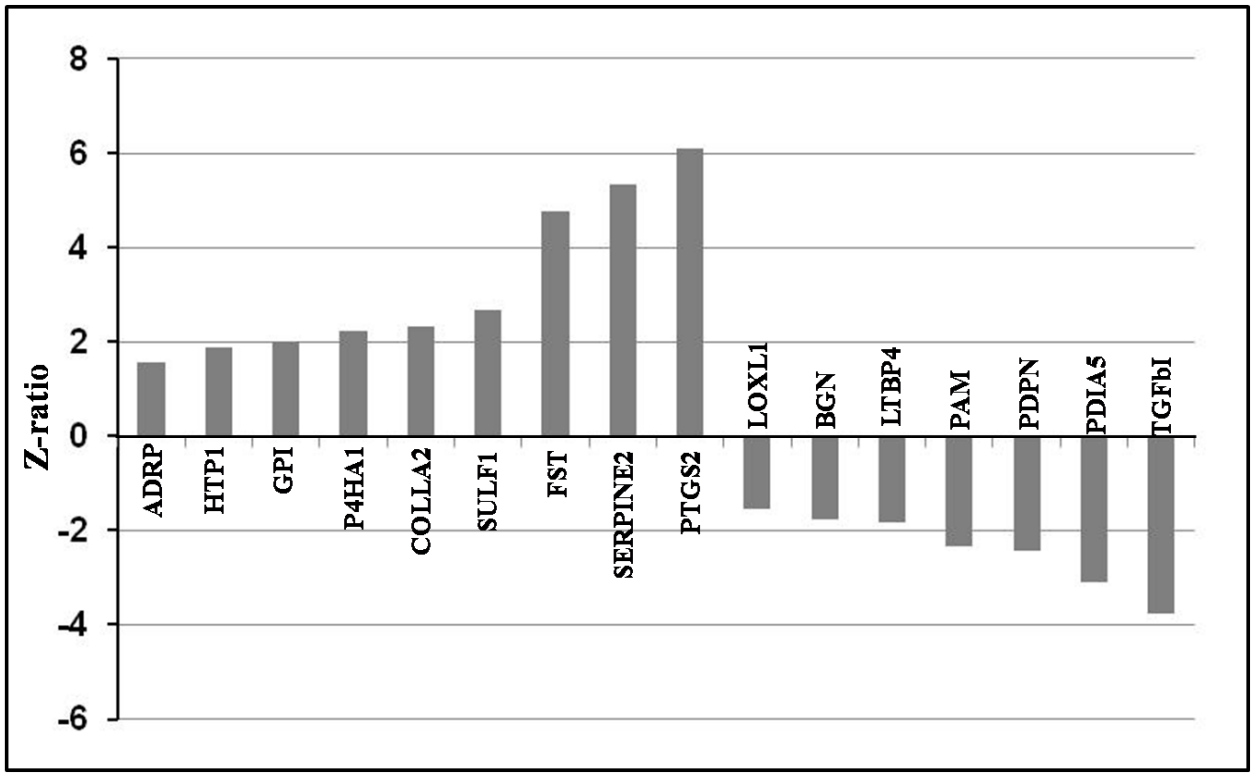


Figure 3.4

Fold changes of differentially expressed genes in the rat UCMSC co-cultured with or without Mat B III cells in qRT-PCR analysis of candidate genes selected initially by microarray analysis. Half shaded bars indicate Z-ratios analyzed by microarray analysis. Dark solid bars represent fold changes determined by real time PCR analysis.

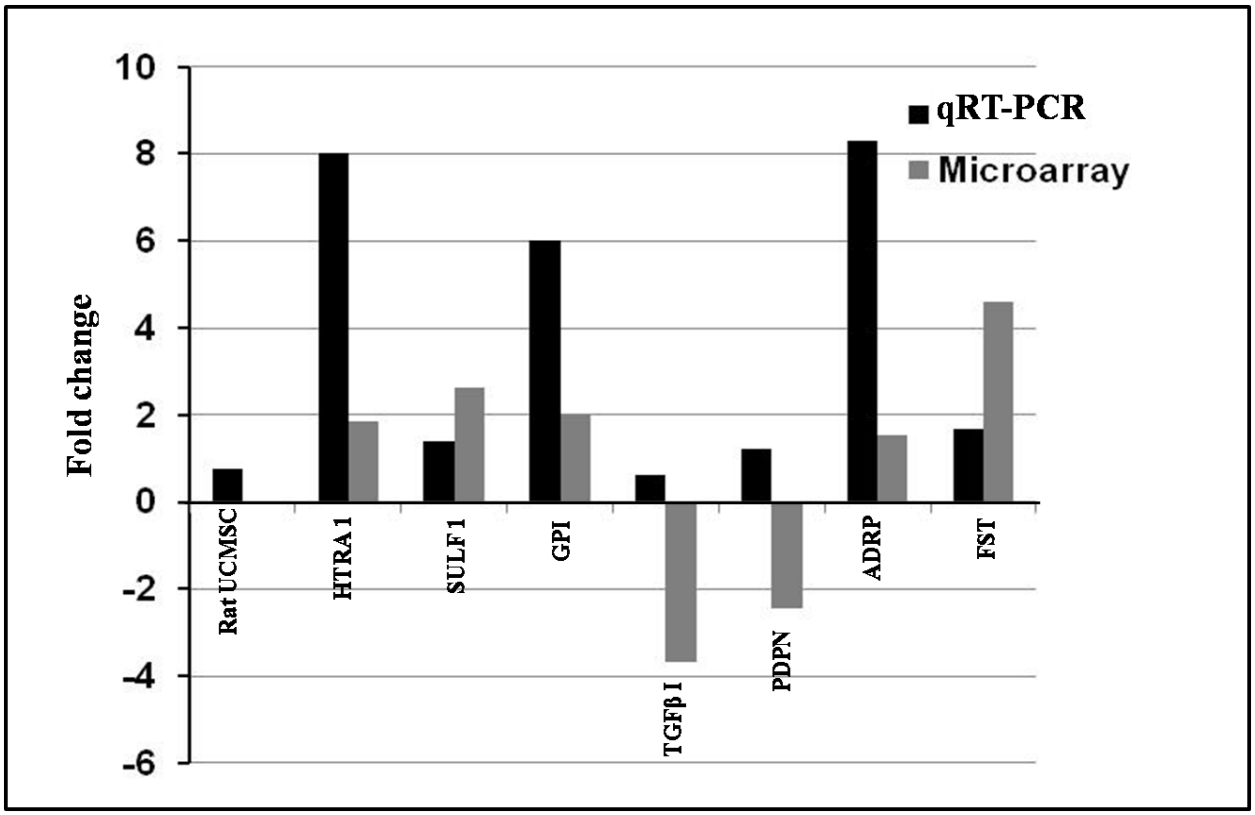


Figure 3.5

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 by rat UCMSC both cells were co-cultured at various ratios as indicated in the figure. [^{3}H]-thymidine uptake was determined at 24 hrs after co-culturing of both cell types. Each value represents means ± SE of three independent experiments. *, $P < 0.05$, compared with rat UCMSC alone or Mat B III alone.

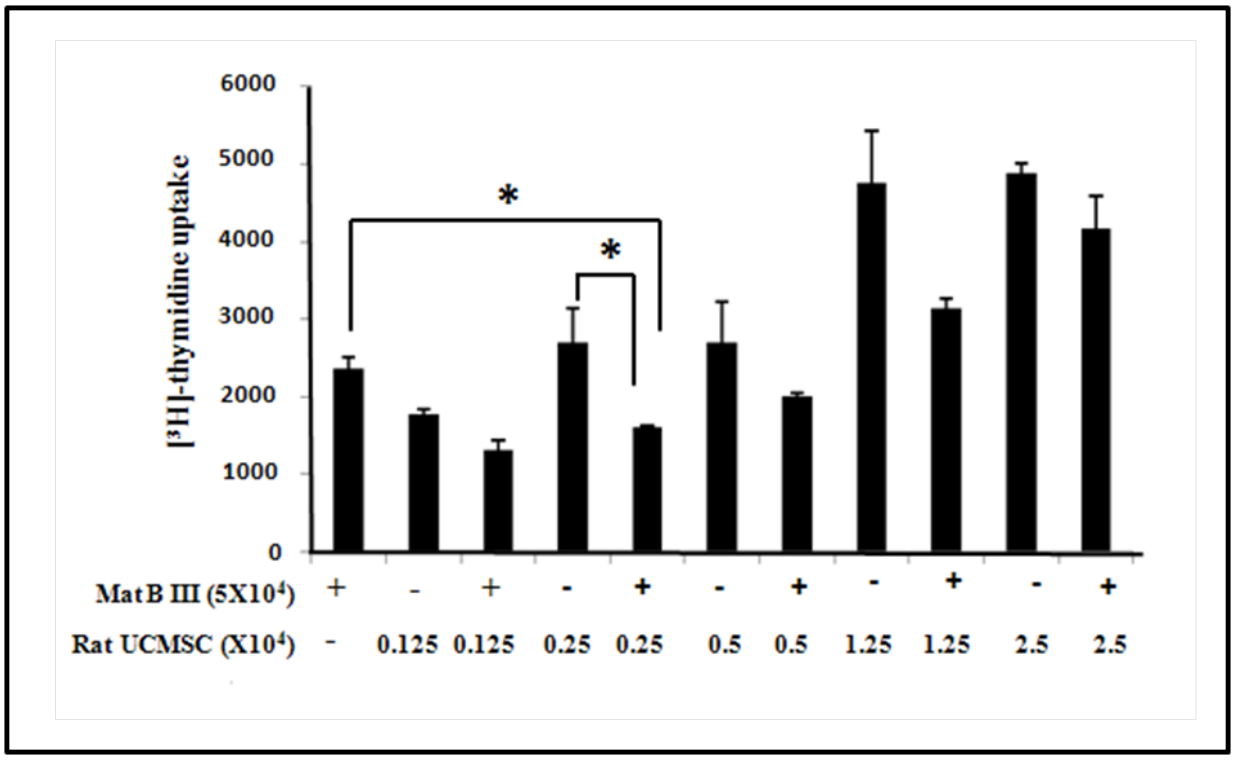


Figure 3.6

The effect of neutralizing antibodies against ADRP (ADFP), SULF-1 and GPI on cell proliferation of Mat B III cells co-cultured with rat UCMSC. [^{3}H]-thymidine uptake was determined at 24 hrs after co-culturing 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 μg/well). Each value represents means ± SE of three independent experiments. *****, *P* value < 0.05, compared with thymidine uptake by Mat B III cells co-cultured with rat UCMSC.

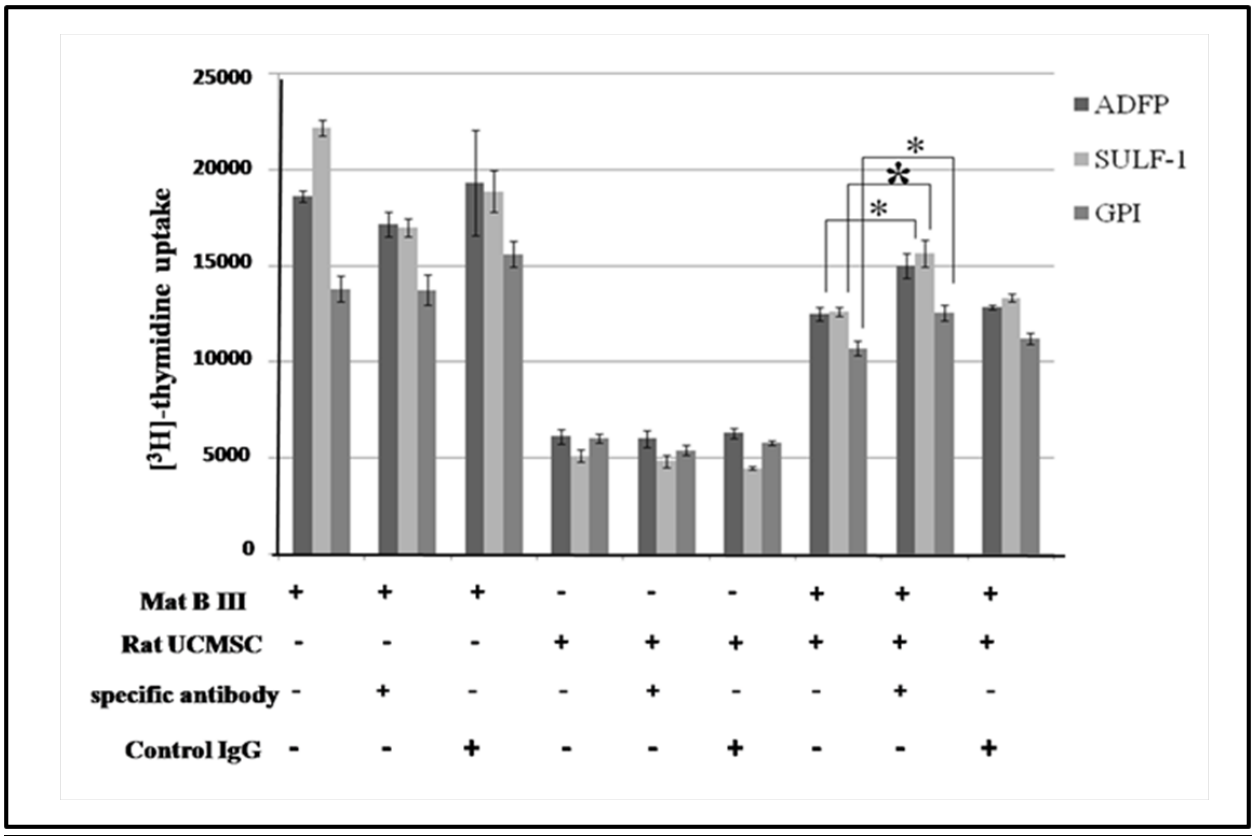


Figure 3.7

The effect of neutralizing antibody cocktail against ADRP, SULF-1 and GPI on cell proliferation of Mat B III cells co-cultured with rat UCMSC. [^{3}H]-thymidine uptake assay was determined at 24 hrs after co-culturing rat UCMSC with Mat B III cells in the presence or absence of anti-ADRP, SULF-1 and GPI antibodies. Each value represents means ± SE of three independent experiments. *, *P* value < 0.05, compared with Mat B III cells co-cultured with rat UCMSC.

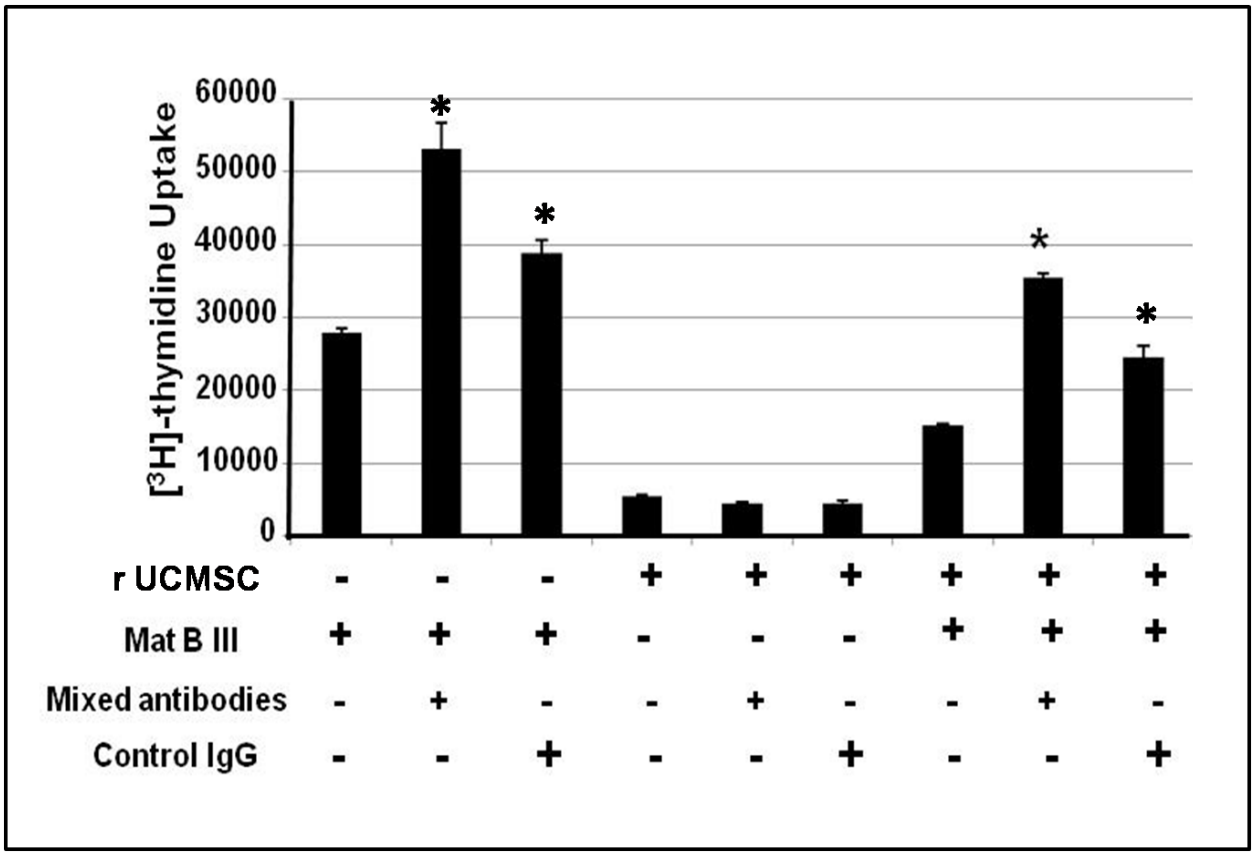


Figure 3.8

The effect of neutralizing antibody against tumor suppressor decorin on cell proliferation of Mat B III cells co-cultured with or without rat UCMSC. The rat UCMSC were co-cultured with Mat B III cells in presence or absence of polyclonal rabbit antibody against Decorin. Since gene expression of decorin in rat UCMSC was unchanged when co-cultured with Mat B III cells; this tumor suppressor gene was used as a control gene. Each value represents means ± SE of three independent experiments. *, *P* value < 0.05, compared with Mat B III cells co-cultured with rat UCMSC.

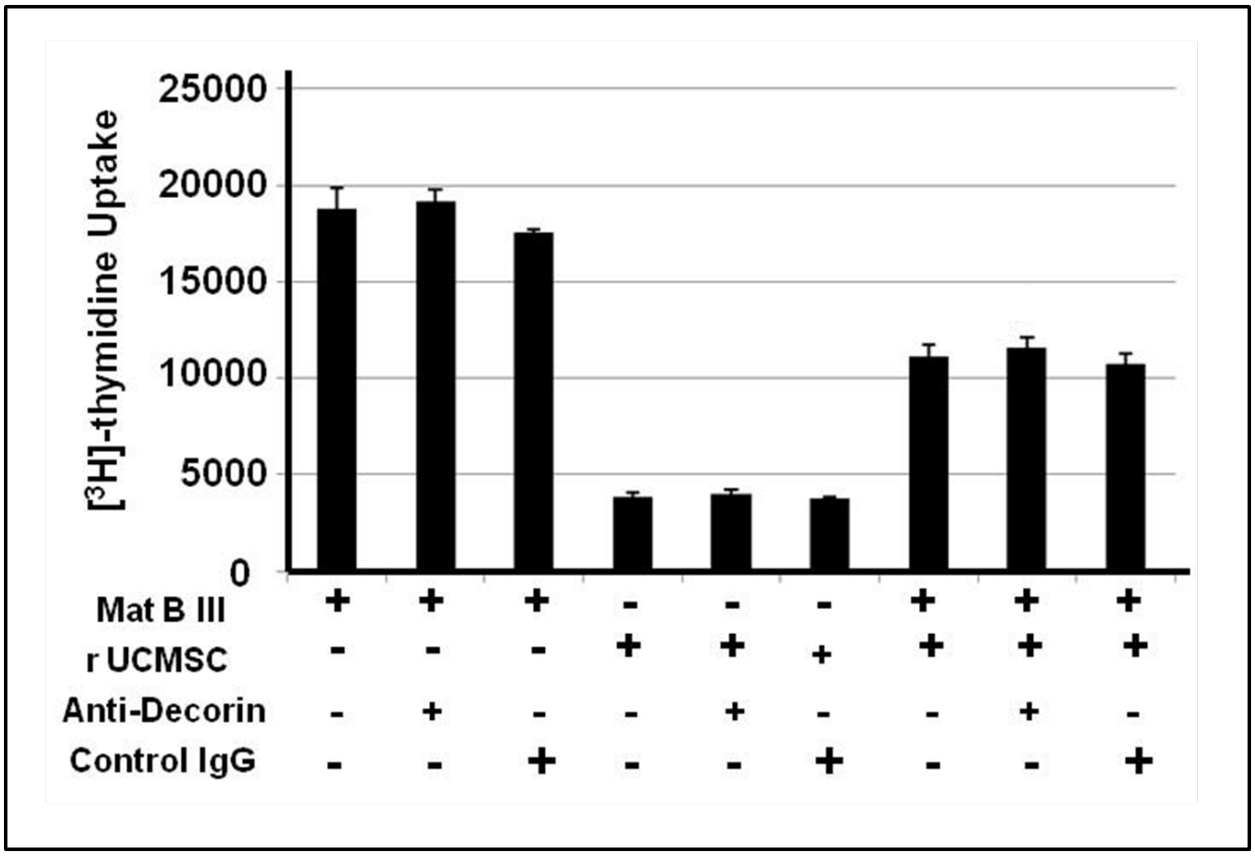
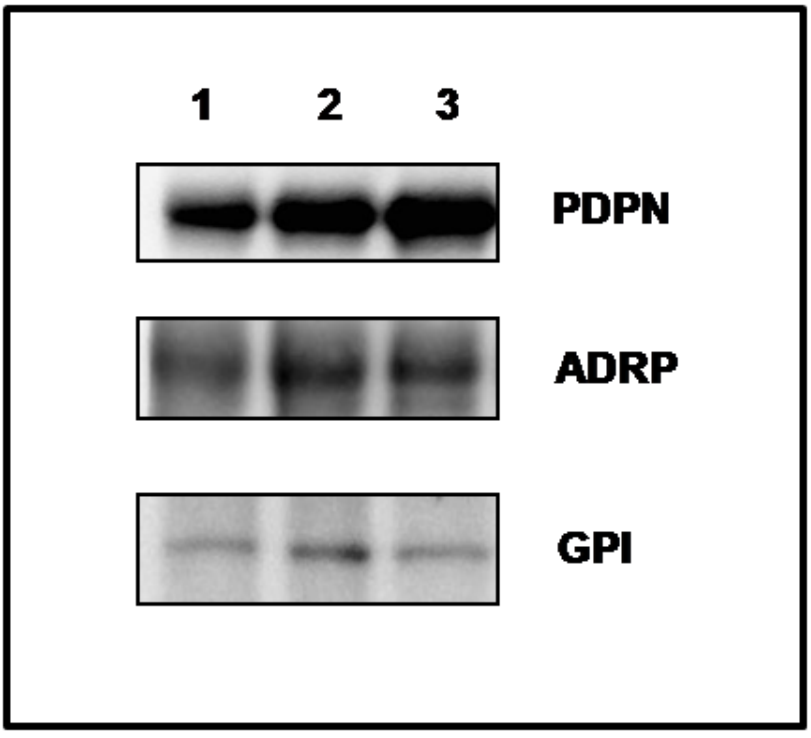


Figure 3.9

Detection of PDPN, ADRP and GPI in the culture medium by Western blot analysis.

Rat UCMSC and Mat B III cells were either cultured individually or co-cultured together in defined medium. Three gene products were immunoprecipitated using specific polyclonal rabbit antibody against PDPN and polyclonal goat antibodies against ADRP and GPI. Each immunoprecipitates were subjected to PAGE and then immunoblotted using specific monoclonal antibody for PDPN or polyclonal antibodies for ADRP and GPI. Lane1, immunoprecipitated protein from the medium cultured with rat UCMSC alone; lane2, immunoprecipitated proteins from the medium cultured with Mat B III cells alone; lane 3, immunoprecipitated proteins from the medium co-cultured rat UCMSC and Mat B III cells at the ratio of 1 to 8.



CHAPTER 4 - Discussion

Increasing evidence suggests that adult stem cells can be effective therapeutic tools for various diseases including cancer [17, 18, 20, 21, 36]. Indeed, multiple adult stem cells engineered to express therapeutic genes have been reported to be very effective in attenuating various cancers [17, 38, 41]. A few papers have also reported that naïve adult stem cells have an intrinsic ability to attenuate growth of several types of cancer cells such as Kaposi's sarcoma and glioma [51-53]. Our colleagues have also reported that un-engineered rat UCMSC attenuate rat mammary carcinoma [18] and mouse pancreatic carcinoma [37] in immunocompetent animals. A drawback of cancer cytotherapy using engineered stem cells is 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[36]. Thus, if naïve 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 for rat malignant breast carcinoma cells *in vitro*. The study provides strong evidence that multiple tumor suppressor genes are involved in rat UCMSC-dependent tumoricidal activity. This evidence can be utilized for future development of a safe cancer-targeted cytotherapy for breast carcinoma.

First, we screened for potential genes involved in the rat UCMSC-dependent tumoricidal activity using cDNA microarray analysis. The microarray analysis provides the means to perform parallel analysis of genome-wide genes in a single assay resulting in a semi quantitative assessment of changes in mRNA expression. Although the Illumina RatRef-12 Expression

BeadChip used for this study does not cover entire genome-wide genes, this was the only available rat microarray chip that covers a larger number of rat genes. From our cDNA microarray analysis we screened only 16 significant genes which are differentially expressed in the rat UCMSC when they were co-cultured for 24 hours with Mat B III rat mammary carcinoma cells (Figure 3.3). 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 (Figure 3.4). Accordingly, seven of the genes screened are differentially expressed; their products are secreted and reported to play a role in cell growth regulation. Differential expression of these seven genes was further confirmed by qRT-PCR using the same RNA samples (Figure 3.4). A remarkable finding in this screening was that six genes are known tumor suppressor genes and one gene is a tumor promoter gene. Up-regulation of multiple tumor suppressor genes in rat UCMSC is a conceivable and reasonable event since our colleagues have found that rat UCMSC, significantly attenuate tumor growth *in vivo* and *in vitro* [18, 37]. Therefore, screening of the intrinsic tumoricidal genes in rat UCMSC by genome wide microarray and confirming their expressions by qRT-PCR analysis appears to have been successfully conducted.

Among seven genes screened, ADRP, SULF-1, and GPI exhibited highest up-regulation when rat UCMSC were co-cultured with mammary carcinoma cells. Accordingly, these three genes were further characterized by their potential tumor suppressor function. For this characterization purpose, [^{3}H] thymidine uptake assay was utilized since this assay is a sensitive evaluation of cell proliferation. As described in the results, individual neutralizing antibodies against three gene products significantly increased [^{3}H] thymidine uptake by Mat B III cells co-cultured with rat UCMSC (Figure 3.6). When all three antibodies were added simultaneously in

the culture medium, this increase was markedly pronounced not only in co-cultured Mat B III cells but also in Mat B III cells alone (Figure 3.7). These results strongly 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 tumor suppressor protein (irrelevant, non-differentially expressed) decorin and control IgG did not show any effect on [^{3}H] thymidine uptake by Mat B III cells. Furthermore, co-culturing rat uterus fibroblasts did not exhibit any effect on 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 3.9). These results strongly suggest that the tumoricidal effect of rat UCMSC is specific and at least, in part, carried out by up-regulation of multiple tumor suppressor genes. Although Larmonier *et al* have reported that nitric oxide plays a significant role in bone marrow mesenchymal stem cell-induced growth alteration of cancer cells [53], rat UCMSC-dependent growth attenuation may not be due to nitric oxide since nitric oxide is very unstable in culture media [54].

In summary, the present study demonstrates that rat UCMSC significantly attenuates growth of Mat B III rat mammary carcinoma cells in culture. Rat UCMSC-dependent tumor cell growth attenuation is associated with up-regulation 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 naïve rat UCMSC is a potential cytotherapeutic tool for breast cancer therapy.

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