HUMAN UMBILICAL CORD MATRIX MESENCHYMAL STEM CELLS SUPPRESS THE GROWTH OF BREAST CANCER BY EXPRESSION OF TUMOR SUPPRESSOR GENES

by

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Abstract

Previous studies have shown that both human and rat umbilical cord matrix mesenchymal stem cells (UCMSC) possess the ability to control the growth of breast carcinoma cells. Comparative analysis of two types of UCMSC suggest that rat UCMSC-dependent growth regulation is significantly stronger than that of human UCMSC. Accordingly, the present study was designed to clarify their different tumoricidal abilities by analyzing gene expression profiles in two types of UCMSC. Gene expression profiles were studied by microarray analysis using Illumina HumanRef-8-V2 and RatRef-12 BeadChip for the respective UCMSC. The gene expression profiles were compared to untreated naïve UCMSC and those co-cultured with species-matched breast carcinoma cells; human UCMSC vs. MDA-231 human carcinoma cells and rat UCMSC vs. Mat B III rat carcinoma cells. The following selection criteria were used for the screening of candidate genes associated with UCMSC-dependent tumoricidal ability; 1) gene expression difference should be at least 1.5 fold between naive UCMSC and those co-cultured with breast carcinoma cells; 2) they must encode secretory proteins and 3) cell growth regulation-related proteins. These analyses screened 17 common genes from human and rat UCMSC. The comparison between the two sets of gene expression profiles identified that two tumor suppressor genes, adipose-differentiation related protein (ADRP) and follistatin (FST), were specifically up-regulated in rat UCMSC, but down-regulated in human UCMSC when they were co-cultured with the corresponding species' breast carcinoma cells. The suppression of either protein by the addition of a specific neutralizing antibody in co-culture of rat UCMSC with Mat B III cells significantly abrogated UCMSC ability to attenuate the growth of carcinoma cells. Over-expression of both genes by adenovirus vector in human UCMSC enhanced their

ability to suppress the growth of MDA-231 cells. In the breast carcinoma lung metastasis model generated with MDA-231 cells, systemic treatment with FST-over-expressing human UCMSC significantly attenuated the tumor burden. These results suggest that both ADRP and FST may play important roles in exhibiting stronger tumoricidal ability in rat UCMSC than human UCMSC and imply that human UCMSC can be transformed into stronger tumoricidal cells by enhancing tumor suppressor gene expression.

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Chapter 1 - Introduction

1.1 Umbilical cord matrix mesenchymal stem cells (UCMSC)

Umbilical cord matrix mesenchymal stem cells (UCMSC) are derived from the gelatinous connective tissue of the umbilical cord, Wharton's jelly (Fig. 1.1). The first isolation of stem cells from the Wharton's jelly was carried out using porcine umbilical cords followed by human umbilical cords (4). UCMSC are collectable from several species, such as rat, pig, human, etc (2, 5). Although UCMSC are a mixture of stromal cells, they exhibit primitive stem cell characteristics which include self-renewability and multipotency. They express similar stem cell markers with those expressed in bone marrow derived mesenchymal stem cells (6). UCMSC can differentiate into cardiomyocytes, neuron-like cells, osteocytes, endothelial cells, and pancreatic islet-like cell clusters (5, 7, 8). They can be cultured for multiple passages, and large quantities of the cells can be obtained without feeder cells because of their high telomerase activity (4, 5). Low immunogenecity is another distinguished character of UCMSC (5, 9, 10). They are known to escape from the immune system by low expression of MHC class I, no expression of MHC class II and an indirect suppression of T cell activation via chemokine expressions, which implies the potential of allogeneic transplantation for disease treatment (8, 10, 11).

1.2 Breast cancer

Breast cancer is one of the most common cancers among women in the United States. It is estimated that 226,870 women are diagnosed with breast cancer and 39,510 are estimated to die in 2012 (1). Etiology of breast cancer is diverse; environmental factors including history of pregnancy, diet, etc, and genetic factors are involved (12). Decisive factors for the treatment of

breast cancer depend upon diagnostic stage, receptor expression of steroids, and growth factors. Mainly the combination of mastectomy, chemotherapy and radiotherapy will be conducted, but these therapies have severe side effects which result in significant reduction of patients' quality of life. Even though campaigns for early detection are expanding, complicated causes of breast cancer are furthering the difficulty of its treatment. Additionally, recurrence and the metastases to bone, lung or liver decrease the 5-year survival rate rapidly. It is on a high demand to explore a novel treatment for breast cancer and metastasized breast cancer which has fewer side effects. In the present study, highly lung metastatic MDA-231 breast adenocarcinoma cells sub-cloned from MDA-MB 231 cells were used, which are ideal model cells for studying lung metastatic breast cancer. MDA-231 cells are estrogen receptor positive and HER-2 receptor and progesterone receptor negative. Mat-B III cells are a rat mammary adenocarcinoma cell line derived from Fischer rats. Their receptor expressions are not yet studied.

1.3 UCMSC on cancer treatment

Application of stem cells on disease treatment is rapidly progressing due to their high potential. In adults, mesenchymal stem cells can be collected from bone marrow, adipose tissue and several other tissues while fetus derived stem cells can be collected from umbilical cord blood or matrix, amniotic fluid, *etc (5, 13, 14)*. Stem cells home to inflammatory regions including cancers, and this is known as tumor tropism, which makes the stem cells useful for the gene delivery by virus and nanoparticles (15, 16). Recent findings show that naïve UCMSC suppress the growth of several kinds of tumors (2, 17, 18). Rat UCMSC completely abolished the growth of rat mammary tumors without recurrence for 100 days *in vivo* (2). The growth of pancreatic and lung cancer xenografts were also significantly suppressed by rat UCMSC therapy in immunocompetent mice (17, 18). The *in vitro* studies showed a decrease in breast cancer cell

growth by indirect co-culture of naïve UCMSC and breast cancer cells. (3). Also, conditioned medium with naïve UCMSC suppressed the growth of multiple types of cancer cells (3, 18). These results were observed in both rat and human UCMSC when they were cocultured with breast, lung, and pancreatic cancer cells (3, 17-20). Some other naïve stem cells are also reported for their possibility for cancer treatment. The growth of Kaposi's sarcoma was attenuated by bone marrow derived mesenchymal stem cells (21). Adipose tissue derived stromal cells strongly inhibited pancreatic ductal adenocarcinoma by cell cycle arrest (22). Additionally, in glioma cells, apoptosis was induced by human umbilical cord blood stem cell treatment (23).

1.3.1 Mechanisms

Mechanisms of tumor suppression by naïve UCMSC are still unclear. Several possibilities are reported, which include an induction of cell cycle arrest and/or apoptosis in tumor cells, and an activation of anti-tumor immune responses in cancer bearing rats (3, 18, 19, 24, 25). These researches suggest that naïve UCMSC communicate with adjacent cancer cells by receiving and sending chemical signals each other: this communication is most likely mediated by cytokines and growth factors. Regarding cell-to-cell communication, it is of interest to note that in tumor bearing rats, rat UCMSC appear to recruit cytotoxic immune cells into the tumor region by secreting MCP-1 thereby attenuating tumor growth which shows the importance of the tumor microenvironment on indirect tumor suppression (25). The importance of the tumor microenvironment is well recognized. It is well known that bone marrow mesenchymal stem cells home to tumors and form the tumor microenvironment which supports tumor growth (26). One aspect of the functional difference between UCMSC and bone marrow mesenchymal stem cells may be suggested in the following study; UCMSC do not transform into tumor associated

fibroblasts when they are cultured with conditioned medium of breast or ovarian cancer cells, while bone marrow mesenchymal stem cells differentiate into tumor associated fibroblasts (27).

Although both human and rat UCMSC can suppress tumor growth, the tumor growth inhibition by human UCMSC is not as strong as that of rat UCMSC. In [^{3}H]-Thymidine uptake assay, rat UCMSC suppressed the growth of rat mammary tumor cells more than 90% in a 1:15 ratio, while human UCMSC suppressed the growth of human breast cancer cells 50% in a 1:2 ratio (Fig 1.2) (2, 3). This difference in cell growth inhibition may be associated with their interaction mechanism with cancer cells; rat UCMSC are shown to suppress the growth of Lewis Lung Carcinoma cells and pancreatic cancer cells by causing G0/G1 cell cycle arrest, while human UCMSC cause G2 cell cycle arrest in breast cancer cells (3, 17, 18). The mechanism for these differences is unknown, but it implies that different cyclin-dependent kinases are modulated by rat and human UCMSC.

1.3.2 Secretory tumor suppressor protein coding genes

Several secreted proteins function to suppress the growth of tumors. In previous studies, it has been shown that the conditioned medium of naïve UCMSC could suppress the growth of tumors (3, 18). Based on these findings, we hypothesized that secreting proteins are the major factors which suppress the growth of tumors.

1.4 Objective and hypothesis of the study

The objective of the study is to identify the key mechanisms by which rat and human UCMSC attenuate tumor growth by comparing gene expressions when UCMSC co-existed with tumor cells. Prior to conducting the study, three hypotheses were set as followed; 1) UMCSC express genes differently when they co-existed with breast carcinoma cells. In support of this

hypothesis, our own preliminary study indicates that human UCMSC preliminarily co-cultured with breast carcinoma cells exhibited significantly stronger breast carcinoma cell suppression ability as compared with naïve UCMSC (data not shown). 2) Tumoricidal activities of rat and human UCMSC are dependent on differentially expressed genes. 3) Over-expression of identified rat UCMSC tumoricidal genes in human UCMSC will generate human UCMSC armed with enhanced tumoricidal ability. The goal of this study is to discover the molecular mechanism by which rat UCMSC significantly attenuate breast carcinoma cells. The principle of the molecular mechanism will be applied for generation of strongly tumoricidal human UCMSC for breast cancer treatment.

Figures

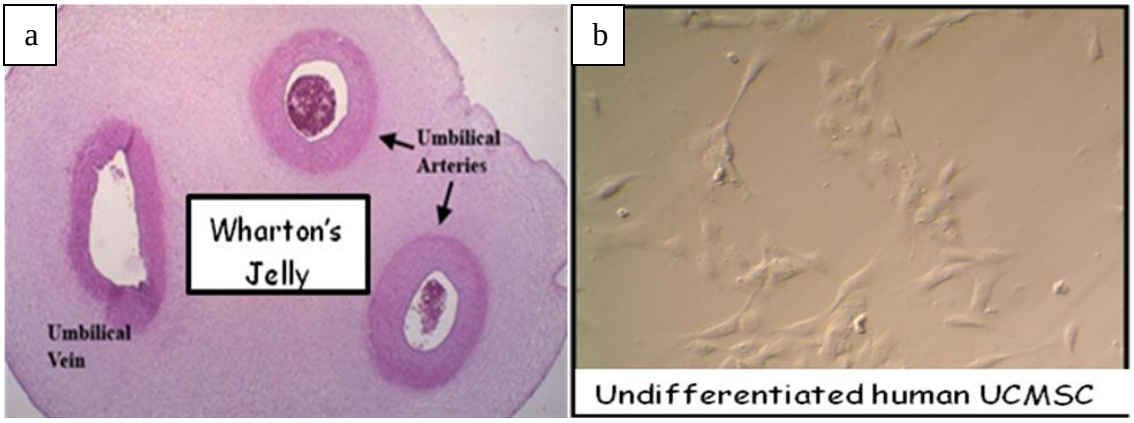

Figure 1.1 Anatomical characteristics of Wharton's Jelly and morphology of human umbilical cord matrix derived mesenchymal stem cells (UCMSC)

a. Cross section of human umbilical cord. UCMSC were collected from Wharton's Jelly of the umbilical cord. b. Morphology of undifferentiatied human UCMSC are in spindle shape when cultured on a culture dish.

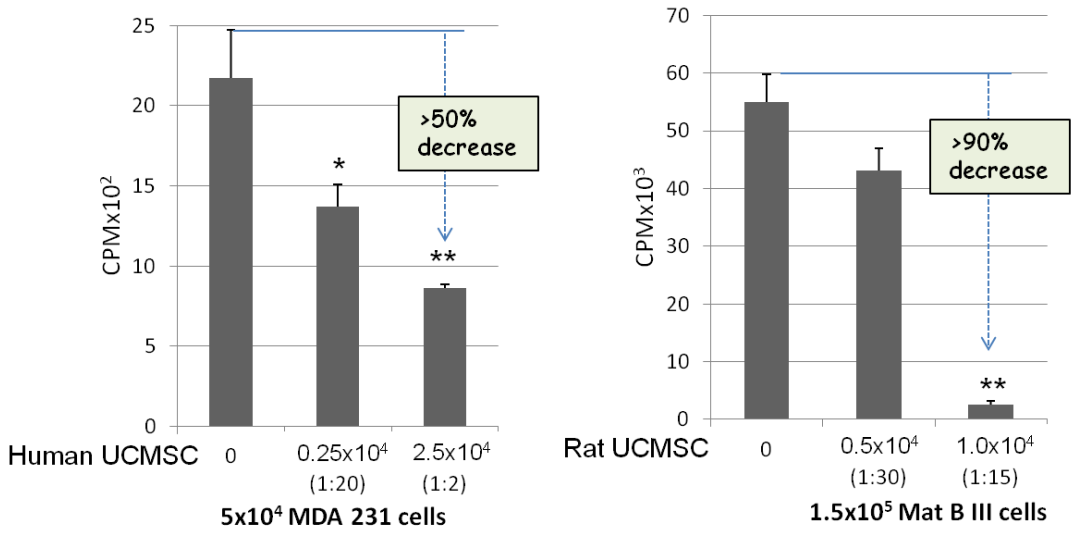

Figure 1.2 Inhibition of DNA synthesis in breast cancer cells by co-cultured rat UCMSC was significantly stronger than that by co-cultured human UCMSC

Human UCMSC were co-cultured with MDA-231 human breast cancer cells ($5x10^4$) directly in 1:20 and 1:2 ratios and rat UCMSC were co-cultured with Mat B III rat mammary tumor cells ($1.5x10^5$) in 1:30 and 1:15 ratios. DNA synthesis was determined by [^{3}H]-thymidine uptake assay. *; $p < 0.05$, **; $p < 0.01$. (2, 3)

Chapter 2 - Microarray analysis of UCMSC

Microarray analysis was conducted to identify the differential gene expression profile between naïve UCMSC and UCMSC co-cultured with breast carcinoma cells. The stepwise process of microarray analysis is schematically illustrated in Figure 2.1.

2.1 Materials and Methods

2.1.1 Cell culture

Human UCMSC were prepared from Wharton's jelly which is the matrix of umbilical cord using enzymatic methods as described previously (6, 28) and rat UCMSC were harvested from E19 pregnant Fisher 344 rats according to the method previously described (2). UCMSC were maintained in defined medium, containing a mixture of 56% low glucose DMEM (Life technologies, Grand Island, NY), 37% MCDB 201 (Sigma, St. Louis, MO), 2% fetus bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA), 1x insulin-transferrin-selenium-X (Life technologies, Grand Island, NY), 1x ALBUMax1, 1x Pen /Strep, 10nM dexamethasone, 100µM ascorbic acid 2-phosphate,10ng/ml epidermal growth factor (EGF), and 10ng/ml platelet derived growth factor-bb; PDGF-BB. Cells were maintained at 37^{o}C in a humidified atmosphere containing 5% CO_2. Rat mammary adenocarcinoma cell line Mat B III cells were purchased from American Type Culture Collection (Manassas, VA). MDA-231 cells, kindly provided from Dr. F. Marini (M.D. Anderson Cancer Center), were maintained in MEM alpha medium (Life technologies) supplemented with 10% FBS and 1x Pen / Strep, 1% Non Essential Amino Acid, 1% L-glutamine at 37 ^{o}C in a humidified atmosphere containing 5% CO_2. The Mat B III cell line was maintained in McCoy's 5A modified medium (Life technologies) supplemented with 10% FBS, 100 units/mL penicillin and 100µg/ml streptomycin.

2.1.2 RNA isolation

An indirect cell culture using Transwell culture dish (Corning, Lowell, MA) was performed to collect RNA samples for microarray study. In brief, human UCMSC (5 x 10^4 cells) or rat UCMSC (1 x 10^5 cells) were seeded in defined medium in the bottom of 6-well or 10cm Transwell culture dish, respectively. After allowing UCMSC to settle for 24 h, MDA-231cells ($5x10^5$ cells) or Mat B III cells ($1.5x10^6$) were added in Transwell inserts (0.4 μm pore size). After 48 h co-culture, total RNA of UCMSC grown in the bottom of Transwell dish and UCMSC cultured alone without carcinoma cells in the insert were collected by TRIzol (Life technologies, Grand Island, NY) following the manufacturer's instruction. Concentration and quality of total RNA were determined by the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA) and Agilent 2100 bioanalyzer (Agilent Technologies, inc. Santa Clara, CA), respectively.

2.1.3 Microarray analysis

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 cRNAs were prepared using an Illumina Total Prep RNA Amplification Kit (Applied Biosystems, Foster City, CA, USA). Briefly, 5μg of total RNA were converted to double stranded cDNA using T7-oligo (dT) primers, followed by an *in vitro* transcription reaction to amplify biotinylated cRNA as described in the manufacturer's instructions (Illumina Inc., San Diego, CA). The biotinylated cRNA from human UCMSC was hybridized to a Human Ref-8 Expression BeadChip platform that contains 24,357 probes. At rat UCMSC-derived biotinylated cRNA was hybridized to a Rat Ref-12 Expression BeadChip that contains 22,519 probes. 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.

2.1.4 Microarray data analysis

The microarray dataset 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 were 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 standard deviation (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 S.D. 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 S.D. to give the z ratios. Further hierarchical cluster analysis was performed using open source software Cluster 3.0 and Java Treeview.

2.1.5 Quantitative real-time 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 the iScript One-Step RT-PCR Kit with SYBR Green (Bio-Rad, Hercules, CA), and the reactions were conducted on the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Carlbad, CA). The qRT-PCR was performed as

follows: 45 cycles with initial incubation at 50^{o}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 Ct values, where Ct 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 Ct method (29) . mRNA expressions of human UCMSC transduced with adenovirus encoding adipose differentiation related protein; ADRP and follistatin; FST were determined as same procedure as mentioned above. The human ADRP primers are described previously (30). Sequences of primers used are described in Table 2.1.

2.1.6 Statistical analysis

All values are expressed as means ± SE for all *in vitro* and *in vivo* experiments. Statistical significance was assessed by ANOVA test and t-test. Statistical significance was set at * $p <$ 0.05.

2.2 Results and Discussion

2.2.1 Comparison of tumor suppressor gene expressions in human UCMSC and rat UCMSC by Microarray analysis

Expressions of tumor suppressor genes in human or rat UCMSC co-cultured with corresponding species' mammary tumor cells were screened by genome-wide microarray analysis using Illumina HumanRef-8 Expression BeadChip for human and RatRef-12 Expression BeadChip for rat, which contains 24,357 and 22,519 distinctive human or rat oligonucleotide probes, respectively. The total RNA were extracted from the human and rat UCMSC either co-cultured with or without MDA-231 cells or Mat B III cells in Transwell culture dishes (cells were separated by a porous membrane with 0.4μm pores). Uniquely expressed genes were screened following the criteria described in the method section and Fig 2.1. By the analysis of Z-normalization of the hybridization signals, 43 genes were selected in human UCMSC and 64 genes from rat UCMSC. By comparing these selected genes between rat and human UCMSC, 17 genes were commonly expressed in both rat and human UCMSC (Fig. 2.2). Eight of these genes were up-regulated in both human and rat UCMSC. Two genes were up-regulated in rat UCMSC but down-regulated in human UCMSC, while the other seven genes were up-regulated in human UCMSC but down-regulated in rat UCMSC (Fig. 2.3). Eight genes were reported as tumor suppressor genes and five genes as tumor promoter genes while the other four genes are related to cell growth but unknown for their functions in tumor growth (Table 2.2). Two tumor suppressor genes, ADRP FST, which were up-regulated in rat UCMSC but down-regulated in human UCMSC were further characterized as potential candidate genes to exhibit stronger tumoricidal activity in rat UCMSC more than that in human UMCSC.

2.2.2 Confirmation of gene expression by qRT-PCR

The mRNA expressions of ADRP and FST in human and rat UCMSC were verified by qRT-PCR. Both ADRP and FST showed constantly upregulated gene expression in rat UCMSC co-cultured with Mat B III cells. In human UCMSC co-cultured with MDA231 cells, although FST expression was down-regulated as it was observed in microarray study, ADRP expression was up-regulated (Fig. 2.4).

Figures and Tables

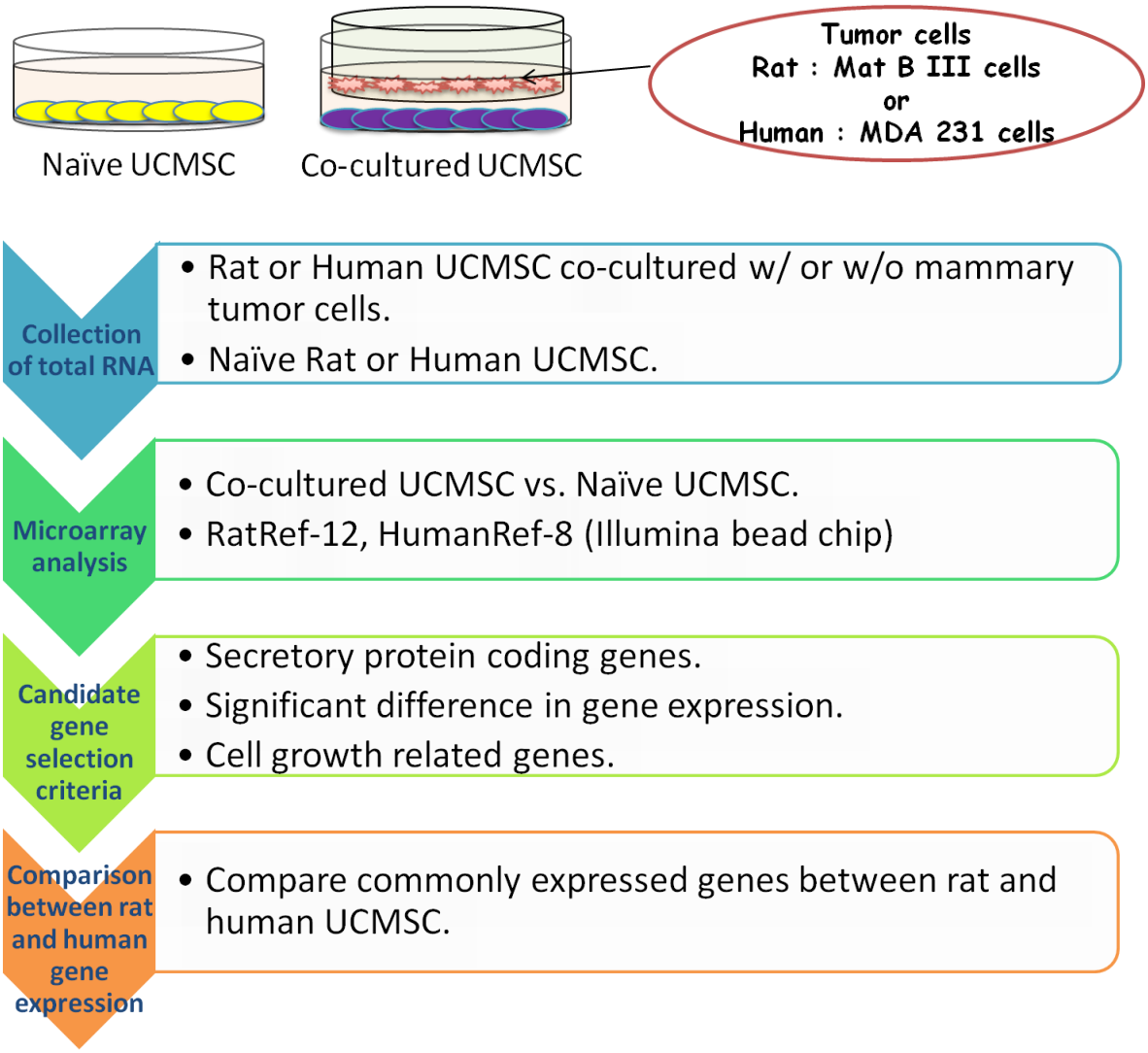

Figure 2.1 Schematic illustrations of the stepwise procedures of microarray analysis

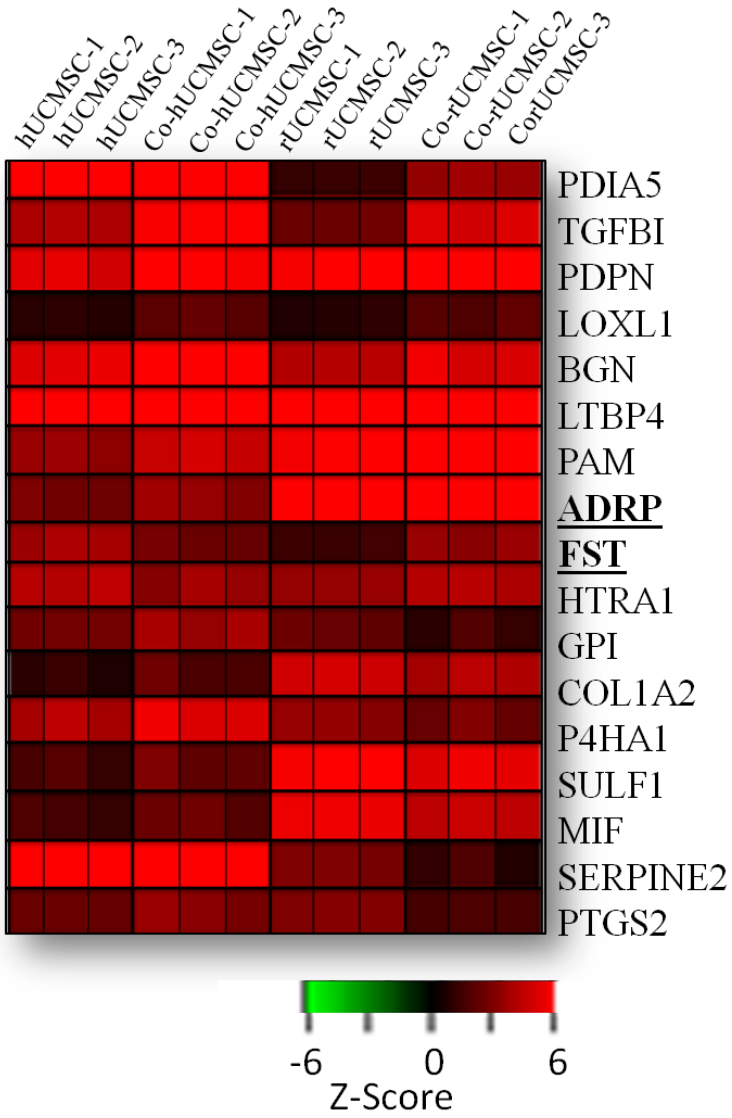

Figure 2.2 Clustering analysis of significant genes

Clustering analysis of significant genes in rat UCMSC (rUCMSC) and human UCMSC (hUCMSC) co-cultured with mammary tumor cells of their respective species, *i.e.*, Mat B III rat breast carcinoma cells or MDA 231 human breast carcinoma cells. The differential gene expression profiles between naive human or rat UCMSC and those co-cultured with mammary tumor cells were compared.

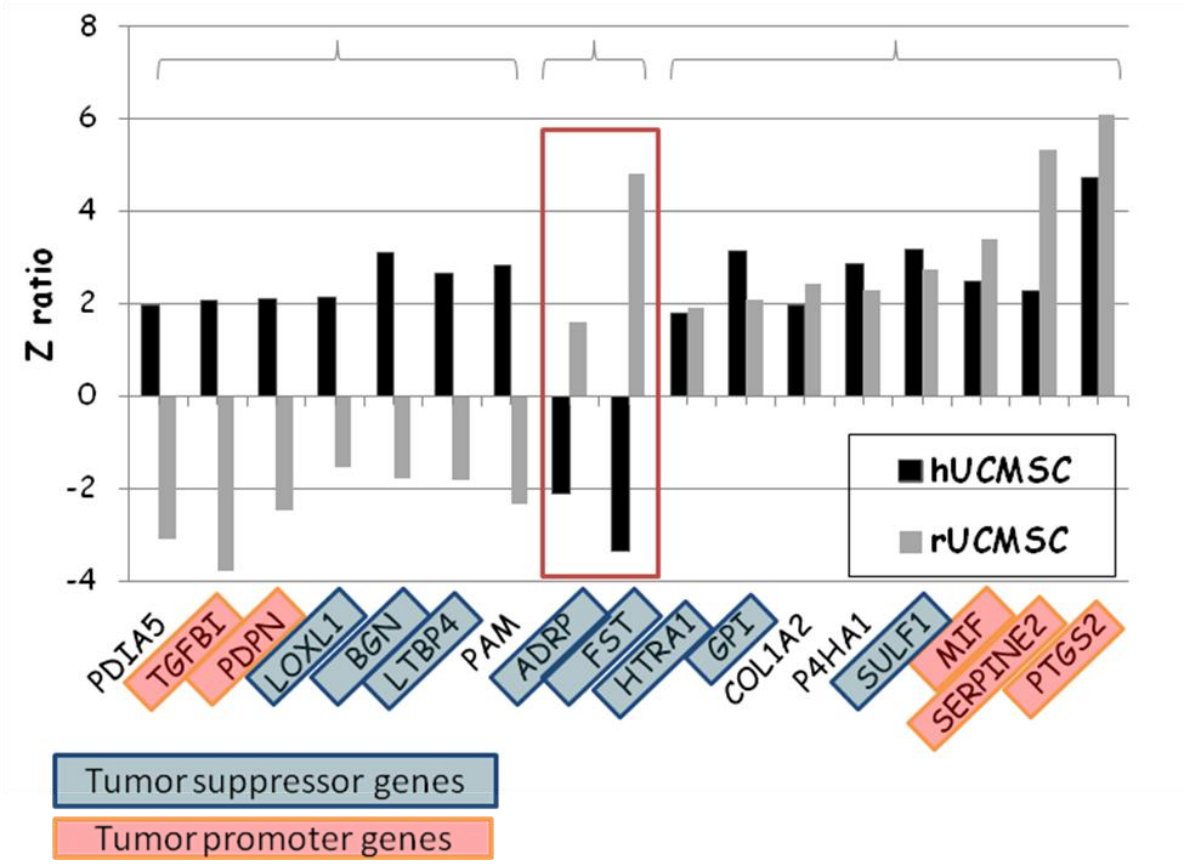

Figure 2.3 Z ratios of the 17 commonly expressed genes in both human and rat UCMSC co-cultured with breast cancer cells.

The solid bars indicate gene expression in human UCMSC (hUCMSC) and the gray bars indicate gene expression in rat UCMSC (rUCMSC). Genes highlighted with blue color indicate tumor suppressor genes whereas genes highlighted with red color indicate tumor promoter genes. Unlabeled genes indicate unclear relationship with tumor growth.

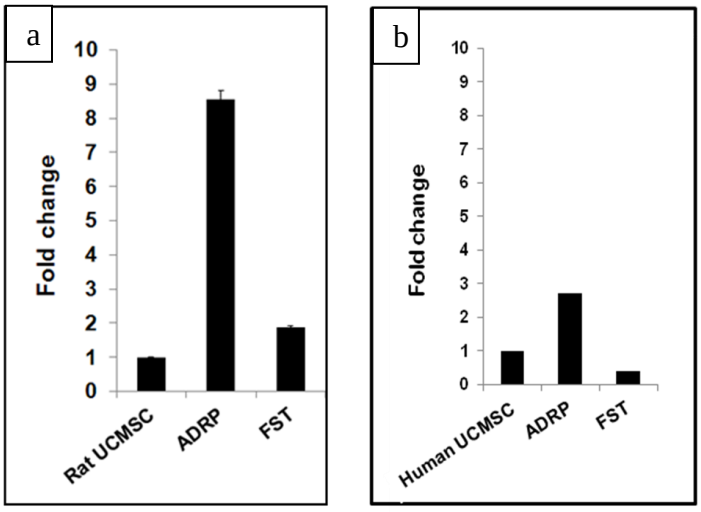

Figure 2.4 Messenger RNA expression levels of ADRP and FST in rat and human UCMSC co-cultured with tumor cells.

The mRNA expression levels of ADRP and FST in rat (a) and human (b) UCMSC co-cultured with Mat B III cells or MDA-231 cells were determined by qRT-PCR. Naïve UCMSC mRNA expression level was set as 1.

Table 2.1 Primers used for qRT-PCR

Gene	Forward (5'-3')	Reverse (5'-3')
Human ADRP (23)	CTCATGGGTAGAGTGGAAA-AGGAGCATTGG	TTGGATGTTGGACAGGAGGG-TGTGGCACGT
Rat ADRP	CATTCAAGACCAGGCCAAAC	AGGAGGTAACATTGCGGAAC
Human FST 344	TGTGCCCTGACAGTAAGTCG	GTCTTCCGAAATGGAGTTGC
Rat FST	TGCTGCTACTCTGCCAATTC	TGCAACACTCTTCCTTGCTC

Table 2.2 Function of differentially expressed genes in human and rat UCMSC

Tumor promoter genes	References	Tumor suppressor genes	References
PTGS2 (COX2) **Rat+/human +**	(31)	**SULF1** **Rat+/human +**	(24, 32)
SERPINE2 **Rat+/human+**	(33, 34)	**GPI** **Rat+/human +**	(24)
MIF **Rat+/human +**	(35)	**HTRA1** **Rat+/human +**	(36)
PDPN **Rat-/human+**	(37)	**ADRP** **Rat+/human-**	(24, 38)
TGFBI **Rat-/human+**	(39)	**FST** **Rat+/human-**	(40)
Unknown genes		**LTBP4** **Human+/rat-**	(41, 42)
P4HA1, COL1A2, **PAM, PDIA5**		**BGN** **Human+/rat-**	(43)
		LOXL1 **Human+/rat-**	(44)

Chapter 3 - Evaluation of the selected genes *in vitro* and *in vivo*

3.1 Materials and Methods

3.1.1 Adenovirus construction and transduction

cDNA plasmids encoding human ADRP and FST were purchased from Open Biosystems (Huntsville, AL). Construction of adenoviral vector for human ADRP and FST genes were done by Vector Biolabs (Philadelphia, NJ). Coding region of cDNA sequences of human ADRP and FST genes were inserted downstream of the GFP gene. Expansion and titeration of adenoviral vector encoding ADRP (Ad-ADRP) or FST (Ad-FST) were done by following the BD Adeno-X Expression system manual (Clontech Laboratories, Inc, Mountain View, CA). Adenovirus encoding GFP was prepared in Dr. F. Marini's lab (University of Texas, Huston, TX) and used as a control vector. The gene transduction to human UCMSC was done by following the procedure described in the previous study (45). Transduction efficiencies of Ad-ADRP and Ad-FST to the human UCMSC were determined by qRT-PCR and under fluoresent microscope as described above.

3.1.2 Evaluation of the effect of conditioned medium obtained from engineered human UCMSC on the growth of human breast carcinoma cells

The effect of ADRP or FST over-expressing human UCMSC on the growth of MDA-231 cells was evaluated by culturing cancer cells in the medium conditioned with either unengineered human UCMSC, ADRP-over-expressing human UCMSC (ADRP-hUCMSC, FST-over-expressing human UCMSC (FST-hUCMSC), GFP-over-expressing human UCMSC (hUCMSC-GFP) or normal defined medium. In brief, different amounts of Ad-ADRP or Ad-FST (50, 100, 300 MOI/cells) were transduced on human UCMSC. The media conditioned with various

engineered human UCMSC were collected after 48h of incubation and diluted with defined medium in a 1:1 ratio. MDA-231 cells seeded in 96 well plates (8000 cells/well) 24h prior were cultured for 3, 5 or 7days in diluted various conditioned media. Viable cell numbers was quantified by MTT assay. Twenty micro liters of MTT solution (5mg/ml) was added 4h before completing the expected time of incubation. Formazan crystals formed were dissolved by adding 100µl solublization buffer (10% SDS containing 0.01N HCl) and incubating overnight in the incubator. The following day, color developed by the reaction was measured at 550 nm and the background absorbance was measured at 630 nm using the Molecular Devices Spectramax 190 plate reader (Global Medical Instrumentation, Inc. Ramsey, MN).

3.1.3 Evaluation of the effect of over-expression of ADRP or FST on two or three dimensional growth of human breast carcinoma cells

Two layer-colony forming assay was carried out as a three dimensional colony growth of human breast carcinoma cells by following the method described previously (3). Briefly, 50 MOI of Ad-ADRP, Ad-FST or Ad-LacZ were transduced into MDA-231 cells. Half ml of 0.9% agar (Sea Plaque agarose, Cambrex Bio Science Rockland, Inc. Rockland, ME) in MEM α medium supplemented with 10% FBS was poured into the wells of a 12-well tissue culture plate as a bottom layer. After solidification, adenoviral vector transduced MDA-231 cells suspended in 0.5ml MEM α medium containing 10% FBS and 0.5% agarose were layered over the bottom layer. The cells were incubated at 37°C with 5% CO_2 for growth of colonies. On days 7 and 12 colony growth was evaluated by an automated phase contrast microscope equipped with Micro Analysis Suite (Olympus CKX41, Center Valley, PA). Colonies with an area greater than 5,000 μm^2 were counted using Micro Suite^{TM} Five software. Human UCMSC transduced with Ad-ADFP, Ad-FST or Ad-GFP in 50 MOI were seeded (2,500 cells) on 12-well plate and allowed to

attach on the plate. On the next day, 0.5 ml of 0.9% agar in defined medium with 5% FBS were poured into the wells of a 12-well tissue culture plate (bottom layer). Engineered human UCMSC and MDA-231 cells ($1x10^4$ cells) were suspended in 0.5ml of defined medium containing 5% FBS and 0.5% agarose, and plated on top of the bottom agar layer.

The standard cell culture in plastic culture dish was carried out as a two dimensional cell growth of human breast carcinoma cells. MDA-231 cells were transduced with Ad-ADRP or Ad-LacZ at MOI of 300. One day after transduction MDA-231 cells were seeded in 24well culture plate at a density of 10,000cells/well. The effect of ADRP over-expression on the growth of MDA-231 cells was evaluated by direct counting of cell number using a haemocytometer 2, 4 and 8 days after transduction.

3.1.5 Evaluation of the effect of FST over-expressing human UCMSC on metastatic tumor growth of human breast carcinoma cells in mouse lung

All mice were housed in an animal care facility and held for 10 days to acclimatize. All animal experiments were carried out under strict adherence with the Institutional Animal Care and Use Committee protocol as set by Kansas State University. A lung metastasis model of MDA-231 cells was developed in immunodeficient CB17/SCID mice (Charles River, Wilmington, MA) by injecting $2x10^6$ cells through the tail vein. Six days after cancer cell inoculation, mice were randomly divided into three treatment groups; (1) PBS, (2) FST over-expressing human UCMSC or (3) LacZ over-expressing human UCMSC. On days 6, 13 and 20 after inoculation with MDA-231 cells, mice received intravenously either 200 µl PBS or gene engineered human UCMSC in 200 µl PBS (human UCMSC group; 30% of cells were stained with PKH26 fluorescent dye). The body weights of the mice were examined every other day. Four weeks after MDA-231 cells transplantation, all mice were sacrificed, lungs were collected

and lung weights were recorded. Numbers of tumor nodules in the lungs were counted under stereomicroscope.3.1.6 Statistical analysis

All values are expressed as means ± SE (standard error) for all *in vitro* and *in vivo* experiments. Statistical significance was assessed by ANOVA and t-test. Statistical significance was set at * $p < 0.05$.

3.2 Results

3.2.1 FST over-expressing human UCMSC inhibited anchorage-dependent growth of MDA-231 cells in vitro

To evaluate the effects of ADRP or FST over-expression in human UCMSC, adenoviral vectors encoding ADRP (Ad-ADRP) and FST (Ad-FST) were individually constructed using GFP encoding expression vector. Gene transduction by Ad-ADRP or Ad-FST was confirmed by observing GFP co-expression in the cells. A good even expression of GFP was detected in human UCMSC under a fluorescent microscope when they were transduced at 50 MOI vectors.

The effect of Ad-FST transduced human UCMSC on the anchorage-dependent growth of MDA-231 cells was evaluated by direct co-culture of the two types of cells. As shown in Fig. 3.1, direct co-culture of FST, but not ADRP, transduced human UCMSC with MDA-231 cells (1:10 ratio) decreased the overall cell growth significantly. The results revealed that relatively small numbers of FST transduced human UCMSC can inhibit the growth of co-cultured MDA-231 cells as compared to LacZ transduced human UCMSC. In addition, to evaluate indirect effect of the gene transduced human UCMSC on the growth of MDA-231 cells, MDA-231 cells were cultured with the conditioned medium obtained from either gene transduced UCMSC and the growth of MDA-231 cells was evaluated by MTT assay (Fig. 3.2). The growth of MDA-231 cells was significantly attenuated when cultured with the conditioned medium from FST transduced human UCMSC as compared to that cultured in LacZ transduced human UCMSC. However, the conditioned medium from ADRP transduced human UCMSC showed only a slight decrease in cell growth. The experiment utilized with the conditioned medium supports that FST appears to be secreted in the culture medium and UCMSC dependent cell growth attenuation is at least in part mediated through proteins secreted from the UCMSC.

3.2.2 Transduction of adeno- FST but not adeno-ADRP attenuated the growth of MDA-231 cells in vitro

Ad-ADRP was transduced into MDA-231 cells in order to observe their tumor suppressing effect. In the 3D culture, after 7 days of virus transduction, there were no significant differences between Ad-LacZ and Ad-FST or Ad-ADRP transduced groups. However, after 12 days from the transduction, the colony size and numbers did not increase in the Ad-FST transduced group, while other groups increased the colony size (Fig. 3.3).

3.2.3 FST transduced human UCMSC inhibited growth of MDA-231 xenografts in mice

FST engineered human UCMSC (FST-hUCMSC) attenuated the growth of MDA-231 grafts in a lung metastasis mammary tumor model. The previous *in vitro* experiments have shown that FST-hUCMSC have significant tumor suppressing ability. In the *in vivo* study, a lung metastasis model of MDA-231 breast cancer cells was used since the lung is the second most frequent site of breast cancer metastasis. Half a million of FST-hUCMSC were administered through the tail vein injection three times every week as a treatment. In lungs, small tumor nodules were observed when mice were sacrificed one week after the last treatment; a total duration was 4 weeks after tumor cell injection (Fig. 3.4A). No tumors were observed in other organs. FST-hUCMSC had a significant effect in reducing tumor nodule numbers in the lung (Fig. 3.4B), however, because of a unexpectedly slower growth of tumors and resultant early sacrificing, there were no significant differences in lung weight among groups. These results suggest that FST-hUCMSC had a tumor suppressing effect in a mouse model.

3.3 Discussion

The current study discovered the change of expression pattern in tumor suppressor genes in rat and human UCMSC when they were co-cultured with tumor cells. Our previous study had revealed that gene expression pattern in rat UCMSC was significantly altered when they were co-cultured with rat breast carcinoma cells (24). However, whether the similar alteration of gene expression in rat UCMSC is also observed in human UCMSC when they were co-cultured with human breast carcinoma cells and the molecular mechanisms by which rat UCMSC markedly attenuate the growth of breast carcinoma cells *in vitro* and *in vivo* were unclear. Previous investigations in the tumoricidal activities of human and rat UCMSC had implied that rat UCMSC have stronger tumoricidal activity than that of human UCMSC, while their mechanistic differences were unclear (2, 3). UCMSC have a significant tumor cells growth suppressing ability in indirect co-culture which appears that UCMSC-dependent tumor cells growth inhibition is partially due to secretory proteins (2, 3). Consequently, we hypothesized that a comparison of gene expressions profiles in rat and human UCMSC co-cultured with breast cancer cells may provide the identity of the key players for the differential tumoricidal abilities in two types of UCMSC. Furthermore, an identification of the key mechanisms in rat UCMSC-dependent strong tumoricidal activity will be utilized to reinforce human UCMSC-dependent tumoricidal activity to the rat UCMSC level. In the present study, two tumor suppressor genes, ADRP and FST were found to be upregulated in rat UCMSC, whereas these two genes were down regulated in human UCMSC when two types of UCMSC were co-cultured with corresponding species' breast carcinoma cells. Therefore, these two gene expression differences in two types of UCMSC may explain their differential tumoricidal activities.

Firstly, ADRP has been reported as a prognosis marker of clear cell renal cell carcinoma (38). Patients with higher expression of ADRP in clear cell renal cell carcinoma tissues had a longer survival rate than those showing lower expression. Although ADRP had an effect to suppress the growth of rat mammary tumors (24), their tumor suppressor effect against human breast cancer was not significant (Fig 3.2). The role of ADRP in tumor suppression has not yet been rigorously studied; however, it is related to the lipid metabolism in several cells. Early adipocyte differentiation was observed in human UCMSC transduced with Ad-ADRP (Fig. 3.5) ADRP dependent lipid accumulation was also observed in murine fibroblasts, which supports our results (46).The initiation of adipocyte differentiation by ADRP in human UCMSC may have weakened their tumoricidal ability.

Secondly, FST, which is known as an activin-binding protein, suppress the metastasis of small cell lung carcinoma (SCLC) (40). FST inhibit the bioactivity of activin A, a TGF beta family protein, which are associated with lung adenocarcinoma proliferation in human patients (47). However, effect of activin A on breast cancer cells are still controversial (48, 49). Although different cell lines were used in this study, FST overexpressing human UCMSC had moderately but significantly reduced the growth of MDA-231 cells *in vitro* which is consistent with the previous study (47). Further studies are needed to determine whether inhibition of activin A is the mechanism by which FST overexpressing human UCMSC and rat UCMSC suppress the growth of tumor cells. Transduction of Ad-FST to MDA-231 cells arrested the growth of MDA-231 cells colonies from Day 7 to Day 12 in colony assay (Fig 3.3). This may indicate that FST expression attenuated the cell growth without inducing cell death (Fig 3.3). Additionally, the growth of MDA-231 cells was significantly attenuated by FST-hUCMSC in *in vivo* studies (Fig 3.4). Further investigation on the circulating level of activin A may enlighten understanding the

mechanism of FST-hUCMSC dependent tumor suppression *in vivo*, since significant increase of circulating activin A was observed in bone metastatic breast cancer patients (49).

As we hypothesized, rat and human UCMSC showed different gene expression profiles (Fig 2.2, 2.3). A comparison of the two gene expression profiles suggests that high expression of two putative tumor suppressor genes in rat UCMSC may be associated with strong tumoricidal activity (Fig 2.3). Over expression of one such gene, FST, in human UCMSC caused a significant suppression of tumor growth in both cell culture and mouse studies. Although the complete depletion of the tumors was not observed by the treatment with FST-over-expressed human UCMSC (Fig 3.4), this study indicates that upregulation of multiple tumor suppressor genes in UCMSC is involved in UCMSC-dependent tumor growth suppression.

The microarray analysis also indicated an upregulation of three tumor promoter genes (prostaglandin-endoperoxide synthase 2; PTGS2, serpin peptidase inhibitor, clade E, member 2; SERPINE2, and macrophage migration inhibitory factor ; MIF) in both rat and human UCMSC when they were co-cultured with breast carcinoma cells. Among these genes, extracellular MIF has been shown to act as a pro-oncogene in breast cancer (35). Since UCMSC derived MIF might play a role as a pro-oncogene, MIF receptor expression in MDA-231 cells and Mat B III cells was investigated (data not shown). MDA-231 cells have a significant level of MIF receptor expression (35), whereas Mat B III cells co-cultured with rat UCMSC did not exhibit significant increase of MIF receptor expression, which implies Mat B III cells are less sensitive to pro-oncogene, MIF. This data suggests different tumoricidal abilities of rat and human UCMSC might be due to different sensitivities of the different breast carcinoma cell to tumor promoter proteins produced by UCMSC; Mat B III cells are poorly sensitive to tumor promoters due to the poor receptor expression, whereas MDA-231 cells are sensitive to those due to a high receptor

expression for tumor promoters. A view from the regulation of the tumor promoter genes also provided a molecular mechanism of differential tumoricidal abilities of two types of UCMSCs. These results also indicate that an investigation in different combination of UCMSC and cancer cells appears to be important in order to fully understand UCMSC-dependent tumoricidal activity.

In conclusion, the tumoricidal ability of UCMSC is a very unique character among many types of stem cells. The present study indicates that rat and human UCMSC dependent tumor growth suppression is due at least in part to the expression modulation of multiple tumor suppressor and promoter genes. This is the first study to describe potential use of human UCMSC engineered with an endogenous tumor suppressor gene over-expression for breast cancer treatment. This study clearly indicates that engineering human UCMSC by endogenous tumor suppressor genes can re-enforce UCMSC-dependent tumoricidal ability. It is apparent that generation of more effective human UCMSC requires further studies for the cell preparation and gene transduction methods.

Figures and Tables

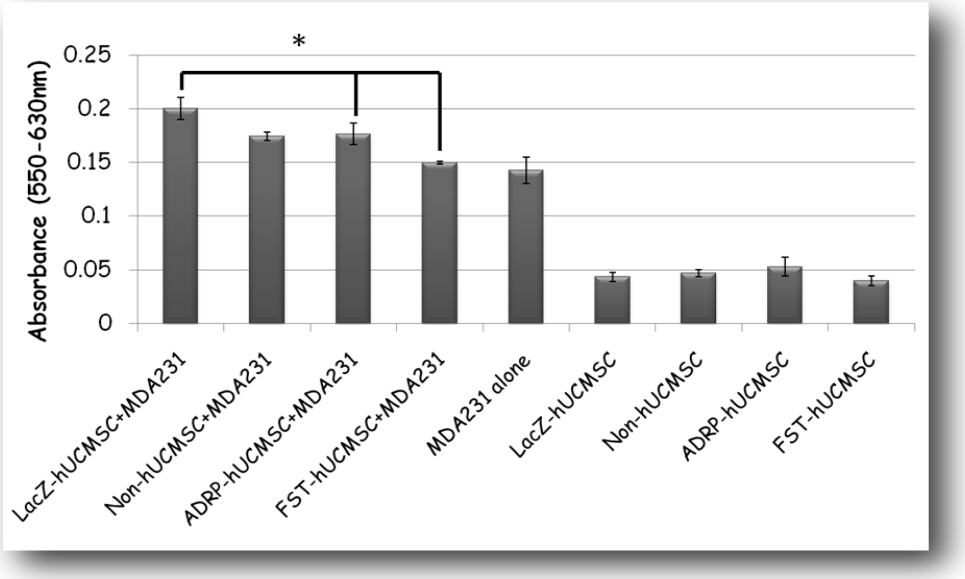

Figure 3.1 The growth of MDA-231 cells was significantly attenuated by the co-culture with ADRP- or FST-hUCMSC.

MDA-231 cells were co-cultured with either LacZ, ADRP- or FST-hUCMSC for 48 h. Cell growth was measured by MTT assay. Either ADRP- or FST over-expressing human UCMSC significantly attenuated growth of co-cultured carcinoma cells. Transduction of adenoviral vector to human UCMSC did not affect the cell growth of human UCMSC. *p<0.05

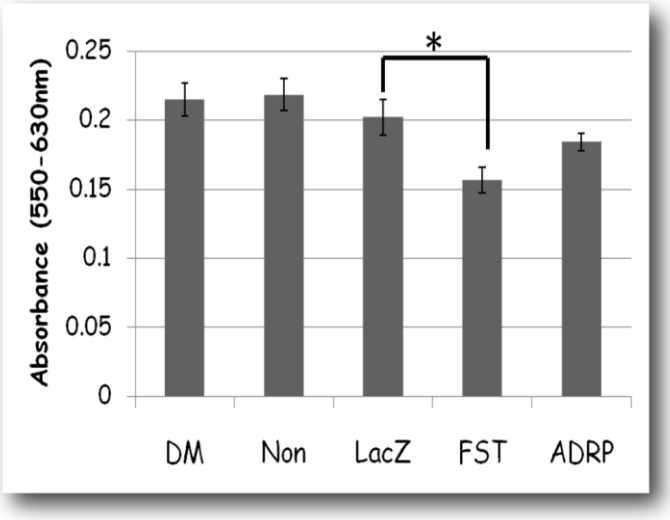

Figure 3.2 The growth of MDA-231 cells was significantly attenuated by culturing with the conditioned medium from Ad-FST but not LacZ or ADRP transduced human UCMSC:

MDA-231 cells were cultured in conditioned media from either 50 MOI Lac Z, ADRP- or FST-transduced human UCMSC for 48 h. Cell growth was determined by MTT assay. *p<0.05. DM; Defined medium, Non; naïve UCMSC, LacZ; LacZ transduced human UCMSC, FST; FST transduced human UCMSC, ADRP; ADRP transduced human UCMSC.

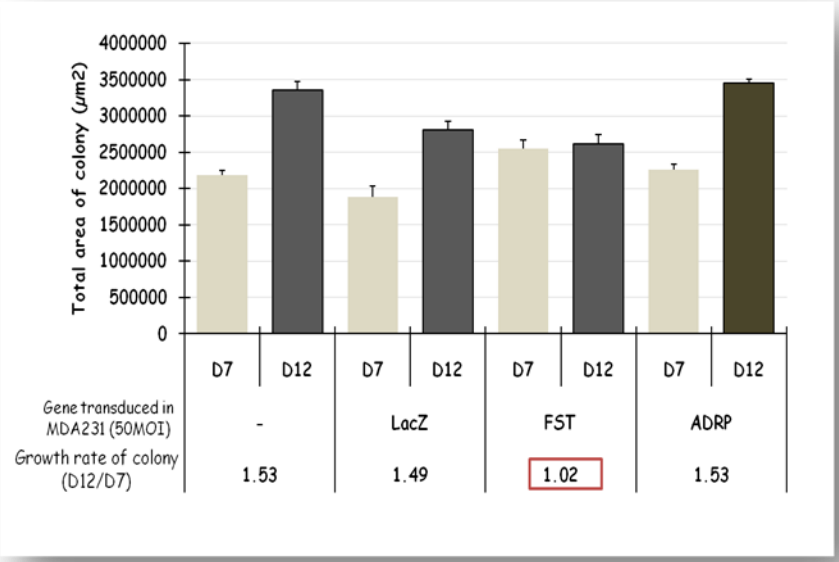

Figure 3.3 The growth of MDA-231 cells was significantly attenuated by the direct transduction of FST but not LacZ or ADRP.

MDA-231 cells were transduced with either 50 MOI Lac Z, ADRP- or FST and cultured in soft agar for the total of 12 days. The rate of the colony growth of MDA-231 cells was determined between the days 7 and 12. The colony growth rate of the FST-transduced cells was 1,02 (no growth), while the growth rates of other gene-transduced cells were approximately 1.5.

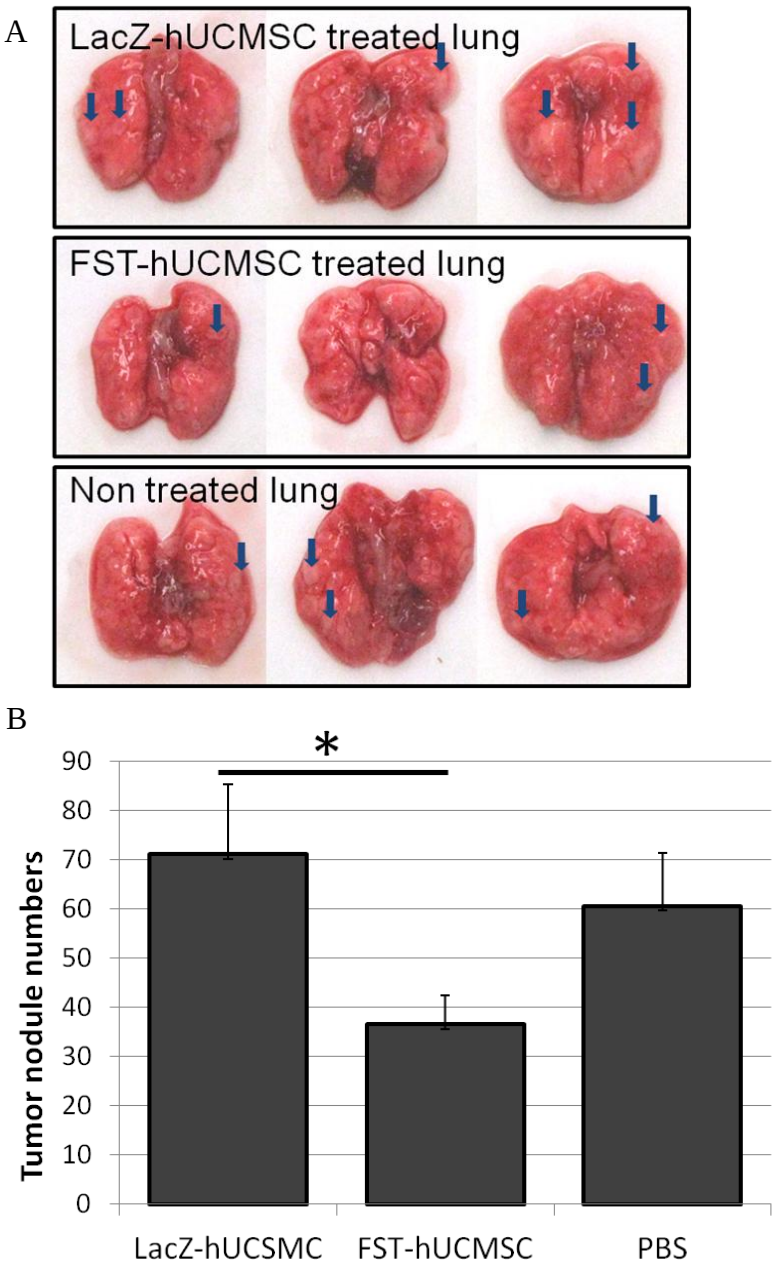

Figure 3.4 Treatment with FST transduced human UCMSC (FST-hUCMSC) significantly attenuated development of metastatic tumor in the mouse lung.

A. Number of tumor nodules in mouse lung was counted under dissection microscope 30 days after the inoculation of the MDA-231 cells. Bar graph represents average number of tumor nodules in each treatment group. B. Macroscopic images of mouse lungs treated with half-

million of LacZ-hUCMSC, FST-hUCMSC and PBS for three times weekly were captured immediately after sacrificing mice. Arrows indicate tumor nodules in the lungs. Fewer numbers of tumor nodules were detected in FST-hUCMSC group. * $p<0.05$ compared to LacZ-hUCMSC treated mice.

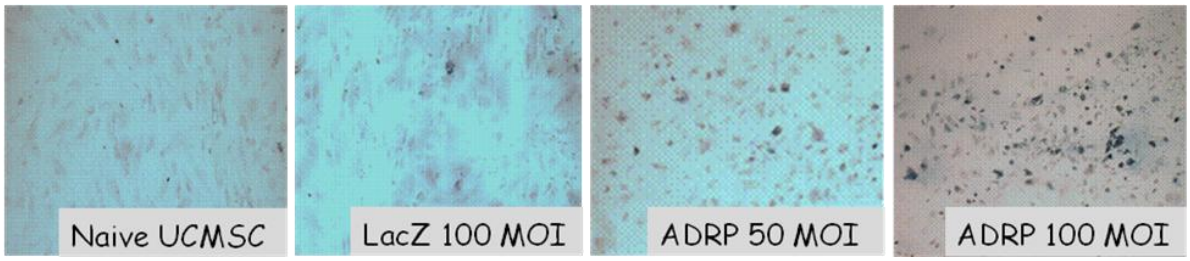

Figure 3.5 ADRP adenovirus vector transduction significantly increased oil droplets in human UCMSC.

Human UCMSC transduced with adeno-ADRP (50 and 100 MOI) exhibited a large number of oil droplets in the cytoplasm of the cells that were stained by Sudan-black staining. However, hUCMSC transduced with adeno-LacZ (100 MOI) showed a negligible amount of oil droplets.

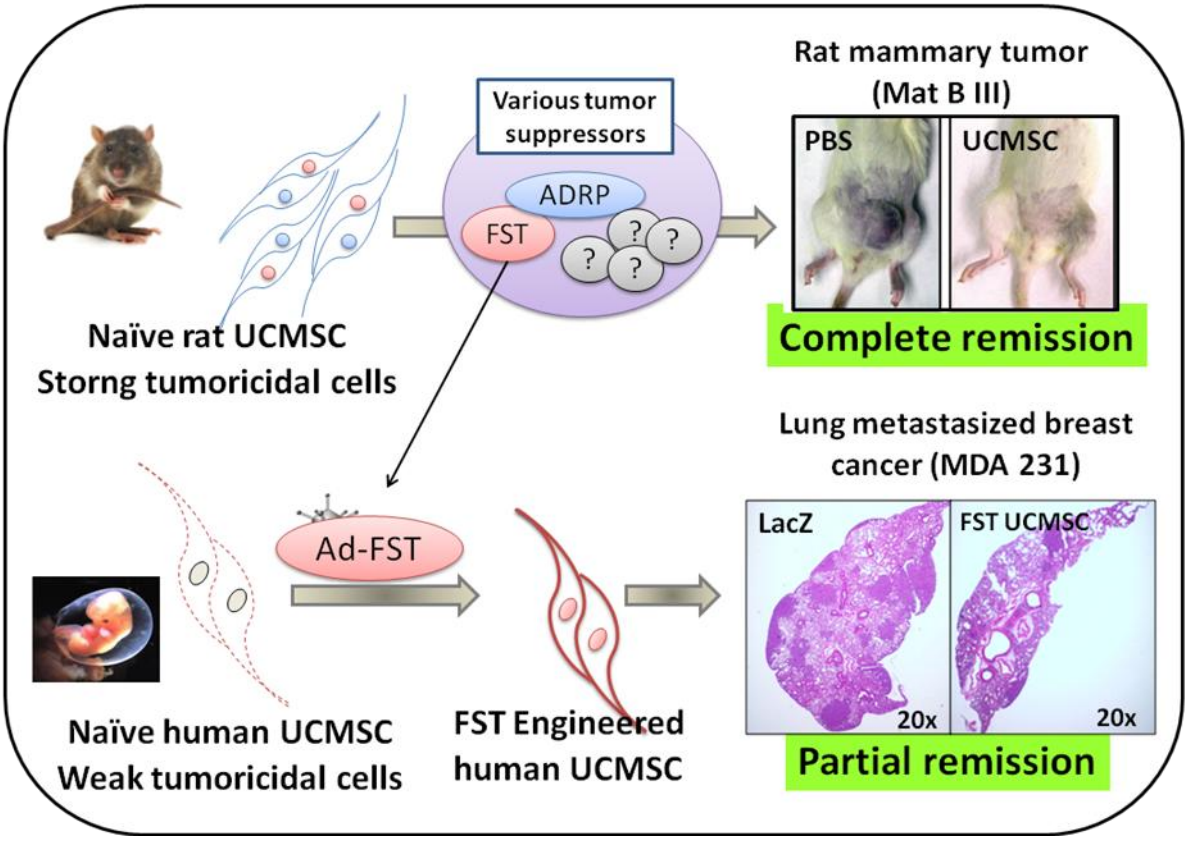

Figure 3.6 Schimatic illustration of the study summary

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