Clinical & Experimental Metastasis (2004) 21: 719–735
DOI 10.1007/s10585-004-8251-4

Caffeine suppresses metastasis in a transgenic mouse model: a prototype molecule for prophylaxis of metastasis

Haiyan Yang[1], Jessica Rouse[1], Luanne Lukes[1], Mindy Lancaster[1], Timothy Veenstra[2], Ming Zhou[2], Ying Shi[3], Yeong-Gwan Park[1] & Kent Hunter[1]
[1]*Laboratory of Population Genetics, Center for Cancer Research, National Cancer Institute, Bethesda, Maryland, USA;* [2]*Laboratory of Proteomics and Analytical Technologies, SAIC-Frederick, National Cancer Institute, Frederick, Maryland, USA;* [3]*Molecular & Clinical Hematology Branch, National Institute of Diabetes & Digestive & Kidney Disease, National Institutes of Health, Bethesda, Maryland, USA*

Received 13 December 2004; accepted in revised form 28 December 2004

Key words: caffeine, 2-D gel analysis, metastasis, microarray, transgenic

Abstract

A significant fraction of cancer patients have occult disseminated tumors at the time of primary diagnosis, which usually progress to become clinically relevant lesions. Since the majority of cancer mortality is associated with metastatic disease, the ability to inhibit the growth of the secondary tumors would significantly reduce cancer-related morbidity and mortality. We have investigated whether caffeine, which has been shown to suppress tumor cell invasiveness and experimental metastasis, can suppress metastasis in a spontaneous transgene-induced mammary tumor model. Chronic exposure to caffeine prior to the appearance of palpable mammary tumors significantly reduced both tumor burden and metastatic colonization. However, when caffeine exposure began after the appearance of frank tumors, caffeine suppressed metastasis without changing primary tumor burden. The means by which caffeine suppressed metastatic activity may be associated with inhibition of malignant transformation of mammary epithelial cells, inhibition of conversion of dormant tumor cells to micrometastases, micrometastases to macrometastases, or inhibition of tumor cell adhesion and motility. Gene and protein expression patterns resulting from caffeine treatment showed that metastasis suppression may be associated with up-regulation the mRNA expression of multiple extracellular matrix genes, including *Fbln1, Bgn, Sparc, Fbn1, Loxl1*, *Col1a1*, *Col3a1*, *Col5a1*, *Col5a2*, *Col5a3*, *Col6a1*, *Col6a2*, and *Col6a3*. These data suggested that caffeine or other methyl xanthine derivatives may improve the clinical outcome in patients prior to and following the diagnosis of metastatic disease, and could potentially reduce the morbidity and mortality associated with disseminated tumors.

Abbreviations: 2-DE – two-dimensional gel electrophoresis; caffeine21 – caffeine given at weaning; caffeine60 – caffeine given at 60 days of age; CID – collision-induced dissociation; CK – cytokeratin; ECM – extracellular matrix; IT – ion trap; PCNA – proliferating cell nuclear antigen; PI3K – phosphatidylinositol-3-kinase; pyMT – polyoma middle T

Introduction

Metastasis is an extraordinarily complex process. To successfully colonize a secondary site, a cancer cell must complete a sequential series of steps before it becomes a clinically detectable lesion. These steps include separation from the primary tumor, invasion through surrounding tissues and basement membranes, entry to and survival in the circulation, lymphatics or peritoneal space, and arresting in a distant target organ, usually, but not always [1] followed by extravasation into the surrounding tissue, survival in the foreign microenvironment, proliferation, and induction of angiogenesis. All of these steps must be performed while evading apoptotic death or immunological responses (reviewed in [2]).

This process is of great relevance to the clinical management of cancer since the majority of cancer mortality is associated with metastatic disease rather than the primary tumor [2]. In most cases cancer patients with localized tumors have significantly better prognoses than those with disseminated tumors. Recent evidence suggests that the first stages of metastasis can be an early event in neoplastic progression [3]

Correspondence to: Kent Hunter, Laboratory of Population Genetics, National Cancer Institute, 41 Library Drive, Bethesda, MD 20892, USA. Tel: +1-301-435-8957; E-mail: hunterk@mail.nih.gov

and that 60–70% of patients have initiated the metastatic process by the time of diagnosis. It is therefore evident that an improved understanding of factors leading to tumor dissemination is of vital importance. However, even patients that have no evidence of tumor dissemination at primary diagnosis are at risk for metastatic disease. Approximately one-third of women who are sentinel lymph node negative at the time of surgical resection of the primary breast tumor will subsequently develop clinically detectable metastatic disease [4]. Even patients with small primary tumors and node negative status (T1N0) at surgery have a significant (15–25%) chance of developing distant metastases [5]. The ability to prevent the growth of dormant disseminated tumor cells into clinically relevant tumors would therefore significantly reduce cancer-related morbidity and mortality.

Caffeine, a methylxanthine, is one of the most commonly used drugs in the world, present in coffee, tea, and many soft drinks. Epidemiological evidence indicates that heavy caffeine consumption is negatively related to cancer-related mortality [6]. Caffeine and other methylxanines have also been shown to inhibit tumor cell invasiveness and experimental metastasis [7–9]. The anti-metastatic effects of caffeine may be related to a decrease in homing rate of primary tumor cells to distant target organs by multiple mechanisms, including by blocking the accessibility of the basement membrane to the tumor cells [7], by suppressing cell adhesion to components of the extracellular matrix (ECM) [8], or by inhibiting complementary binding of tumor cells to endothelial cells [8, 9], etc. Furthermore, caffeine can inhibit tumor cell growth and induce substantial differentiation in HL-60 cells [10]. However, the *in vivo* effects of caffeine on autochthonous metastasis have not yet been reported.

Caffeine has multiple biochemical activities. It inhibits phosphodiesterase activities, alters intracellular calcium mobilization, inhibits phosphatidylinositol-3-kinase (PI3K) activity, and antagonizes adenosine receptors (reviewed in [9, 11, 12]). These activities may all contribute to the modulation of the metastatic process that has previously been attributed to caffeine. Given the complex activities of caffeine and the consequent effects on carcinogenesis, the high level of caffeine consumption by human populations on a daily basis, and the association of caffeine consumption with fibrocystic breast disease and other benign breast disease [13, 14], we initiated a comprehensive study of the *in vivo* functions of caffeine.

In the current study, we examined the effect of caffeine on tumorigenesis and metastasis using a highly metastatic autochthonous transgene-induced mammary tumor model. These transgenic mice carry the MMTV-polyoma middle T (MMTV-PyMT) antigen. Female virgins develop palpable mammary tumors within 60 days of birth and more than 90% of which develop pulmonary metastases by 100 days [15]. We showed for the first time the *in vivo* anti-metastatic activity of caffeine against autochthonous metastasis. The mechanisms by which caffeine inhibited tumorigenesis and metastasis were explored on a global molecular level, using mRNA microarray and 2D gel analysis followed by protein identification using mass spectrometry (MS) sequencing. The analyses of the global gene and protein expression pattern may be useful for hypothesis-generation regarding the pathways modulated by caffeine that might be targeted for metastasis prophylaxis, and as a proof-of-principle that small molecule agents might be useful for reducing the morbidity and mortality associated with metastatic disease.

Materials and methods

Animals

FVB/N-TgN (MMTV-PyVT)^{634Mul} male mice obtained from W. Muller (McMaster University, Hamilton, Ontario, Canada) were bred to female FVB/NJ mice [15]. Transgene positive female offspring, as determined by PCR amplification of weanling tail biopsy DNA [16], were randomly assigned to one of the three experimental groups: caffeine exposure commenced at weaning (designated as caffeine21), caffeine exposure commenced at 60 days of age (caffeine60), or control, with 17 mice in each group. Caffeine (purity $>98.5\%$, Sigma) was added into the drinking water at a concentration of 0.44 mg/ml, similar to the concentration of caffeine in 0.6% tea solution [17]. The presence of primary tumors in animals was screened by palpation on alternate day, and the diagnosis was confirmed by examination for an additional week. To permit development of metastases, the mice were euthanized 40 days following primary tumor diagnosis. Mice were weighed and mammary tumors were completely dissected, weighed, snap frozen in liquid nitrogen, and stored at -80 °C; lungs were collected for histological examination. Statistical analysis of tumor burden was performed using the non-parametric Mann–Whitney U test.

Histological analysis

The lung tissues were fixed in 10% neutral buffered formalin and paraffin-embedded. Three non-adjacent serial sections were hematoxylin and eosin stained and examined as previously described [18]. Briefly, lung sections were examined under $12\times$ magnification and scored using a Leica Q500IW image analysis system. Both macroscopic and microscopic lesions were enumerated. The minimal size of a lesion considered a metastasis by this protocol was spherical nests of $\geq \sim 100$ tumor cells. The number and size of metastases in each sample were quantified, and the results normalized to the size of the lung tissue scored to reduce errors due to variations in sample sizes present on each slide. Metastatic index, the average metastases per unit

area of lung analyzed, was averaged for each caffeine treatment group and compared to that of the control group by the non-parametric Mann–Whitney U test to determine statistical significance.

Western blotting

Tumor samples were homogenized in PBS, pH 7.4 with 1% Triton x-100, 0.5% deoxycholate, 0.1% SDS, 0.004% NaF, 2 mM PMSF, 2 mM sodium orthonovate, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A. Protein concentration was determined by BCA assay (Pierce). Ten micrograms of each sample protein were separated on SDS-PAGE pre-cast gels (BioRad) and then transferred to an Immobilon-P membrane (Millipore). Western blotting was performed as previously described [16]. Membranes were incubated with the primary antibody solution of proliferating cell nuclear antigen (PCNA), Ki-67, ATM, p53, phosph-p53 (Ser15), or phosph-p53 (Ser20), and then with the secondary antibody (anti-mouse or anti-goat IgG conjugated with HRP) solution. The presence of the antigen was detected using ECL-Plus Kit (Amersham) following the manufacturer's protocol.

RNA extraction and processing for Affymetrix GeneChip® analysis

Frozen tumor samples were pulverized on dry ice. Total RNA was extracted from the pulverized samples using TRIzol® Reagent (Life Technologies) according to the standard protocol. The quantity and quality of the RNA were determined using an Agilent Technologies 2100 Bioanalyzer (Bio Sizing Software version A.02.01., Agilent Technologies) and/or a GeneQuant Pro (Amersham Biosciences). Samples containing high-quality total RNA with A_{260}/A_{280} between 1.8 and 2.1 were purified with the RNeasy Mini Kit (Qiagen). An on-column digest was performed as part of this purification step using the RNase-Free DNase Set (Qiagen). Purified total RNA for each treatment group was then pooled to produce a uniform sample containing 8 μg RNA.

Double stranded cDNA was synthesized from this preparation using the SuperScript™ Choice System for cDNA Synthesis (Invitrogen) according to the protocol for Affymetrix GeneChip® Eukaryotic Target Preparation (Affymetrix). Double stranded cDNA was purified using the GeneChip® Sample Cleanup Module (Qiagen). Synthesis of Biotin-Labeled cRNA was obtained by *in vitro* transcription of purified template cDNA using an Enzo BioArray High Yield RNA Transcript Labeling Kit (T7) (Enzo Life Sciences). Target cRNA was purified by using a GeneChip® Sample Cleanup Module (Qiagen). A 1 : 10 dilution was made with 1 μl of the purified IVT reaction product, then run on the Agilent Technologies 2100 Bioanalyzer (Bio Sizing Software, Version A.02.01., Agilent Technologies) to determine the quality and quantity of the labeling reaction. The quantity of cRNA was subsequently adjusted according to the Affymetrix protocol prior to fragmentation using a GeneChip® Sample Cleanup Module (Qiagen). One microliter was removed from the fragmentation reaction, and sample quality was checked prior to hybridization using an Agilent Technologies 2100 Bioanalyzer (Bio Sizing Software, Version A.02.01., Agilent Technologies) to confirm proper fragmentation.

Hybridization cocktails from each fragmentation reaction were prepared according to the Affymetrix GeneChip® protocol. The hybridization cocktail was applied to an Affymetrix Murine Genome MOE430 GeneChip® Arrays, processed on an Affymetrix® Fluidics Station 400 then analyzed on an Agilent GeneArray Scanner using Affymetrix Microarray Suite version 5.0.0.032 software. Data were analyzed using BRB ArrayTool v 3.2.0 (http://linus.nci.nih.gov/BRB-ArrayTools.html). Spots with a signal less than 50 were not included in the analysis. Normalization was performed by using median signal over entire array for each array with the median array as reference. The gene expression level of each group was determined by averaging the signal of the three samples from each group. Differentially expressed genes were determined using a cut-off value of >1.5-fold and $P < 0.005$.

Quantitative real-time RT-PCR

Template cDNA for PCR amplification was synthesized from mRNA (3–4 samples per group) using a Superscript kit (Invitrogen Corp., Carlsbad, CA). Real-time PCR was performed using SYBR green reagent (PE applied Biosystems, Foster City, CA) on an ABI Prism 7900 HT Sequence Analyzer (PE Applied Biosystems) per manufacture's protocol. Gene-specific primers (sequences available upon request) were designed with Primer3 software to amplify fragments of 150–500 base pairs. Each reaction was run in triplicate. Amplification of target genes was normalized to *Gapdh* amplification using the rodent *Gapdh* primers (PE Applied Biosystems).

Two-dimensional gel electrophoresis (2-DE)

Protein was extracted from frozen tumor tissues (five samples per group) using Cellular and Organelle Extraction Reagent (Sigma) per manufacture's protocol. Six hundred microliters of solubilized protein samples was diluted to a total volume of 450 μl with rehydration buffer (8 M urea, 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 0.002% bromophenol blue, 0.5% IPG buffer, 0.28% dithiothreitol), and applied to ready-to-use immobiline DryStrips, pH 3–10 (Amersham, Piscataway, NJ). After rehydration for 12 h at 20 °C, the strips were run at 500 V for 1 h followed by at 1000 V for 1 h and at 8000 V for 8 h 20 min for isoelectric focus.

DryStrips were incubated in a solution of 10 mg/ml of DTT in an equilibration buffer (50 mM Tris–HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, bromophenol blue) for 15 min, and subsequently in a solution of 45 mg/ml of iodoacetamide in the same equilibration buffer for 15 min. For the second dimension the proteins were separated on a 12.5% SDS homogenous pre-cast gel (Amersham) at 2.5 W/gel for 30 min, followed by 17 W/gel until the frontline reached the bottom of the gels. The gels were stained with Collodial Coomassie Blue and then scanned using GS-700 Imaging Densitometer (Bio-Rad, Hercules, CA). Evaluation of the 2-D gels was performed using PD Quest software (Bio-Rad). Differentially expressed protein spots identified by image analysis and confirmed by visual inspection were excised from the gels for protein identification by mass spectrometric analysis. All protein samples were analyzed in duplicates.

Protein identification by mass spectrometric analysis

Coommassie blue stained 2-D protein gel spots were digested with trypsin and peptides extracted essentially as described previously [19]. The samples were desalted with C18 Zip Tips (Millipore, Bedford, MA) per manufacturer's protocols and stored at −20 °C until analysis.

The samples were analyzed by microcapillary reversed-phase liquid chromatography using an Agilent 1100 capillary LC system (Agilent Technologies, Palo Alto, CA) coupled online to an ion trap (IT) mass spectrometer (LCQ Deca XP, Thermo Finnigan, San Jose, CA). Reversed-phase separations were performed using 75 μm i.d. × 360 μm o.d. × 10 cm long capillary columns (Polymicro Technologies Phoenix, AZ) that were slurry packed in house with 5 μm Jupiter C-18 stationary phase (Phenomenex, Torrence, CA). After sample injection, a 20 min wash with 95% buffer A (0.1% v/v formic acid in water) was applied and peptides were eluted using a linear gradient of 5% solvent B (0.1% v/v formic acid in acetonitrile) to 50% solvent B over 45 min with a constant flow rate of 0.5 μl/min.

The IT-MS was operated in a data-dependent mode where each full MS scan was followed by three tandem MS scans in which the three most abundant peptide molecular ions were dynamically selected for collision-induced dissociation (CID) using a normalized collision energy of 38%. The temperature of heated capillary and electrospray voltage (applied on column base) were 180 °C and 1.8 kV, respectively. The CID spectra were searched against the NCBI non-redundant mouse protein database using SEQUEST (Thermo Finnigan, San Jose, CA).

Results

Suppression effect of caffeine on tumor growth and lung metastases

On average the PyMT mice consumed ~ 2.75 ml water per day (data not shown), which corresponded to a daily intake of 1.21 mg caffeine per mouse. This is equivalent to a daily consumption of ~ 2750 mg caffeine for a 60-kg woman on a body weight basis. However, since mice metabolize caffeine much faster than human beings [20], the corrected value of 1.21 mg of caffeine per mouse per day, according to metabolic body size [21], was equivalent therefore to 636 mg of caffeine per 60-kg woman per day. A cup of coffee contains approximately 100 mg caffeine [22], thus the caffeine dose received by mice in the treatment group was equivalent to 6–7 cups of coffee per day in a human, close to that consumed by a heavy coffee drinker. No significant differences were observed in the body weight in the carcasses of control and caffeinated mice (Table 1) after tumor resection. Since the body weight of the mice reflects their food intake, the consistency in body weight across all groups indicated that central nervous system stimulatory effect of caffeine exposure likely did not affect the food intake significantly.

Mice in all groups developed mammary tumors. Furthermore 100% of the control and 76–82% in the caffeine treatment groups subsequently developed lung metastases (Table 1). Control animals developed an average of 9.3 g tumor burden (Figure 1a) with an average latency of 49.4 days (range 44–66 days, SD = 6.6). Mice given caffeine at weaning developed significantly less tumor burden (6.6 g; Figure 1a) than the controls ($P = 0.002$) without significantly changing the tumor latency (average 53.3 days, range 40–62 days, SD = 6.5). Animals given caffeine at 60 days (after the onset

Table 1. Effect of caffeine exposure on animal body weight, tumor incidence, tumor burden, and metastasis incidence.

Group	Number of mice per group	Number of mice bearing tumors	Number of mice with lung metastases	Average tumor burden (g)	*P*-value vs control	Average carcass weight (g)	*P*-value vs control
Control	17	17	17	9.19	N/A	22.9	N/A
Caffeine21	17	17	13	7.23	0.002	24.6	0.12
Caffeine60	17	17	14	8.48	0.39	22.3	0.26

Caffeine was given to mice at weaning (caffeine21) or at 60 days of age (caffeine60). No significant difference was observed in the body weight of animal in the three groups. Mice receiving caffeine from weaning developed smaller mammary tumors ($P = 0.002$). Less animals developed lung metastases in caffeine-treated groups, however, the difference was not significant.

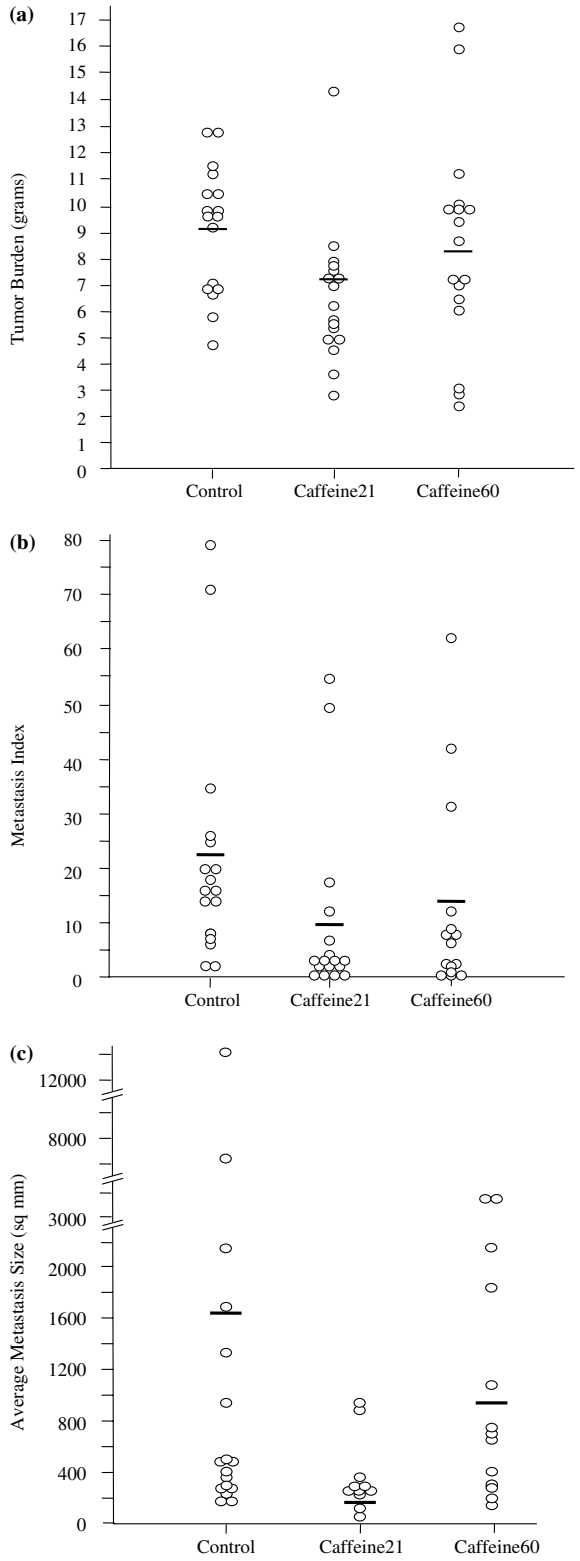

Figure 1. Tumor burden (a), metastasis index (b) and the average size of the lung metastases (c) in the control FVB mice and mice treated with caffeine. Caffeine exposure starting from weaning significantly decreases the tumor burden, metastasis index as well as the size of the lung metastases, whereas caffeine given at 60-day of age decreases metastasis index significantly but has not effect on tumor burden nor the size of lung metastases.

of palpable tumors) developed 8.5 g tumor burden with an average latency of 49.5 days (range 36–70 days, SD = 9.0), not significantly different from those of control animals (Figure 1a). Metastatic index of the control group, as measured by the density of tumor lesions per area of lung tissue scored in non-adjacent serial sections, was 21.9 (range 2.2–79.7, SD = 21.44; Figure 1b). Both caffeine groups had significantly lower metastatic index compared to controls. Metastatic index was decreased by 55% in mice receiving caffeine at weaning (average metastatic index 9.8, range 0–54.9, SD = 16.6, $P = 0.002$) and by 35% in those receiving caffeine at 60 days of age (average metastatic index 14.3, range 0–62.3, SD = 19.2, $P = 0.03$). These data showed that caffeine administration commencing from weaning inhibited both tumor burden and metastasis, whereas caffeine given at 60 days specifically inhibited pulmonary metastasis without affecting primary tumors.

To elucidate the mechanism underlying the inhibitory effect of caffeine in tumorigenesis and metastasis, the frequency of mammary gland hyperplastic foci and pulmonary metastasis foci was examined. Mice given caffeine at weaning, but not at 60 days of age, developed fewer hyperplastic foci than the control mice, as observed in the whole mounts of the mammary gland (Figure 2). This observation suggested that caffeine given at the early stage of tumorigenic process may suppress the malignant transformation of mammary epithelial cells. This hypothesis was consistent with the fact that caffeine given at 60 days, after the formation of frank mammary tumor, did not alter the primary tumor burden nor the frequency of hyperplastic foci. Quantitative analysis of the lung metastases revealed that exposure to caffeine from weaning significantly decreased metastasis size ($P = 0.02$; Figure 1c). For animals receiving caffeine from 60 days of age, the size of metastatic foci did not change (Figure 1c). These observations suggested that caffeine suppresses the formation of metastases, either by inhibiting extravasation or/and survival of single disseminated cells or by delaying or preventing the conversion of dormant cells to proliferative lesions.

Caffeine does not alter Atm *signaling pathway*

Caffeine has been shown to influence cell proliferation and apoptosis via a variety of signal transduction pathways, including the ATM pathway [23–25]. In a previous study, we identified a metastatic suppressor genomic locus on chromosome 9. This genomic region is linked to the *Atm* gene. Based on these observations we hypothesized that *Atm* gene may be a metastatic suppressor candidate gene [26]. To investigate whether the inhibitory effect of caffeine was due to its effect on *Atm* signal pathway, we examined the expression and activation of *Atm* and its downstream targets *p53* and cell proliferation in the mammary tumors. Cell proliferation rate, determined by PCNA and Ki-67 immunoblotting, did not show significant changes upon caffeine treatment (data not shown). The expression and/or activation of *Atm* and *p53* also did not change with caffeine exposure (data not shown). Therefore the

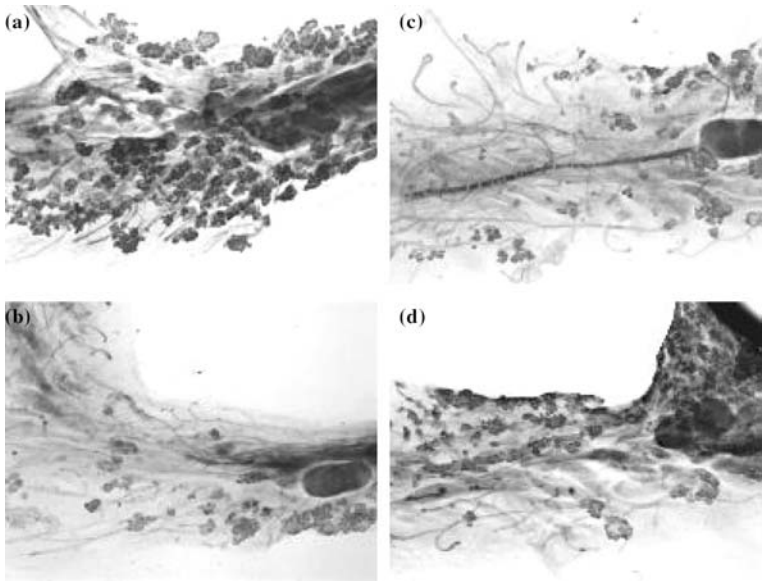

Figure 2. Representative whole-mounts of mammary glands from female FVB/PyMT mice. (a) from control mouse; (b–d) from mice receiving caffeine at weaning (21 days of age). Mice receiving caffeine from weaning appeared to have less mammary hyperplastic foci than the control mice.

suppression of metastatic capacity by caffeine was likely to be *Atm*-independent.

Molecular profiling of tumor samples from control and caffeine-treated animals

To identify potential molecules and molecular pathways involved in suppressing tumorigenic and metastatic cascades upon caffeine treatment, we initiated a systems biology approach to determine global changes in gene and protein expressions associated with caffeine-mediated tumor and metastases suppression. To identify these genes whose expressions were regulated by caffeine, gene expression profiles of mammary tumors from each treatment group were analyzed using Affymetrix microarrays and compared with that from control group. One hundred and three genes were detected showing more than 1.5-fold difference ($P < 0.005$) by caffeine21 and 79 genes by caffeine60 (Appendices), respectively, among which 21 genes were differentially regulated by both treatments. Using the Gene Ontology Tool of the NetAffy Analysis Center of Affymetrix, we classified 62 out of 103 cafffeine21-regulated genes and 36 out of 79 caffeine60-regulated genes into functional groups (Tables 2 and 3).

Gene expression patterns showed that both caffeine treatments significantly changed the expression of genes involved in ECM remodeling, signal transduction pathways, transcriptional regulation, cytoskeleton regulation, immune response, and cell polarity. Most significantly, a number of ECM genes were overexpressed, including *Fbln1, Bgn, Sparc, Fbn1, Loxl1*, *Col1a1*, *Col3a1*, *Col5a1*, *Col5a2*, *Col5a3*, *Col6a1*, *Col6a2*, and *Col6a3*, the majority of which have been implicated in metastasis. Caffeine21 and caffeine60 showed high consistent regulation of ECM gene expression. These findings suggested that modulation of ECM composition, and subsequently inhibition of cell migration may be one mechanism by which caffeine interfered with the metastasis. Consistent with the observed overexpression of collagen gene family, caffeine21 down-regulated the expression *Mmp12* and *Adamts10* which are involved in proteolysis [27, 28], and up-regulated the expression of extracellular protease inhibitors including *Serping1* and *Serpinf1*.

Confirmation of array result using quantitative real-time RT-PCR

To verify the results of microarray experiments, real-time RT-PCR was performed. mRNA samples used in the Affymetrix microarray analysis were subjected to reverse transcription and subsequent quantification by real-time PCR. Figure 3 showed side-by-side comparisons of expression levels measured by microarray analysis and real-time PCR for 11 randomly chosen genes. Twelve of the 15 pairs of comparisons showed a consistent expression pattern in real-time PCR and array analysis, confirming the reliability of microarray data. The comparisons of expression patterns for three genes, *Col5a3*, *Serpinf1*, and *Fbln1*, were discordant following caffeine60 exposure. Since the same mRNA samples were used for both assays, the discrepancy was unlikely due to animal variations in gene expression. One possible explanation was that the two assays may measure the expression level of different alternative splicing variants of these genes. To explore such a possibility, we examined the existence and location of alternative splicing variants of these genes using BLAST search engine. We identified multiple alternative splicing

Table 2. Differentially regulated genes (>1.5-fold; $P > 0.005$) from mammary tumors of the PyMT transgenic mice exposed to caffeine from weaning (caffeine21), determined by DNA microarray analysis.

Gene symbol	Probe set	Protein	Caffeine21/control
Extracellular matrix remodeling			
Bgn	1448323_a_at	Biglycan	2.31
Col15a1	1448755_at	Procollagen, type XV	2.06
Col1a1	1423669_at	Procollagen, type I, alpha 1	2.31
Col1a2	1423110_at	Procollagen, type I, alpha 2	2.24
Col3a1	1427884_at	Procollagen, type III, alpha 1	2.24
Col5a1	1416741_at	Procollagen, type V, alpha 1	3.78
Col5a2	1422437_at	Procollagen, type V, alpha 2	2.08
Col6a1	1448590_at	Procollagen, type VI, alpha 1	2.17
Col6a2	1452250_a_at	Procollagen, type VI, alpha 2	2.59
Col6a3	1424131_at	Procollagen, type VI, alpha 3	2.46
Ecm1	1448613_at	Extracellular matrix protein 1	1.87
Fbln1	1422540_at	Fibulin 1	1.91
Fbn1	1425896_a_at	Fibrillin 1	2.06
Loxl1	1451978_at	Lysyl oxidase-like 1	2.15
Pcolce	1437165_a_at	Procollagen C-proteinase enhancer protein	2.23
Sftpd	1420378_at	Surfactant associated protein D	0.47
Sparc	1416589_at	Secreted acidic cysteine rich glycoprotein	1.77
Spon1	1451342_at	Spondin 1, (f-spondin) extracellular matrix protein	2.72
Tmlhe	1452500_at	Trimethyllysine hydroxylase, epsilon	1.63
Proteolysis			
Adam19	1418402_at	A disintegrin and metalloproteinase domain 19 (meltrin beta)	2.00
Adamts10	1441309_at	A disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 10	0.29
Ctsk	1450652_at	Cathepsin K	1.82
Mmp12	1449153_at	Matrix metalloproteinase 12	0.38
Serpinf1	1416168_at	Serine (or cysteine) proteinase inhibitor, clade F), member 1	1.92
Serping1	1416625_at	Serine (or cysteine) proteinase inhibitor, clade G, member 1	1.89
Usp18	1418191_at	Ubiquitin specific protease 18	0.35
Immune response			
G1p2	1431591_s_at	Interferon, alpha-inducible protein	0.47
Ifit1	1450783_at	Interferon-induced protein with tetratricopeptide repeats 1	0.30
Ifit2	1418293_at	Interferon-induced protein with tetratricopeptide repeats 2	0.33
Ifit3	1449025_at	Interferon-induced protein with tetratricopeptide repeats 3	0.34
Signaling pathway			
F2r	1437308_s_at	Coagulation factor II (thrombin) receptor	1.71
Gnb1	1425908_at	Guanine nucleotide binding protein, beta 1	1.80
Grb10	1425458_a_at	Growth factor receptor bound protein 10	1.99
Lrp1	1448655_at	Low density lipoprotein receptor-related protein 1	1.97
Mknk1	1417631_at	MAP kinase-interacting serine/threonine kinase 1	0.62
Olfr56	1417292_at	Olfactory receptor 56	0.58
Pdgfrb	1436970_a_at	Platelet derived growth factor receptor, beta polypeptide	2.18
Socs2	1449109_at	Suppressor of cytokine signaling 2	0.51
Txnip	1415996_at	Thioredoxin interacting protein	1.72
Apoptosis			
Tnfaip3	1450829_at	Tumor necrosis factor, alpha-induced protein 3	0.51
Cytoskeleton organization			
Krt1-15	1422667_at	Keratin complex 1, acidic, gene 15	0.47
Plekhh1	1452517_at	Pleckstrin homology domain containing, family H (with MyTH4 domain) member 1	1.62
Transcription regulation			
Irf7	1417244_a_at	Interferon regulatory factor 7	0.40
Mist1	1418800_at	Muscle, intestine and stomach expression 1	0.48
Msx1	1448601_s_at	Homeo box, msh-like 1	1.94

Table 2. Continued.

Gene symbol	Probe set	Protein	Caffeine21/control
Nkx2-6	1452018_at	NK2 transcription factor related, locus 6 (Drosophila)	0.40
Stat1	1450033_a_at	Signal transducer and activator of transcription 1	0.57
Tcf7l2	1420747_at	Transcription factor 7-like 2, T-cell specific, HMG-box	2.91
Usf1	1426164_a_at	Upstream transcription factor 1	0.56
Cell polarity			
Stau1	1450741_at	Staufen (RNA binding protein) homolog 1 (Drosophila)	1.84
Zbp1	1429947_a_at	Z-DNA binding protein 1	0.51
Other			
Bcl11a	1447334_at	B-cell CLL/lymphoma 11A (zinc finger protein)	0.29
Csng	1420633_a_at	Casein gamma	0.17
Cyp2e1	1415994_at	Cytochrome P450, family 2, subfamily e, polypeptide 1	3.29
Fstl1	1416221_at	Follistatin-like 1	2.04
Ier5	1460009_at	Immediate early response 5	1.62
Morf4l1	1437801_at	Mortality factor 4 like 1	0.56
Rad23a	1422964_at	RAD23a homolog (S. cerevisiae)	0.62
S3-12	1418595_at	Plasma membrane associated protein, S3-12	3.28
Stx1bl	1457875_at	Syntaxin 1 b-like	0.26
Tmlhe	1452500_at	Trimethyllysine hydroxylase, epsilon	1.63
Zc3hdc1	1426774_at	Zinc finger CCCH type domain containing 1	0.55

Caffeine21/Control is the ratio of normalize group average signal of caffeine21 vs control.

variants for *Fbln1* and *Serpinf1* and that the Affymetrix probe sets of *Fbln1* gene were located in alternative splicing regions. Therefore, the differences shown by RT-PCR and array analysis may result from different expression level of alternative splicing variants. Since the inconsistency was only observed in the samples from mice given caffeine at 60 days of age, we hypothesized that caffeine60 may influence the process of alternative splicing. This hypothesis remains to be tested.

2-DE protein expression pattern in the mammary tumors of PyMT transgenic mice

Since changes in gene expression are not always translated into changes in protein level, protein profiles of control and caffeine treated samples were resolved and compared by 2-DE analysis to identify proteins regulated by caffeine. The overall protein profiles of control and caffeine-treated samples appeared similar. However, by using computer-assisted differential analysis, we detected 18 and 23 protein spots displaying over 2-fold differences as a result of exposure to caffeine from weaning or from the age of 60 days, respectively. Caffeine21 increased the expression of 12 proteins and decreased that of six proteins, with majority of proteins identified by MS sequencing involved in stress regulation and ECM remodeling (Table 4). Caffeine60 up-regulated 8 proteins and down-regulated 15 proteins, 10 of which were identified by MS sequencing. These 10 proteins represented components of chaperon and cytoskeletal regulation, cell proliferation and apoptosis (Table 4). Many of the differentially regulated proteins, including keratin 18 and 19 [29, 30], tropomyosin beta 2 [31], and peroxiredoxin I [32, 33], have been reported to be involved in cancer or metastasis. Altered expression of a number of proteins including GRP78, PDI, tropomysin beta 2, and peroxiredoxin I correlated well with the decreased metastatic propensity of mammary tumors [34, 35] following caffeine60 treatment.

Discussion

There is pressing need for effective and low-toxicity chemotherapeutic and chemopreventive agents against disseminated tumors. In the current study, we have demonstrated the inhibitory effect of caffeine on neoplasm development and dissemination in a spontaneously arising, highly metastatic mammary tumor model. To our knowledge, this is the first report examining the *in vivo* effect of caffeine on spontaneous metastasis occurring in a natural context. Previous investigations of caffeine in metastasis have been limited to studies using tumor cell lines or experimental metastasis models. Although these approaches have proven valuable, they are limited in that they do not encompass all stages of tumorigenesis and the metastasis cascade [7, 9]. Our finding that caffeine consumption initiated after primary tumor formation efficiently suppressed the spontaneous metastasis provided proof-of-principle that the application of small molecules such as caffeine or other compounds might be utilized in chemotherapy and metastasis intervention to reduce morbidity and mortality associated with neoplastic disease. This possibility was especially compelling

Table 3. Differentially regulated genes (>1.5-fold differences, $P < 0.005$) from mammary tumors of the PyMT transgenic mice exposed to caffeine from age of 60 days (caffeine60), determined by DNA microarray analysis.

Gene symbol	Probe set	Protein	Caffein60/control
Extracellular matrix			
Bgn	1416405_at	Biglycan	1.76
Col1a1	1423669_at	Procollagen, type I, alpha 1	2.30
Col5a1	1416741_at	Procollagen, type V, alpha 1	3.80
Col5a2	1422437_at	Procollagen, type V, alpha 2	1.85
Col5a3	1419703_at	Procollagen, type V, alpha 3	1.97
Col6a3	1424131_at	Procollagen, type VI, alpha 3	2.29
Fbln1	1422540_at	Fibulin 1	1.93
Fbn1	1425896_a_at	Fibrillin 1	2.13
Loxl1	1451978_at	Lysyl oxidase-like 1	1.95
Nid1	1448469_at	Nidogen 1	2.78
Sparc	1416589_at	Secreted acidic cysteine rich glycoprotein	1.76
Thbs2	1422571_at	Thrombospondin 2	1.89
Transcriptional regulation			
Mef2a	1421252_a_at	Myocyte enhancer factor 2A	2.32
Nssr	1418526_at	Neural-salient serine/arginine-rich	0.58
Tcf7l2	1420747_at	Transcription factor 7-like 2, T-cell specific, HMG-box	2.14
Usf1	1426164_a_at	Upstream transcription factor 1	0.56
Cytoskeleton organization			
Myl1	1452651_a_at	Myosin, light polypeptide 1, alkali; atrial, embryonic	1.70
Mylk	1425504_at	Myosin, light polypeptide kinase	2.40
Cell Cycle			
Pmp22	1417133_at	Peripheral myelin protein	1.96
Proteolysis			
Serpinf1	1416168_at	Serine (or cysteine) proteinase inhibitor, clade F), member 1	2.18
2310046G15Rik	1437671_x_at	RIKEN cDNA 2310046G15 gene	2.26
Signaling tranduction pathway			
Acvr2	1457451_at	Activin receptor IIA	2.52
Grb10	1425458_a_at	Growth factor receptor bound protein 10	2.00
Neto1	1425132_at	Neuropilin (NRP) and tolloid (TLL)-like 1	2.02
Nkd2	1419466_at	Naked cuticle 2 homolog (Drosophila)	1.70
Pld1	1425739_at	Phospholipase D1	1.70
Other			
Adh1	1416225_at	Alcohol dehydrogenase 1 (class I)	2.22
B4galt6	1450913_at	UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 6	1.98
Cd164l1	1417439_at	CD164 sialomucin-like 1	2.35
Fkbp10	1415951_at	FK506 binding protein 10	1.77
Hsp105	1425993_a_at	Heat shock protein 105	0.51
Mid2	1450537_at	Midline 2	2.32
Mrc2	1421045_at	Mannose receptor, C type 2	2.89
Prg4	1449824_at	Proteoglycan 4 (megakaryocyte stimulating factor, articular superficial zone protein)	1.90
Snai1	1448742_at	Snail homolog 1 (Drosophila)	1.94
Usp7	1454949_at	Ubiquitin specific protease 7	3.91

Caffeine60/control is the ratio of normalize group average signal of caffeine60 vs control.

since high caffeine intake has been associated with decreased cancer mortality in human populations [6]. Exploring the underlying mechanism of caffeine-induced metastatic suppression may enable the identification of novel targets for the development of more effective and specific anti-metastatic drugs.

Preliminary data from an experimental metastasis model showed that caffeine exposure starting before tumor cell dissemination reduced metastasis size and the number of metastasis foci induced by orthotopic implantation of Mvt2 [36], a highly metastatic mouse mammary tumor cell line (unpublished data). Similarly,

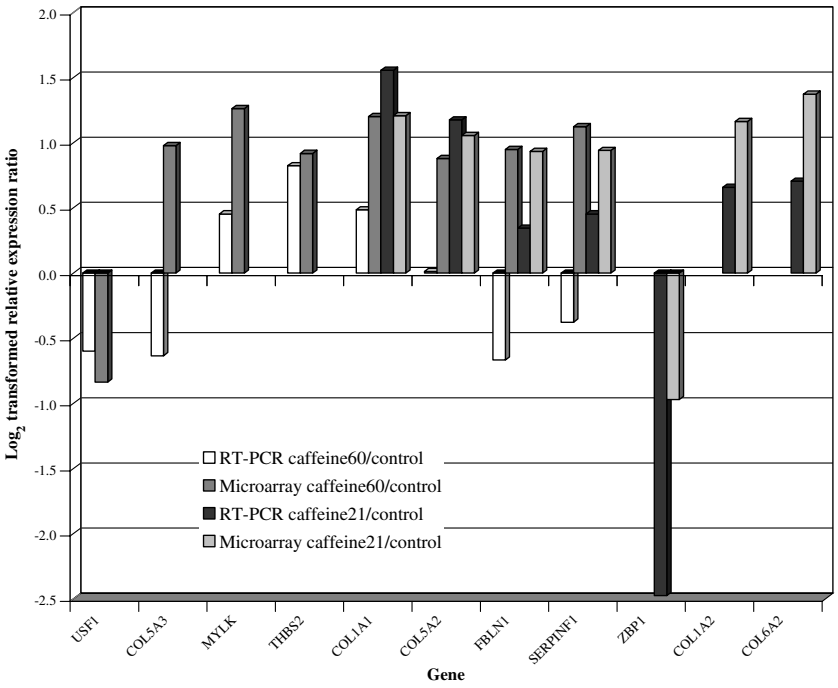

Figure 3. Validation of microarray results for a subset of genes by real-time PCR. Each sample were run in triplicate and normalized to the amplification of *Gapdh*. The relative expression of each gene (caffeine/control) determined by real-time PCR and microarray was log transformed and plotted side-by-side. The relative expression of 80% of the genes was consistent in the two assays.

caffeine-treated tumor cells induce significantly smaller metastasis lesions and less metastatic foci [37]. In addition, we showed that caffeine decreased tumor burden probably by inhibiting formation of hyperplastic foci. Taken together, our data suggested two potential chemopreventive mechanisms of caffeine: (1) caffeine consumption starting at the early stage of tumorigenesis reduced tumor burden by inhibiting the transformation of epithelial cells to malignant cells thus limiting the number of viable malignant lesions; (2) caffeine suppressed tumor metastasis via inhibiting the conversion of dormant tumor cells to micrometastasis, and of micrometastasis to macrometastasis, but likely did not effect established proliferating macrometastases.

Data from DNA microarray and 2-D gel analysis showed prominent changes in gene expression both at the mRNA and protein level as a consequence of caffeine treatment, and revealed a number of novel targets of caffeine. Alteration of expression level of ECM and cytoskeleton genes suggested that caffeine modulated metastasis in a variety of ways, including cell-ECM interaction, chemotaxis (modulated by processing of ECM components), tumor cell polarity, and survival of disseminating tumor cells in the circulation. Out of the 21 common-regulated genes in both caffeine treatments, nine were involved in ECM remodeling. The ECM provides a microenvironment for both tumor growth and metastasis. Cell motility in this context is largely dependent on interaction between the tumor cell and ECM components [38]. For example, *Fbln1*, a gene up-regulated in the caffeine21 group inhibits cell attachment and motility promoted by fibronectin [39]. Another such example was provided with *Sparc*, which is an extracellular glycoprotein that influences the deposition of ECM and modulates the interaction between cell and ECM. Expression of *Sparc* is associated with high content of collagen fibers [40], inhibition of cell proliferation, and induction of apoptosis [41]. *In vivo* experiment shows that SPARC expression in ovarian cancer cells is inversely correlated with the degree of malignancy [41]. Up-regulation of collagen genes in the caffeine21 group suggested that tumors that arose in these cohorts may have a higher collagen content, which has previously been demonstrated to be negatively related to cell-ECM adhesion and tumor cell motility [42]. Overexpression of ECM components following caffeine exposure strongly supported the hypothesis that enhancement of ECM expression was related to a less-invasive phenotype. Indeed, this is consistent with previous studies that have determined expression patterns observed in a non-metastatic mammary tumor cell line compared to a high-metastatic counterpart [42]. Furthermore, these data are in accordance with the *in vitro* observation that caffeine inhibits tumor cell motility [37].

Our data also support previous studies that have shown that vimentin and members of the cytokeratin (CK) gene family play a prominent role in tumor metastasis. Expression of vimentin is linked to more aggressive tumor characteristics including high histopathologic grade and poor differentiation [43, 44]. The *de novo* expression of vimentin is frequently involved in the epithelial-to-mesenchymal transition which is associated with increased invasive/migratory properties of epithelial cells [45]. Keratin family proteins are

Table 4. Differentially regulated proteins identifies using 2-DE followed by MS protein sequencing.

Accession number	Protein	Gene symbol	Caffeine/control
Differentially regulated by caffeine give at weaning			
Stress-related proteins			
NP_112442	Heat shock 70 kD protein 8	Hspa8	2.04
NP_080405	Endoplasmic reticulum protein Erp29 precursor	Erp29	2.14
P17879	Heat shock 70 kD protein 1	Hspa1	2.24
P14602	Heat shock protein 27	Hspb1	3.95
NP_033955	Heat shock protein 47	Serpinh1	3.34
Cytoskeleton regulation			
Q9WVA4	Transgelin 2	Tagln2	2.13
B25819	Actin		12.48
NP_032905	L-Plastin 2	Lcp1	0.38
A43803	Vimentin	Vim	0.48
P17182	Alpha enolase	Eno1	0.38
P10107	Annexin I	Anxa1	2.34
Other			
XP_125429	Similar to infereron-inducible protein 10	LOC236937	2.79
Differentially regulated by caffeine give at 60 days			
Stress-related proteins			
P09103	Protein disulfide isomerase precursor	P4hb	0.44
A37048	dnaK-type molecular chaperon grp78 precursor	Grp78	0.23
Q922R9	RIKEN cDNA 2410002K23	2410002K23RIK	0.33
Cytoskeleton regulation			
NP_034794	Keratin complex 1, acidic, gene 18	Krt1-18	2.32
A44131	Tropomysin beta 2	Tpm-2	2.31
P19001	Keratin, type I cytoskeletal 19	Krt1-19	0.43
Cell proliferation and Apoptosis			
Q06830	Peroxiredoxin 1	Prdx1	14.83
EFMS1	Translation elongation factor eEF-1 alpha chain		0.37
Other			
Q91X72	Hemopexin	Hpx	13.18
NP_032933	Peptidylprolyl isomerase A	Ppia	0.47

involved in cell migration, cell invasiveness, plasminogen activity, as well as drug and radiation resistance [29]. Additionally, expression phenotype of the keratin gene family varies with tumor metastatic potential. A highly metastatic tumor cell line MTLn3 predominately expresses CKs 7, 8, 14, and 19, while normal mammary epithelium co-expresses CKs 5, 8, 14, and 18 [30]. Up-regulation of keratin 18 and down-regulation of keratin 19 have been associated with tumors of decreased metastatic capacity, which again, is consistent with the outcome of current study.

In addition to ECM and cytoskeleton components, other genes and proteins may also functionally relate to metastasis inhibition. Of particular interest on this regard was peroxiredoxin I (Prxd1) whose expression was dramatically increased by 15-fold in animals receiving caffeine from age of 60 days. *Prxd1* is a putative tumor suppressor [46] which is involved in cellular redox regulation and acts to protect cells from reactive oxygen species and eliminate peroxides generated as a consequence of normal cellular metabolic functions. *Prxd1* regulates signal transduction pathways involving c-Abl, caspases, nuclear factor-kappaB and activator protein-1 by regulating the intracellular concentrations of H_2O_2, and therefore has a very important role in regulating cell proliferation, differentiation and apoptosis [47, 48]. Mice deficient in *Prxd1* develop multiple malignant tumors at a high incidence [32]. Expression of *Prxd1* is negatively related to metastatic efficacy of tumor cell lines [33], consistent with our observation.

Previous studies from our laboratory demonstrate that the metastatic efficiency of this model is significantly influenced by the genetic background upon which it arose [49]. Microarray analysis also demonstrated that the gene expression profile of these tumors was also significantly affected by genetic background effects (H. Yang et al., submitted). Since pulmonary metastasis were suppressed in the caffeine60 group without affecting the development of primary tumor, a comparison of gene expression pattern from caffeine60 treatment with those from low-metastatic inbred strains DBA and NZB was currently underway. Preliminary

analyses revealed that certain genes associated with the low-metastatic phenotype as a consequence of caffeine60 treatment were also implicated in suppression of metastatic efficacy by the DBA and NZB genetic background. This provided further supporting evidence for the role of these genes and pathways in metastatic progression, as well as suggesting that caffeine and genetic modulation of metastatic potential may impact the same molecular pathways.

In conclusion, this study demonstrated that caffeine had a strongly inhibitory effect on both tumorigenesis and metastasis in the transgenic mouse model. Genes and proteins involved in ECM remodeling, signal transduction, transcriptional regulation, and cytoskeletal regulation were identified as novel targets of caffeine and were potentially associated with tumorigenesis or metastasis. Clarification of the precise relationship between these genes and the biochemical functions of caffeine may help to decipher the molecular pathways by which caffeine exerts its anti-tumorigenic and anti-metastatic activities. While the neurological effects of caffeine and lack of specificity make the use of caffeine somewhat undesirable in a clinical setting, this study demonstrated that small molecule agents could be efficacious for metastasis chemo-prophylaxis. Identification of other less toxic agents that can be administered prophylactially to patients following the primary diagnosis might significantly reduce cancer morbidity and mortality by reducing metastatic burden.

Acknowledgements

We would like to thank Dr Nigel Crawford for the critical review and editing of the manuscript. Microarray analyses were performed using BRB ArrayTools developed by Dr Richard Simon and Amy Peng Lam.

Appendix

Table A1. Differentially regulated genes (>1.5-fold differences; $P < 0.005$) from mammary tumors of the PyMT transgenic mice exposed to caffeine from age of 21 days, determined by microarray analysis. Caffeine21/control is the ratio of normalize group average signal of caffeine21 vs control.

Gene symbol	Probe set	Description	Caffeine21/control
1110014P06Rik	1451305_at	RIKEN cDNA 1110014P06 gene	0.58
2310002A05Rik	1456248_at	RIKEN cDNA 2310002A05 gene	0.15
2900040J22Rik	1433129_at	RIKEN cDNA 2900040J22 gene	3.33
4930560O18Rik	1433017_at	RIKEN cDNA 4930560O18 gene	0.19
6330412A17Rik	1430003_at	RIKEN cDNA 6330412A17 gene	3.04
A430056A10Rik	1423555_a_at	RIKEN cDNA A430056A10 gene	0.49
A730098P15	1436628_at	Hypothetical protein A730098P15	2.38
AA409132	1426774_at	Expressed sequence AA409132	0.55
Adam19	1418402_at	A disintegrin and metalloproteinase domain 19 (meltrin beta)	2.00
Adamts10	1441309_at	A disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 10	0.29
AI504298	1439381_x_at	Expressed sequence AI504298	1.85
AW123240	1438713_at	Expressed sequence AW123240	2.27
Bgn	1448323_a_at	Biglycan	2.31
Bicc1	1423484_at	Bicaudal C homolog 1 (Drosophila)	2.32
Cenpb	1426051_a_at	Centromere autoantigen B	0.44
Col15a1	1448755_at	Procollagen, type XV	2.06
Col1a1	1423669_at	Procollagen, type I, alpha 1	2.31
Col1a2	1423110_at	Procollagen, type I, alpha 2	2.24
Col3a1	1427884_at	Procollagen, type III, alpha 1	2.24
Col5a1	1434479_at	Procollagen, type V, alpha 1	1.88
Col5a2	1422437_at	Procollagen, type V, alpha 2	2.08
Col6a1	1448590_at	Procollagen, type VI, alpha 1	2.17
Col6a2	1452250_a_at	Procollagen, type VI, alpha 2	2.59
Col6a3	1424131_at	Procollagen, type VI, alpha 3	2.46
Crlf1	1418476_at	Cytokine receptor-like factor 1	2.45
Csng	1420633_a_at	Casein gamma	0.17
Ctsk	1450652_at	Cathepsin K	1.82
Cyp2e1	1415994_at	Cytochrome P450, family 2, subfamily e, polypeptide 1	3.29
D130043N08Rik	1418507_s_at	RIKEN cDNA D130043N08 gene	0.60
D130051D11Rik	1439839_at	RIKEN cDNA D130051D11 gene	4.93
D330035F22Rik	1424415_s_at	RIKEN cDNA D330035F22 gene	2.25

Table A1. Continued.

Gene symbol	Probe set	Description	Caffeine21/control
D630024D12Rik	1452517_at	RIKEN cDNA D630024D12 gene	1.62
Ecm1	1448613_at	Extracellular matrix protein 1	1.87
F2r	1437308_s_at	Coagulation factor II (thrombin) receptor	1.71
Fabp5	1416021_a_at	Fatty acid binding protein 5, epidermal	0.63
Fbln1	1422540_at	Fibulin 1	1.91
Fbn1	1425896_a_at	Fibrillin 1	2.06
Fstl	1416221_at	Follistatin-like	2.04
G1p2	1431591_s_at	Interferon, alpha-inducible protein	0.47
Gnb1	1425908_at	Guanine nucleotide binding protein, beta 1	1.80
Grb10	1425458_a_at	Growth factor receptor bound protein 10	1.99
Ier5	1460009_at	Immediate early response 5	1.62
Ifi47	1417292_at	Interferon gamma inducible protein	0.58
Ifit1	1450783_at	Interferon-induced protein with tetratricopeptide repeats 1	0.30
Ifit2	1418293_at	Interferon-induced protein with tetratricopeptide repeats 2	0.33
Ifit3	1449025_at	Interferon-induced protein with tetratricopeptide repeats 3	0.34
Iigp-pending	1419042_at	Interferon-inducible GTPase	0.40
Irf7	1417244_a_at	Interferon regulatory factor 7	0.40
Krt1–15	1422667_at	Keratin complex 1, acidic, gene 15	0.47
Loxl	1451978_at	Lysyl oxidase-like	2.15
Lrp1	1448655_at	Low density lipoprotein receptor-related protein 1	1.97
Mist1	1418800_at	Muscle, intestine and stomach expression 1	0.48
Mknk1	1417631_at	MAP kinase-interacting serine/threonine kinase 1	0.62
Mmp12	1449153_at	Matrix metalloproteinase 12	0.38
Morf4l1	1437801_at	Mortality factor 4 like 1	0.56
Mrc2	1421045_at	Mannose receptor, C type 2	2.61
Msx1	1448601_s_at	Homeo box, msh-like 1	1.94
Nkx2–6	1452018_at	NK2 transcription factor related, locus 6 (Drosophila)	0.40
Pcolce	1437165_a_at	Procollagen C-proteinase enhancer protein	2.23
Pdgfrb	1436970_a_at	Platelet derived growth factor receptor, beta polypeptide	2.18
Ppnr-pending	1420747_at	Per-pentamer repeat gene	2.91
Prss18	1448982_at	Protease, serine, 18	0.80
Rad23a	1422964_at	RAD23a homolog (S. cerevisiae)	0.62
Rap140-pending	1457034_at	Retinoblastoma-associated protein 140	3.15
Retn	1449182_at	Resistin	2.42
S3-12-pending	1418595_at	Plasma membrane associated protein, S3-12	3.28
Serpinf1	1416168_at	Serine (or cysteine) proteinase inhibitor, clade F), member 1	1.92
Serping1	1416625_at	Serine (or cysteine) proteinase inhibitor, clade G, member 1	1.89
Sftpd	1420378_at	Surfactant associated protein D	0.47
Sgy1-pending	1417787_at	Soggy 1	2.71
Smarcd3	1418467_at	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily d, member 3	2.40
Socs2	1449109_at	Suppressor of cytokine signaling 2	0.51
Sparc	1416589_at	Secreted acidic cysteine rich glycoprotein	1.77
Spon1	1451342_at	Spondin 1, (f-spondin) extracellular matrix protein	2.72
Ssg1-pending	1424186_at	Steroid sensitive gene 1	2.12
Stat1	1450033_a_at	Signal transducer and activator of transcription 1	0.57
Stau1	1450741_at	Staufen (RNA binding protein) homolog 1 (Drosophila)	1.84
Tmlhe	1452500_at	Trimethyllysine hydroxylase, epsilon	1.63
Tnfaip3	1450829_at	Tumor necrosis factor, alpha-induced protein 3	0.51
Txnip	1415996_at	Thioredoxin interacting protein	1.72
Usf1	1426164_a_at	Upstream transcription factor 1	0.56
Usp18	1418191_at	Ubiquitin specific protease 18	0.35
Wwox	1431960_at	WW domain-containing oxidoreductase	1.91
Xbp1	1434279_at	X-box binding protein 1	0.56
Zbp1	1429947_a_at	Z-DNA binding protein 1	0.51
	1427086_at	*M. musculus*, clone IMAGE:5028619, mRNA	2.67
	1431498_at	*M. musculus* transcribed sequence	0.26

Table A1. Continued.

Gene symbol	Probe set	Description	Caffeine21/control
	1435137_s_at	*M. musculus* RIKEN cDNA 1200015M12 gene, mRNA (cDNA clone IMAGE:5009304), partial cds	1.81
	1443393_at	*M. musculus*, clone IMAGE:3709354, mRNA	2.27
	1444851_at	*M. musculus*, Similar to zinc finger protein, clone MGC:51479 IMAGE:4012958, mRNA, complete cds	2.66
	1445489_at		0.25
	1445920_at	*M. musculus* transcribed sequence with weak similarity to protein ref:NP_076996.1 (H.sapiens) hypothetical protein MGC5297 [Homo sapiens]	0.27
	1445925_at	*M. musculus* transcribed sequences	0.20
	1446638_at	*M. musculus* transcribed sequences	0.63
	1447020_at	*M. musculus* 7 days neonate cerebellum cDNA, RIKEN full-length enriched library, clone:A730027B14 product:unknown EST, full insert sequence.	3.88
	1447334_at	*M. musculus* transcribed sequence	0.29
	1447826_x_at	*M. musculus* transcribed sequences	0.19
	1447836_x_at	*M. musculus* transcribed sequences	4.00
	1456547_at	*M. musculus* transcribed sequences	2.69
	1457334_at	*M. musculus* 12 days embryo male wolffian duct includes surrounding region cDNA, RIKEN full-length enriched library, clone:6720481A07 product:nucleolar protein GU2, full insert sequence.	9.41
	1457875_at	*M. musculus* transcribed sequence with strong similarity to protein sp:Q15531 (H.sapiens) ST1B_HUMAN Syntaxin 1B	0.26
	1459127_at	*M. musculus* transcribed sequences	3.63
	1459368_at	*M. musculus* 9 days embryo whole body cDNA, RIKEN full-length enriched library, clone:D030017N12 product:unknown EST, full insert sequence	0.21

Table A2. Differentially regulated genes (> 1.5-fold differences; $P < 0.005$) from mammary tumors of the PyMT transgenic mice exposed to caffeine from age of 60 days, determined by microarray analysis.

Gene symbol	Probe set	Description	Caffeine60/control
1500016H10Rik	1426386_at	RIKEN cDNA 1500016H10 gene	0.65
1700003M02Rik	1429851_at	RIKEN cDNA 1700003M02 gene	0.24
1700009P17Rik	1429181_at	RIKEN cDNA 1700009P17 gene	3.14
2010305K11Rik	1444590_at	RIKEN cDNA 2010305K11 gene	1.65
2310046G15Rik	1437671_x_at	RIKEN cDNA 2310046G15 gene	2.26
4930458D05Rik	1453488_at	RIKEN cDNA 4930458D05 gene	2.60
4933406K04Rik	1432252_a_at	RIKEN cDNA 4933406K04 gene	3.10
4933434M16Rik	1431895_at	RIKEN cDNA 4933434M16 gene	2.51
5730446C15Rik	1433412_at	RIKEN cDNA 5730446C15 gene	0.28
Adh1	1416225_at	Alcohol dehydrogenase 1 (class I)	2.22
AI849053	1436642_x_at	Expressed sequence AI849053	3.00
B4galt6	1450913_at	UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 6	1.98
Bgn	1416405_at	Biglycan	1.76
Col1a1	1423669_at	Procollagen, type I, alpha 1	2.30
Col5a1	1416741_at	Procollagen, type V, alpha 1	3.80
Col5a2	1422437_at	Procollagen, type V, alpha 2	1.85
Col5a3	1419703_at	Procollagen, type V, alpha 3	1.97
Col6a3	1424131_at	Procollagen, type VI, alpha 3	2.29
D030041N04Rik	1443076_at	RIKEN cDNA D030041N04 gene	0.26
Efna3	1455344_at	Ephrin A3	3.42
Fbln1	1422540_at	Fibulin 1	1.93
Fbn1	1460208_at	Fibrillin 1	1.69
Fbn1	1425896_a_at	Fibrillin 1	2.13
Fkbp10	1449632_s_at	FK506 binding protein 10	2.30

Table A2. Continued.

Gene symbol	Probe set	Description	Caffeine60/control
Fstl	1416221_at	Follistatin-like	2.03
Grb10	1425458_a_at	Growth factor receptor bound protein 10	2.00
Hsp105	1425993_a_at	Heat shock protein 105	0.51
Loxl	1451978_at	Lysyl oxidase-like	1.95
Lpo	1448998_at	Lactoperoxidase	1.55
Matn2	1419442_at	Matrilin 2	1.71
Mef2a	1421252_a_at	Myocyte enhancer factor 2A	2.32
Mid2	1450537_at	Midline 2	2.32
Mrc2	1421045_at	Mannose receptor, C type 2	2.89
Myl1	1452651_a_at	Myosin, light polypeptide 1, alkali; atrial, embryonic	1.70
Mylk	1425504_at	Myosin, light polypeptide kinase	2.40
Neto1	1425132_at	Neuropilin (NRP) and tolloid (TLL)-like 1	2.02
Nid1	1448469_at	Nidogen 1	2.78
Nkd2	1419466_at	Naked cuticle 2 homolog (Drosophila)	1.70
Nssr	1418526_at	Neural-salient serine/arginine-rich	0.58
Pld1	1425739_at	Phospholipase D1	1.70
Pmp22	1417133_at	Peripheral myelin protein	1.96
Ppnr-pending	1420747_at	Per-pentamer repeat gene	2.14
Prg4	1449824_at	Proteoglycan 4 (megakaryocyte stimulating factor, articular superficial zone protein)	1.90
Rap140-pending	1457034_at	Retinoblastoma-associated protein 140	3.03
Salf-pending	1453376_at	Stoned B/TFIIA-alpha/beta-like factor	2.96
Sec61a	1434986_a_at	SEC61, alpha subunit (S. cerevisiae)	0.58
Serpinf1	1416168_at	Serine (or cysteine) proteinase inhibitor, clade F), member 1	2.18
Snai1	1448742_at	Snail homolog 1 (Drosophila)	1.94
Sparc	1416589_at	Secreted acidic cysteine rich glycoprotein	1.76
Spon1	1451342_at	Spondin 1, (f-spondin) extracellular matrix protein	2.63
Ssg1-pending	1424187_at	Steroid sensitive gene 1	2.20
Tem1-pending	1417439_at	Tumor endothelial marker 1 precursor	2.35
Thbs2	1422571_at	Thrombospondin 2	1.89
Usf1	1426164_a_at	Upstream transcription factor 1	0.56
	1458054_at	*M. musculus* transcribed sequences	0.24
	1445802_at	*M. musculus* transcribed sequences	0.33
	1446935_at	*M. musculus* transcribed sequences	0.33
	1441721_at	*M. musculus* transcribed sequences	0.33
	1446031_at	*M. musculus* transcribed sequence with weak similarity to protein ref:NP_081764.1 (M.musculus) RIKEN cDNA 5730493B19 [*Mus musculus*]	0.36
	1442460_at	*M. musculus* transcribed sequences	0.43
	1445498_at	*M. musculus* transcribed sequences	0.49
	1426114_at		0.50
	1447641_at	*M. musculus* transcribed sequences	0.51
	1421754_at		0.58
	1436404_at	*M. musculus*, clone IMAGE:3586350, mRNA, partial cds	1.62
	1427216_at	*M. musculus* RIKEN cDNA 6030405N23 gene, mRNA (cDNA clone IMAGE:4488589), partial cds	1.89
	1442698_at	*M. musculus* 7 days neonate cerebellum cDNA, RIKEN full-length enriched library, clone:A730053D01 product:unknown EST, full insert sequence	2.31
	1456547_at	*M. musculus* transcribed sequences	2.32
	1452433_at		2.38
	1457451_at	*M. musculus* transcribed sequence with strong similarity to protein sp:P27037 (H.sapiens) AVR2_HUMAN Activin receptor type II precursor (ACTR-II) (ACTRIIA)	2.52
	1455088_at	*M. musculus* transcribed sequences	2.62
	1420311_s_at		2.82

Table A2. Continued.

Gene symbol	Probe set	Description	Caffeine60/control
	1445688_at	*M. musculus* transcribed sequence with weak similarity to protein ref:NP_081764.1 (*M.musculus*) RIKEN cDNA 5730493B19 [*M musculus*]	2.98
	1446481_at	*M. musculus* 0 day neonate lung cDNA, RIKEN full-length enriched library, clone:E030036K09 product:unknown EST, full insert sequence	3.26
	1458383_at	*M. musculus* adult male pituitary gland cDNA, RIKEN full-length enriched library, clone:5330435K21 product:unclassifiable, full insert sequence	3.57
	1455743_at	*M. musculus* transcribed sequences	3.89
	1454949_at	*M. musculus* 10 days neonate cerebellum cDNA, RIKEN full-length enriched library, clone:B930099F18 product:Ubiquitin specific Protease 7, full insert sequence	3.91
	1459127_at	*M. musculus* transcribed sequences	4.28
	1447113_at	*M. musculus* transcribed sequences	5.15

Caffeine60/control is the ratio of normalize group average signal of caffeine60 vs control.

References

1. Al Mehdi AB, Tozawa K, Fisher AB et al. Intravascular origin of metastasis from the proliferation of endothelium-attached tumor cells: A new model for metastasis. Nat Med 2000; 6(1): 100–2.
2. Liotta LA, Stetler-Stevenson WG. Principles of molecular cell biology of cancer: Cancer metastasis. In DeVita SHV, Rosenberg SA (eds): Cancer: Principles & Practice of Oncology, 4th edition. Philadelphia, PA: J.B. Lippincott, 1993; 134–49.
3. Schmidt-Kittler O, Ragg T, Daskalakis A et al. From latent disseminated cells to overt metastasis: Genetic analysis of systemic breast cancer progression. Proc Natl Acad Sci USA 2003; 100(13): 7737–42.
4. Heimann R, Lan F, McBride R et al. Separating favorable from unfavorable prognostic markers in breast cancer: The role of E-cadherin. Cancer Res 2000; 60(2): 298–304.
5. Heimann R, Hellman S. Clinical progression of breast cancer malignant behavior: What to expect and when to expect it. J Clin Oncol 2000; 18(3): 591–9.
6. Grossarth-Maticek R, Eysenck HJ. Coca-Cola, cancers, and coronaries: Personality and stress as mediating factors. Psychol Rep 1991; 68: 1083–7.
7. Gude RP, Menon LG, Rao SG. Effect of caffeine, a xanthine derivative, in the inhibition of experimental lung metastasis induced by B16F10 melanoma cells. J Exp Clin Cancer Res 2001; 20(2): 287–92.
8. Cohen MC, Bereta M, Bereta J. Effect of cytokines on tumour cell-endothelial interactions. Indian J Biochem Biophys 1997; 34(1–2): 199–204.
9. Bereta M, Bereta J, Georgoff I et al. Methylxanthines and calcium-mobilizing agents inhibit the expression of cytokine-inducible nitric oxide synthase and vascular cell adhesion molecule-1 in murine microvascular endothelial cells. Exp Cell Res 1994; 212(2): 230–42.
10. Bang BE, Ericsen C, Aarbakke J. Effects of cAMP and cGMP elevating agents on HL-60 cell differentiation. Pharmacol Toxicol 1994; 75(2): 108–12.
11. Welsch CW. Caffeine and the development of the normal and neoplastic mammary gland. Proc Soc Exp Biol Med 1994; 207(1): 1–12.
12. Shafer SH, Phelps SH, Williams CL. Reduced DNA synthesis and cell viability in small cell lung carcinoma by treatment with cyclic AMP phosphodiesterase inhibitors. Biochem Pharmacol 1998; 56(9): 1229–36.
13. Minton JP, Foecking MK, Webster DJ et al. Response of fibrocystic disease to caffeine withdrawal and correlation of cyclic nucleotides with breast disease. Am J Obstet Gynecol 1979; 135(1): 157–8.
14. Minton JP, Foecking MK, Webster DJ et al. Caffeine, cyclic nucleotides, and breast disease. Surgery 1979; 86(1): 105–9.
15. Guy CT, Cardiff RD, Muller WJ. Induction of mammary tumors by expression of polyomavirus middle T oncogene: A transgenic mouse model for metastatic disease. Mol Cell Biol 1992; 12(3): 954–61.
16. Le Voyer T, Lu Z, Babb J et al. An epistatic interaction controls the latency of a transgene-induced mammary tumor. Mamm Genome 2000; 11(10): 883–9.
17. Lu YP, Lou YR, Lin Y et al. Inhibitory effects of orally administered green tea, black tea, and caffeine on skin carcinogenesis in mice previously treated with ultraviolet B light (high-risk mice): relationship to decreased tissue fat. Cancer Res 2001; 61(13): 5002–9.
18. Lifsted T, Le Voyer T, Williams M et al. Identification of inbred mouse strains harboring genetic modifiers of mammary tumor age of onset and metastatic progression. Int J Cancer 1998; 77(4): 640–4.
19. Wilm M, Shevchenko A, Houthaeve T et al. Femtomole sequencing of proteins from polyacrylamide gels by nano-electrospray mass spectrometry. Nature 1996; 379 (6564): 466–9.
20. Labedzki A, Buters J, Jabrane W et al. Differences in caffeine and paraxanthine metabolism between human and murine CYP1A2. Biochem Pharmacol 2002; 63(12): 2159–67.
21. Gilbert RM. Caffeine as a drug abuse. In Gibbins RJ, Israll Y, Kolant H et al. (eds): Recent Advances in Alcohol and Drug Problems. New York: John Wiley 1976; 49–75.
22. Bunker ML, McWilliams M. Caffeine content of common beverages. J Am Diet Assoc 1979; 74(1): 28–32.
23. Browaeys-Poly E, Cailliau K, Vilain JP. Transduction cascades initiated by fibroblast growth factor 1 on Xenopus oocytes expressing MDA-MB-231 mRNAs. Role of Grb2, phosphatidylinositol 3-kinase, Src tyrosine kinase, and phospholipase Cgamma. Cell Signal 2001; 13(5): 363–8.
24. Foukas LC, Daniele N, Ktori C et al. Direct effects of caffeine and theophylline on p110 delta and other phosphoinositide 3-kinases. Differential effects on lipid kinase and protein kinase activities. J Biol Chem 2002; 277(40): 37124–30.
25. Zhou BB, Chaturvedi P, Spring K et al. Caffeine abolishes the mammalian G(2)/M DNA damage checkpoint by inhibiting ataxia-telangiectasia-mutated kinase activity. J Biol Chem 2000; 275(14): 10342–8.
26. Hunter KW, Broman KW, Voyer TL et al. Predisposition to efficient mammary tumor metastatic progression is linked to the breast cancer metastasis suppressor Gene Brms1. Cancer Res 2001; 61(24): 8866–72.

27. Agapova OA, Ricard CS, Salvador-Silva M et al. Expression of matrix metalloproteinases and tissue inhibitors of metalloproteinases in human optic nerve head astrocytes. Glia 2001; 33(3): 205–16.
28. Somerville RP, Jungers KA, Apte SS. Discovery and characterization of a novel, widely expressed metalloprotease, ADAM-TS10, and its proteolytic activation. J Biol Chem 2004; 279(49): 51208–17.
29. Baribault H, Wilson-Heiner M, Muller W et al. Functional analysis of mouse keratin 8 in polyoma middle T-induced mammary gland tumours. Transgenic Res 1997; 6(6): 359–67.
30. Lichtner RB, Julian JA, North SM et al. Coexpression of cytokeratins characteristic for myoepithelial and luminal cell lineages in rat 13762NF mammary adenocarcinoma tumors and their spontaneous metastases. Cancer Res 1991; 51(21): 5943–50.
31. Wirth PJ. Two-dimensional polyacrylamide gel electrophoresis in experimental hepatocarcinogenesis studies. Electrophoresis 1994; 15(3–4): 358–71.
32. Neumann CA, Krause DS, Carman CV et al. Essential role for the peroxiredoxin Prdx1 in erythrocyte antioxidant defence and tumour suppression. Nature 2003; 424(6948): 561–5.
33. Jiang D, Ying W, Lu Y et al. Identification of metastasis-associated proteins by proteomic analysis and functional exploration of interleukin-18 in metastasis. Proteomics 2003; 3(5): 724–37.
34. Wulfkuhle JD, Sgroi DC, Krutzsch H et al. Proteomics of human breast ductal carcinoma *in situ*. Cancer Res 2002; 62 (22): 6740–9.
35. Bini L, Magi B, Marzocchi B et al. Protein expression profiles in human breast ductal carcinoma and histologically normal tissue. Electrophoresis 1997; 18(15): 2832–41.
36. Pei XF, Noble MS, Davoli MA et al. Explant-cell culture of primary mammary tumors from MMTV-c-Myc transgenic mice. *In Vitro* Cell Dev Biol Anim 2004; 40(1): 14–21.
37. Lentini A, Kleinman HK, Mattioli P et al. Inhibition of melanoma pulmonary metastasis by methylxanthines due to decreased invasion and proliferation. Melanoma Res 1998; 8(2): 131–7.
38. Liotta LA, Rao CN, Barsky SH. Tumor invasion and the extracellular matrix. Lab Invest 1983; 49(6): 636–49.
39. Twal WO, Czirok A, Hegedus B et al. Fibulin-1 suppression of fibronectin-regulated cell adhesion and motility. J Cell Sci 2001; 114(24): 4587–98.
40. Puolakkainen PA, Brekken RA, Muneer S et al. Enhanced growth of pancreatic tumors in SPARC-Null mice is associated With decreased deposition of extracellular matrix and reduced tumor cell apoptosis. Mol Cancer Res 2004; 2(4): 215–24.
41. Yiu GK, Chan WY, Ng SW et al. SPARC (secreted protein acidic and rich in cysteine) induces apoptosis in ovarian cancer cells. Am J Pathol 2001; 159(2): 609–22.
42. Wang W, Wyckoff JB, Frohlich VC et al. Single cell behavior in metastatic primary mammary tumors correlated with gene expression patterns revealed by molecular profiling. Cancer Res 2002; 62(21): 6278–88.
43. Hu L, Lau SH, Tzang CH et al. Association of Vimentin overexpression and hepatocellular carcinoma metastasis. Oncogene 2004; 23(1): 298–302.
44. Sommers CL, Byers SW, Thompson EW et al. Differentiation state and invasiveness of human breast cancer cell lines. Breast Cancer Res Treat 1994; 31(2–3): 325–35.
45. Grille SJ, Bellacosa A, Upson J et al. The protein kinase Akt induces epithelial mesenchymal transition and promotes enhanced motility and invasiveness of squamous cell carcinoma lines. Cancer Res 2003; 63(9): 2172–8.
46. Mu ZM, Yin XY, Prochownik EV. Pag, a putative tumor suppressor, interacts with the Myc Box II domain of c-Myc and selectively alters its biological function and target gene expression. J Biol Chem 2002; 277(45): 43175–84.
47. Butterfield LH, Merino A, Golub SH et al. From cytoprotection to tumor suppression: The multifactorial role of peroxiredoxins. Antioxid Redox Signal 1999; 1(4): 385–402.
48. Wen ST, Van Etten RA. The PAG gene product, a stress-induced protein with antioxidant properties, is an Abl SH3-binding protein and a physiological inhibitor of c-Abl tyrosine kinaseáactivity. Genes Dev 1997; 11(19): 2456–67.
49. Hunter K, Welch DR, Liu ET. Genetic background is an important determinant of metastatic potential. Nat Genet 2003; 34(1): 23–4.