

Discrepancy of biologic behavior influenced by bone marrow derived cells in lung cancer

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Abstract. Disseminated cancer cells may initially require local nutrients and growth factors to thrive and survive in bone marrow. However, data on the influence of bone marrow derived cells (BMDC, also called bone stromal cells in some publications) on lung cancer cells is largely unexplored. This study explored the mechanism of how bone stromal factors contribute to the bone tropism in lung cancer. The difference among lung cancer cell lines in their abilities to metastasize to bone was found using the SCID animal model. Supernatant of bone marrow aspiration (BM) and condition medium from human bone stromal cells (BSC) were used to study the activity of bone stromal factors. We found bone stromal factors significantly increased the proliferation, invasion, adhesion and expression of angiogenesis-related factors, and inhibited the apoptosis for high bone metastasis H460 lung cancer cells. These biologic effects were not seen in SPC-A1 or A549 cells, which are low bone metastasis lung cancer cells. Adhesion of H460 cells to surface coated with bone stromal cells can activate some signal transduction pathways, and alter the expression of adhesion associated factors, including integrin β 3 and ADAMTS-1, two potential targets related with bone metastasis. We concluded that bone marrow derived cells had a profound effect on biological

behavior of lung cancers, therefore favoring the growth of lung cancer cells in bone.

Introduction

Bone is composed of different cell types in varying ratios, including osteoblastic cells, osteoclastic cells, bone stromal cells and endothelial cells. Bone seems a milieu particularly favorable for the growth, vascularization and survival of lung, breast and prostate cancer cells, but not colon or gastric cancer cells (1,2). Disseminated cancer cells may initially require local nutrients and growth factors to thrive and survive in bone marrow (3). Bone marrow derived cells (BMDC), also called bone stromal cells, can secrete many growth factors and cytokines. The lists include, but are not limited to TGF- β , HGF, IL-6, CXCR4, GM-CSF, and G-CSF (4-6).

Extra-cellular matrix (ECM) in the bone is rich in fibronectin, laminin, osteopontin and collagen. Before growing in the bone microenvironment, circulating tumor cells must adhere to extra-cellular matrix, and invade the interstitial space of the target organs, which is essential for development of bone metastasis. Therefore, it is postulated adhesion effect plays an important role for the bone tropism of cancer (7-9).

Malignant cells express molecular pathways that are probably also expressed by bone marrow derived cells. This Tumor-cell fusion is a source of myeloid traits in cancer, and a unifying explanation for bone metastasis (10,11). There have been some studies specifically looking at the interaction between endothelial/osteoblastic/osteoclastic cells and cancer cells (12,13). Recently, Idboufker *et al* reported osteoblast-like cells increased bone related gene expression (RANKL and PTHrP) and invasive gene (MMP-2) in breast cancer cells. Thus, osteoblast can modulate the ability of breast cancer to invade the bone matrix and to adapt to the bone microenvironment (14). However, data on the influence of bone stromal cells on lung cancer cells is largely unexplored. In order to explore the mechanism of how bone stromal factors contribute to the bone tropism, we use supernatant of bone marrow aspiration (BM) and condition medium from human

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bone stromal cells (BSC) to investigate their effects on lung cancer cells.

Materials and methods

Cell lines and reagents. Lung cancer cell lines, A549, H460, gastric cancer cell line SGC-7901 were obtained originally from ATCC. SPC-A1 lung cancer cell line, and tongue cancer cell line T8113 were already established in our laboratory and have been reported previously. These cells were cultured in pheno-red-free RPMI-1640/10% fetal calf serum. Supernatant of bone marrow aspiration (BM) and condition medium from human bone stromal cells (BSC) were used to study the activity of bone stromal factors. Bone marrow samples (10 ml) were obtained from the sternum intra-operatively, and stored in a tube with heparin, centrifuged at 2000 x g for 10 min to separate plasma, which was stored at -80°C until use. We did not dialyze BM. Supernatant was diluted 1:5 by regular medium for treatment. Human bone marrow derived cells (or human bone stromal cells) were purchased from Sciencell Research Laboratories (Cat. No.7500, Carlsbad, CA). Regular medium (RM), condition medium from fibroblast cells (FC) were chosen as controls.

MTT assay. MTT assay was applied to analyze these different treatments on proliferation of lung cancer cells and controls. Cells (5×10^4 /ml) were seeded on 96-well plate on D0 in triplicate manner, and treated by RM, BSC, BM consecutively for 7 days. Every other day, medium was changed and OD was measured using MTT.

PI staining for cell cycle analysis. Flow cytometry analysis of DNA content was performed to assess the cell cycle phase distribution. Cells were harvested and stained for DNA content using propidium iodide fluorescence. The computer program Multicycle from Phenix Flow System (San Diego, CA) was used to generate histograms, which were used to determine the cell cycle phase distribution.

Apoptosis assay by double staining with annexin V-FITC and propidium iodide. Double staining of annexin V-FITC and propidium iodide (PI) were conducted according to manufacturer's instruction (Alexis Biochemicals, San Diego, CA). In brief, 5×10^5 cells were rinsed with 1X binding buffer provided by kit, stained with 5 μ l annexin V (20 μ g/ml in Tris-NaCl) and 10 μ l PI (50 μ g/ml in 1X binding buffer) in triplicate manner. After incubation at room temperature for 10 min, the cells were immediately analyzed in a FACScan (Becton-Dickinson, San Jose, USA). Ten thousand cells were gated for analysis on the basis of forward and side scatter using FACScan software, and compensation values were adjusted according to annexin V alone and PI staining alone. Annexin⁺/PI⁻ stands for early apoptosis and annexin⁺/PI⁺ stands for late apoptosis/secondary necrosis.

Adhesion assay. Ninety-six cell tissue culture plates were coated with RM or BM at 37°C overnight. Cells were trypsinized and resuspended in RPMI-1640/0.1% BSA at concentration of 5×10^5 /ml. After adhesion for 1 h, the suspended cells were gently washed away by PBS. MTT was used to evaluate the quantity of cells remaining on the plate.

Real-time PCR. Real-time PCR was used to analyze expression of angiogenesis related factors induced by BM/BSC in H460 cells. Total RNA was extracted from two independent experiments by TRIzol reagent (Life Technologies, Inc., Rockville, MD) according to the manufacture's instructions. The first strand cDNA was synthesized with Superscript II reverse transcriptase and oligo(dT) primers. The primers were designed to span different exons to avoid amplification of genomic DNA. Real-time PCR was done in triplicate in a final volume of 10 μ l containing 0.1 μ l forward primer (10 ng/ml), 0.1 μ l reverse primer, 0.1 μ l SYBRA Green, 4.7 μ l Mix (Applied Biosystems, Foster City, CA). ABI 7900 sequence detector (Applied Biosystems) and SDS 2.2 software were used for PCR reaction and data analysis. Cycle threshold numbers (Ct) of all genes were normalized to β -actin. Samples without template were set up to exclude the measurement of primer dimers.

The following primers were designed: IL-8: upstream TTGGCAGCCTTCCTGATTTC, downstream TTAGCACTCCTTGGCAAACTG; VEGF: upstream CACCATGCAGATTATGCGGA, downstream CATCTCTCCTATGTGCTGGCCT; PDGF: upstream AAGGTGGCCAAGGTGGAATAC, downstream TCCAAATGCTCCTCTAACCTCAC.

Microarray procedures. We used the Affymetrix gene chip U133A 2.0 to study the gene expression profile of H460 cells, after exposure to secreted proteins from bone stromal cells. Experiments were conducted in triplicate manner at 2 and 24 h time-points. Total RNA of the samples were isolated as described previously and further purified with RNeasy Mini kit (Qiagen, Valencia, CA). The cRNA labeling was performed according to protocols from Affymetrix, Inc. (Santa Clara, CA). The labeled cRNA was subsequently hybridized to human U133 133 A 2.0 oligonucleotide probe array (Affymetrix, Inc.) according to standard protocols done by Shanghai Biochip Co. Gene expression was considered to be significantly changed when 2-fold or more difference was detected and $P < 0.05$, calculated by t-test between different time-points.

Some genes were selected for real-time PCR analysis to confirm microarray data. Real-time PCR was conducted as above. The following primers were designed: ADRb2: upstream 5'-GATTGCCTTCCAGGAGCTTCT-3', downstream 5'-CCATTCCCATAGGCCTTCAA-3'; BIRC3: upstream 5'-TGGTTTCCAAGGTGTGAGTACTTG-3', downstream 5'-AACTTGACGGATGAACTCCTGTC-3'; ADAMTS1: upstream 5'-TTTTGCAGCCCAAGGTTGTAG-3', downstream 5'-GATGCGATCACAACCAGCTTT-3'.

Invasion assay. Matrigel was diluted by pheno-free RPMI-1640 to the concentration of 8.9% mg/ml on the ice. Cell culture inserts, 8.0 μ mm pore size (Becton-Dickinson Labware, Franklin Lakes, NJ) were covered with Matrigel at 37°C, and placed into 24-well plates. Cells (10^6) were added to the upper chamber of inserts, and regular medium/BM to the lower chamber. After 48 h, cell culture inserts were fixed with 3.7% paraformaldehyde/PBS for 15 min. Cells in the upper chamber were then removed with a cotton swab and the filters stained using a Diff-Quick stain (HEMA-3[®] stain set, Biochemical Sciences, Swedesbord, NJ). Cells in 3 represen-

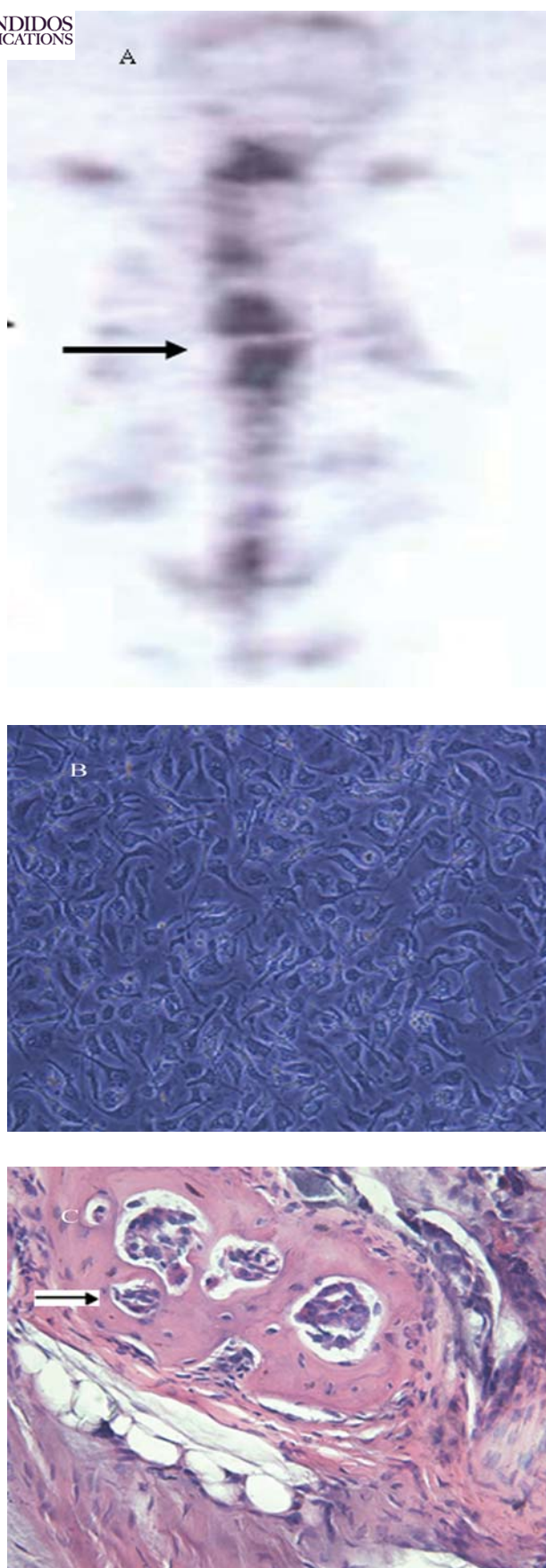


Figure 1. Bone metastasis of nude mice injected by H460. (A) SCID was injected with H460 cells through the tail vein. Eight weeks later, hot spot was detected by bone scan. (B) Tissues from spine were spliced and cultured, and cancerous cells were found. (C) Bone metastasis was confirmed further by pathological examination.

tative x200 microscopic fields were counted. Invading ratio was defined as invading cells in control group divided by that in BM group. Assays were repeated 2 times with similar results.

Evaluation of bone metastasis potential of different lung cancer cell lines using bone scan and pathological examination. Different cell lines were injected into NIH-BNX nude mice via tail vein. Animal experimentation was in accordance with institutional guidelines. Bone metastasis was evaluated with bone scan 8 weeks after cell injection. Before dissection, 148 MBq ^{99}Tc -MDP 3mCo ^{99}mTc -MDP (Shanghai Xinke Co., Shanghai) was injected into the mice, and bone scan using SPECTE was performed to ascertain the overall distribution of the radio-tracer. Bone metastasis was defined when hot spots were detected. To confirm these hot spots were real bone metastasis, bone tissues from specious areas were biopsied, sliced, and primary cell culture was conducted. Additionally, part of bone-metastasis-suspicious lesions were infiltrated into a 10% solution of formalin immediately, and delivered for the pathologic procedure within 24 h. Section 4- μm thick was cut for routing HE staining.

Results

The difference among lung cancer cell lines in ability to metastasize to bone. SCID was injected with lung cancer cells through tail vein. Eight weeks later, hot spots were detected by bone scan in all three mice injected with H460 cells (Fig. 1A). Tissues from specious spine were then sliced, and cancerous cells through primary culture indicated the bone contained metastasis (Fig. 1B). Additionally, bone metastasis was also confirmed by pathological examination. So H460 cells have high bone metastasis potential shown by our nude mice model. This result was consistent with report by Yang *et al* (15). With a similar experiment we found one out of 9 mice with SPC-A1 cells, and one out of 10 mice injected with A549 developed bone metastasis. Our results showed SPC-A1 and A549 cells are low bone metastasis lung cancer cells, in accordance with literature reports (16,17).

Bone stromal factors promoted proliferation of lung cancer cells with high bone metastasis potential. Both supernatants from BM and BSC significantly increased the proliferation of H460 lung cancer cells, which had wide-spread skeletal metastatic potential shown in the above animal experiment (Fig. 2A). This activity was not shown in A549 and SPC-A1 lung cancer cells (Fig. 2B and C). BM did not improve the proliferation of tongue cancer cells T8113, which had very low bone metastasis potential, and even inhibited the growth of SGC7901, a gastric cancer cell line with rare metastasis to bone (Fig. 2D and E). Bone stromal-cell-induced proliferation in H460 cells was blocked by Zometa (bisphosphonate), but not by PI3-K inhibitor Wartmannin in time-course and dose-dependent studies (Fig. 2F).

Flow cytometry analysis showed that both BM and BSC reduced early and late apoptosis in H460 cells (Fig. 3), but did not affect cell cycle distribution. It could be the underlying mechanism by which BM and BSC promoted H460 cells proliferation. In the low bone metastasis lung cancer

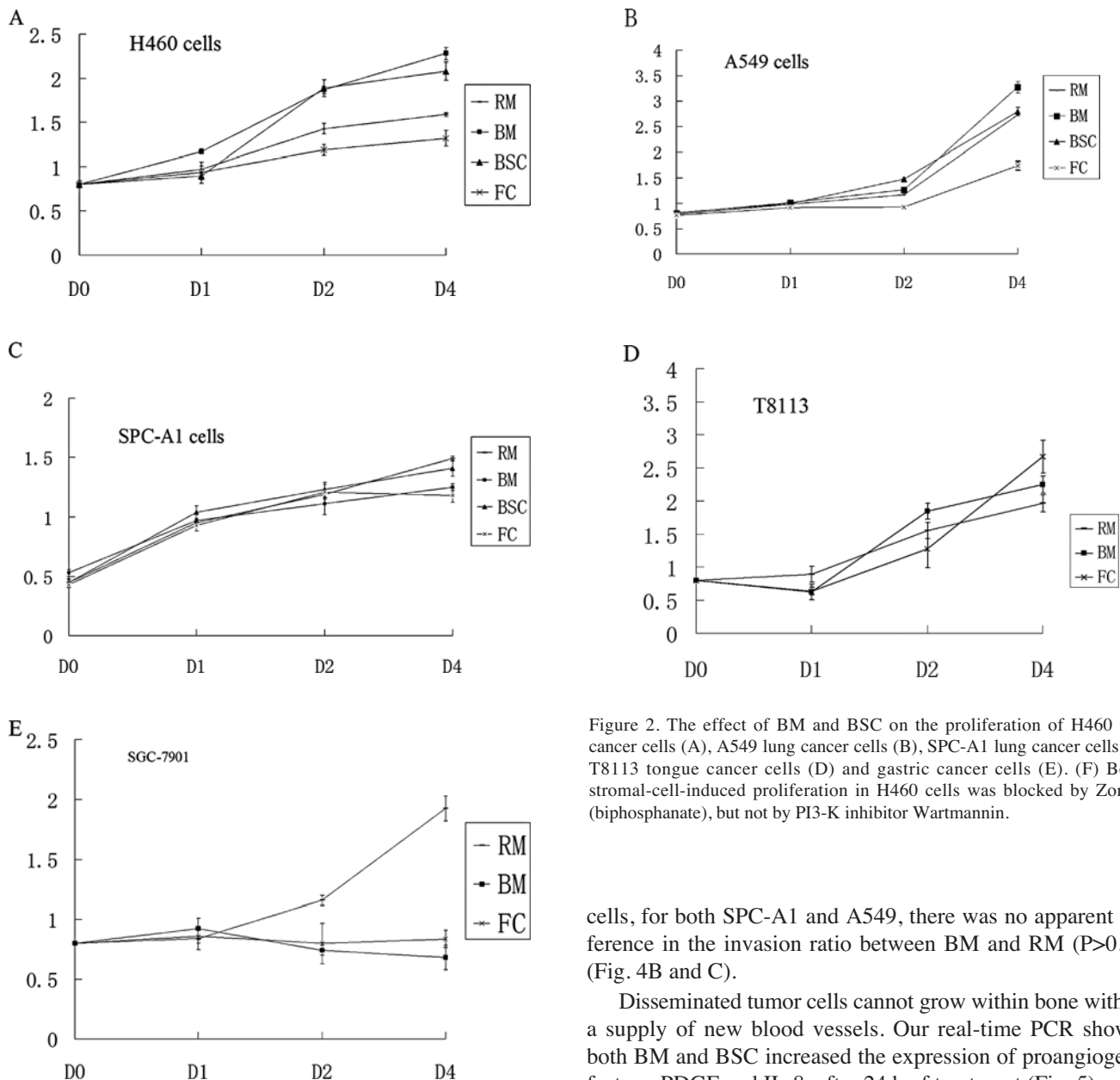


Figure 2. The effect of BM and BSC on the proliferation of H460 lung cancer cells (A), A549 lung cancer cells (B), SPC-A1 lung cancer cells (C), T8113 tongue cancer cells (D) and gastric cancer cells (E). (F) Bone-stromal-cell-induced proliferation in H460 cells was blocked by Zometa (biphosphanate), but not by PI3-K inhibitor Wartmannin.

cells, for both SPC-A1 and A549, there was no apparent difference in the invasion ratio between BM and RM ($P>0.05$) (Fig. 4B and C).

Disseminated tumor cells cannot grow within bone without a supply of new blood vessels. Our real-time PCR showed both BM and BSC increased the expression of proangiogenic factors, PDGF and IL-8, after 24 h of treatment (Fig. 5).

Exposure to bone stromal proteins may activate downstream signaling in H460 cells. Secreted protein from bone stromal cells might facilitate the homing of tumor cells. After adhering to the plate coated with the condition medium from bone stromal cells for 2 h, 97 genes were significantly up-regulated. Analysis using Ontoexpress software indicated these genes were associated with protein and amino acid biosynthesis. After adhering for 24 h, 145 genes were significantly up-regulated, these are associated with signal transduction, cell adhesion and regulation of transcription.

Microarray showed a total of 31 genes that were consistently up-regulated in H460 cells (Table I). Some of these genes, including ADAMTS1, BIRC3, were confirmed by real-time PCR. Ontoexpress showed that the main function of the 31 genes were associated with signal transduction pathways (5 genes) and adhesion molecules (5 genes). Our study showed adhesion of H460 cells to bone stromal cells can activate some signal transduction pathways and alter the expression of adhesion associated factors, and thus favoring

cells SPC-A1, neither BM nor BSC affected its apoptosis and cell cycle distribution (data not shown).

Bone stromal factors promote adhesion, invasion and expression of angiogenesis associated factors in lung cancer cells with high bone metastasis potential. We investigated whether different bone metastasis potential of cell lines was related to their adhesive capability to bone stromal cells. Through adhesion assay, we found H460 cells were more likely to adhere to the plates coated by supernatant from bone stromal cells than regular medium. In contrast, SPC-A1 cells showed equivalent adhesive capability to supernatant from bone stromal cells and regular medium (Fig. 4A).

Invasive capability was closely associated with bone metastasis potential. Differences between lung cancer cell lines were also tested by invasion assay. There were significantly more H460 cells, which invaded through Matrigel to BM compared with (P=0.001). In the low bone metastasis

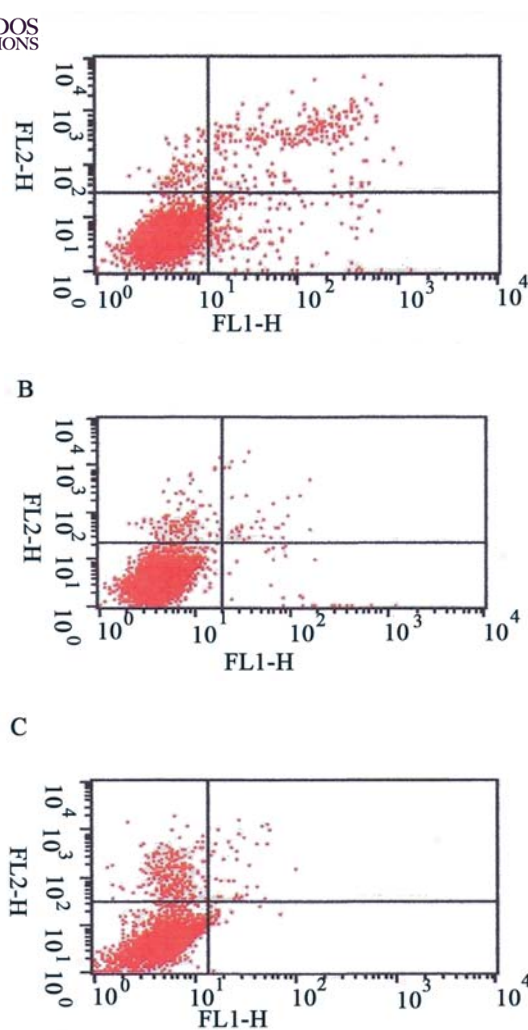


Figure 3. Double staining of Annexin V-FITC and propidium iodide (PI) to examine apoptosis in H460 cells (A) 24 h after the treatment of regular medium, (B) 24 h after the treatment of condition medium from human bone stromal cells, (C) 24 h after the treatment of supernatant of bone marrow. x-axis, annexin V staining and y-axis, PI staining; PI⁻/annexin V⁺ early apoptosis and PI⁺/annexin V⁺ late apoptosis. Compared with RM treatment, percentage of both early apoptosis (PI⁻/annexin V⁺, 1.08 ± 0.47 and 3.17 ± 0.06 , $P=0.0015$) and late apoptosis/secondary necrosis (PI⁺/annexin V⁺, 0.827 ± 0.215 and 2.11 ± 0.36 , $P=0.0061$) in BM group was significantly less. Similarly, after treatment of BSC, both early apoptosis (0.7 ± 0.31 and 3.17 ± 0.06 , $P=0.0068$) and late apoptosis/secondary necrosis (0.497 ± 0.08 and 2.11 ± 0.361 , $P=0.0001$) were also decreased.

the growth of cancer cells with high potential for bone metastasis.

Discussion

Bone metastasis occurs in about 20% of patients and causes pain and detrimental complications including spinal cord compression and pathologic fracture (18,19). Consensus recommendations of the second Cambridge conference in 2008 (20) indicated that one of the priorities that needs further study and should be a major focus of research within the next five years is, modulation of the effects of bone cells on other cell types and their interactions with the bone microenvironment. The purpose of this current study is to

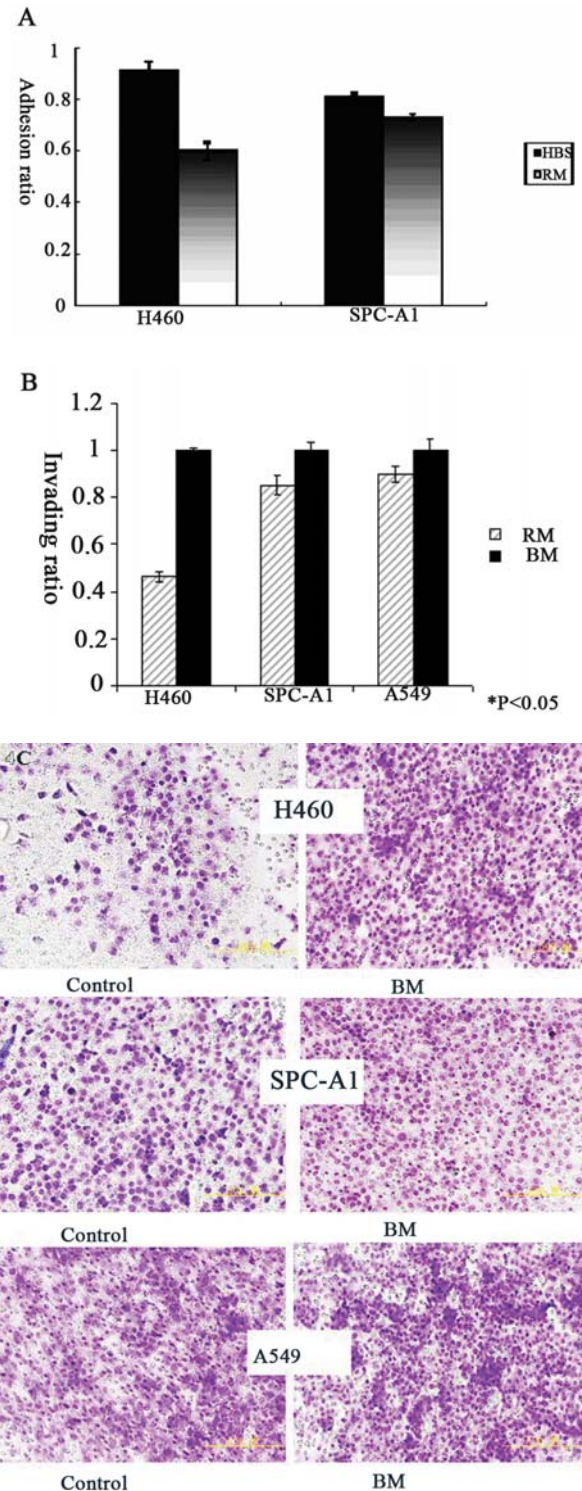


Figure 4. (A) Different bone metastasis potential of cell lines was related to their adhesive capability to bone stromal cells. The plates were coated by either supernatant from bone stromal cells or regular medium. The adhesion ratio was defined as remaining cells after adhesion for 1 h compared by total cell number. HBS, human bone stromal cell. (B) Difference among lung cancer cell lines shown by invasion assay. Invading ratio was defined as invading cells in control group divided by that in BM group. Assays were conducted in triplicates, and mean/SD is presented. * $P<0.05$. (C) Cell culture inserts were fixed with 3.7% paraformaldehyde/PBS for 15 min and were then stained using a Diff-Quick stain. Cells in 3 representative $\times 200$ microscopic fields were counted.

contribute to a better understanding of the interaction of tumor and bone microenvironment.

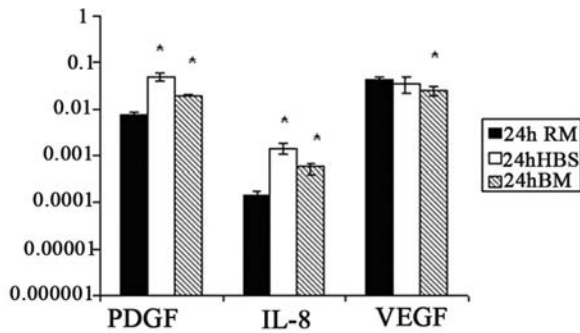


Figure 5. Real-time PCR was used to analyze the regulation of angiogenesis related factors for H460 after 24 h different treatments. Experiment was done in triplicate with SYBRA Green Master Mix. ABI 7900 sequence detector and SDS 2.0 software were used for PCR reaction and data analysis. x-axis, different angiogenesis related factors and y-axis, the relative values normalized to β -actin. * $P < 0.05$.

BMDC-tumor fusion has been detected in numerous animal models and recently in human cancer (11). Although a supporting role of bone stromal factors on metastatic breast or prostate cancer cells have been repeatedly proposed, it is largely unknown what the mechanism of bone tropism is for lung cancer. In our study, we found bone stromal factors favored the growth of cancer cells with bone tropism by increasing proliferation, reducing apoptosis, promoting angiogenesis, invading capability, but not affecting cell cycle. Bone stromal cells secrete some growth factors which promote the proliferation of high bone metastasis potential cells. Our hypothesis is supported by the finding that flt-1 signaling in bone marrow derived cells was required for its activity on the growth of glioma (21).

Zometa has been proved to exert multiple biological effects, including inhibition of proliferation, cell adhesion

Table I. Up-regulated genes of H460 after adhering to the supernatants of bone stromal cells (increased >2 -fold, $P < 0.05$).

Gene title	Public ID	Fold change 2 h/0 h	Fold change 24 h/2 h	t-test
CTP synthase	NM_001905	2.15	1.70	0.0002
Matrix metalloproteinase 7	NM_002423	2.17	1.45	0.0002
HSD3B1	NM_000862	5.38	1.24	0.0106
INPP4A	NM_004027	2.15	1.33	0.0464
Integrin β 3	NM_000212	4.60	1.18	0.0374
FGF binding protein 1	NM_005130	2.08	1.03	0.0002
Forkhead box J1	NM_001454	2.18	1.16	0.0136
Adrenergic, β -2-, receptor, surface	NM_000024	2.03	1.22	0.0064
Calcium channel γ subunit 1 (CACNG1)	NM_000727	2.32	1.47	0.0042
Jagged 1	U73936	3.31	4.94	0.0063
Tissue factor pathway inhibitor 2	L27624	2.08	2.32	0.0033
Phosphatidic acid phosphatase type 2B	AB000889	2.03	1.11	0.0294
Adlcan	AF245505	3.59	2.12	0.0194
Baculoviral IAP repeat-containing 3	U37546	2.07	3.36	0.0249
Flavoprotein oxidoreductase MICAL2	BE965029	2.31	1.39	0.0055
TNF receptor superfamily, member 21	BE568134	3.08	2.40	0.0021
HMT1 hnRNP methyltransferase-like 1	AL050065	4.52	1.33	0.0309
Jagged 1 (Alagille syndrome)	U77914	2.57	4.78	0.0410
Laminin α 2	AK026829	4.82	1.79	0.0244
PHLDA1	NM_007350	3.03	3.25	0.0335
TNF receptor superfamily, member 12A	NM_016639	2.06	1.90	0.0190
Chromosome 12 open reading frame 5	NM_020375	2.85	1.12	0.0004
Lipase, endothelial	NM_006033	3.55	1.13	0.0121
Sphingosine kinase 1	NM_021972	2.13	2.41	0.0008
Aldehyde dehydrogenase 8 family member A1	NM_022568	6.51	1.32	0.0317
Heat shock 70 kDa protein 12A	NM_025015	2.02	2.67	0.0310
Syntaxin binding protein 6 (amisyn)	NM_014178	4.96	1.30	0.0175
ADAMTS-1	AK023795	2.06	1.97	0.0010
SCAN domain containing 2	AF244812	3.07	1.48	0.0301
Inter- α (globulin) inhibitor H4	D38535	3.01	1.22	0.0472
Adenosylmethionine decarboxylase 1	M21154	2.02	1.06	0.0007



SPANDIDOS PUBLICATIONS x invasion of tumor cells, and increasing apoptosis.

anism of its effect is to inhibit farnesyl diphosphate synthase, thus blocking the prenylation of small signaling proteins essential for cell function and survival. In our study, we found Zometa inhibited the proliferation-promoting activity of BM. We are going to use cytokine array to investigate its molecular pathway.

The pathways of metastasis have been under intense scientific scrutiny and much is now known about the steps involved. However, the actual genes involved in bone metastasis are not fully understood. Integrin anchors on ECM and may secondarily trigger the downstream signaling. Its role in mediating bone metastasis has been repeatedly investigated (22,23). Prostate cancer cells selected *in vitro* for collagen I binding (LNCaPcol) are highly motile and acquired the capacity to grow within the bone compared to non-tumorigenic LNCaP parental cells. The expression of integrin $\alpha 2\beta 1$, RhoC guanosine triphosphatase was increased in high bone metastasis, collagen-binding LNCaPcol and C4-2B cells, compared to parental LNCaP cells (24,25). Integrin $\alpha \nu \beta 3$ was associated with breast cancer bone metastasis, and its inhibition by S247 can reduce bone metastasis (26-28). Our study showed adhesion of H460 cells to bone stromal cells can activate some signal transduction pathways, and alter the expression of adhesion associated factors, therefore favoring the growth of cancer cells with high bone metastasis potential. One of the increased genes, integrin $\beta 3$, is a member of integrin family. Lung cancer variably expressed integrin $\alpha \nu \beta 3$ and there was no data on its expression in metastatic lung cancer in the bone (29). So the role of $\beta 3$ integrin in bone metastasis of lung cancer needs to be further verified.

Another interesting gene is ADAMTS-1 (a disintegrin and metalloprotease with thrombospondin type I motifs), one of the 19 members of a family of genes for metalloproteinase. It is located at chromosome 16C3-5 region in mice and at chromosome 21 in human. It comprises of 6 regions: pro-metalloproteinase, metalloproteinase, disintegrin-like region, TSP-I thrombospondin, homologous domain containing TSP type I motif, spacer region and TSPCOOH-terminal TSP submotifs (30). Liu *et al* reported the role of ADAMTS-1 in lung metastasis from SCID models injected with breast and lung cancer cell lines (31). Rock *et al* compared the ADAMTS-1 expression of lung cancer with surrounding normal tissue and found higher expression in tumor cells (32). Parathyroid hormone (PTH) was able to up-regulate ADAMTS-1 expression and increase osteoblastic activities via the type I collagen processing (33), indicating the active role of ADAMTS-1 in bone microenvironment. We are presently verifying if ADAMTS-1 could be associated with bone metastasis.

Acknowledgements

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