Comparative analysis of gene regulatory networks of highly metastatic breast cancer cells established by orthotopic transplantation and intra-circulation injection

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Abstract. Metastasis signature genes in breast cancer have been studied comparing transcriptomic profiles of highly metastatic cancer cell lines established by intra-circulation injection with that of their parental cell line. However, this method is not suitable to analyze the initial steps of metastasis including invasion into local tissues and the circulatory system. To characterize the molecular mechanisms of early metastasis, we established highly metastatic MDA-MB-231 cell lines that metastasized to lung by the two animal transplantation models: the orthotopic transplantation method, which mimics all steps of metastasis, or intra-circulation injection method. We then performed data-mining and network analysis of gene expression profiles of metastatic cell lines established by each transplantation method. Transcriptome analysis of seven metastatic cell lines revealed novel lung metastasis signature genes, including known metastasis promoting genes and signature genes. In the OXconc (orthotopic xenograft concentration) signature, 'chemotaxis' and 'cell adhesion' terms were enriched. In the TVIconc (tail vein injection concentration) signature, 'antigen recognition' and 'cell adhesion' were enriched. Furthermore, network analysis of the metastasis signature genes highlighted hub genes in the gene regulatory network. Our findings show that expression profiles of highly metastatic cell lines were different between the orthotopic transplantation and intra-circulation injection method. It also indicates that some metastatic signature genes have been missed in previous studies. Characterization of metastasis genes using the orthotopic transplantation method will be helpful in understanding the multi-step mechanisms of metastasis. Signature genes in OXconc may have the potential to become prognostic markers.

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Introduction

Most metastatic cancer cannot be cured with current treatment. Thus, understanding the biological mechanism of metastasis is critical for development of effective clinical treatment. The invasion-metastasis cascade consists of: i) invasion into local tissue and induction of angiogenesis/lymphanginogenesis; ii) intravasation; iii) survival in the circulation; iv) arrest at a distant organ site; v) extravasation; vi) micrometastasis formation; and vii) metastatic colonization. Metastatic process is believed to be driven by metastasis genes that are involved in at least one of these steps (1). Metastasis signature genes and metastatic organ tropism have been analyzed in clinical studies by transcriptomic analysis using DNA microarrays (2-5). Previous studies showed that some metastasis signature genes are involved in specific steps of the invasion-metastasis cascade (6-8). In some cases of metastasis genes, co-activation with other genes was required for metastasis in distant organs (6).

The human breast carcinoma cell line MDA-MB-231 is an ideal tool for metastatic study because it exhibits metastatic activities to distant organs by the xenograft model using immunodeficient mice. For example, the intra-cardiac injection method produced metastases to lung, bone and brain, whereas, the tail vein injection (TVI) method metastasizes to lung because injected cells tend to be trapped in the lung (5). Highly metastatic cells can be established by repetition of recovery of cancer cells from metastatic lesions followed by their injection into blood vessels. In the present study, we refer to this procedure as 'concentration of metastatic activities'. Organ specific-metastatic signature genes were previously identified by transcriptomic analysis in highly metastatic cancer cells (3,5,9).

In the orthotopic xenograft (OX) model, tumor cells are injected into organs from which they are derived. In the case of breast cancer, the tumor cells are transplanted into the fat pad. Transplanted breast cancer cells form primary tumors and metastases to lung and bone, as well as liver and lymph node (3,10,11). Therefore, the OX model mimics the overall steps of the invasion-metastasis cascade in contrast to the TVI method, which only mimics the steps after extravasation.

In the present study we generated highly metastatic cell lines using the OX or TVI method and compared both gene expression profiles to determine the characteristics of early steps of metastasis, including local invasion and leaking out into vessels.

Materials and methods

Cell culture. MDA-MB-231 cells (ATCC, Manassas, VA, USA) were cultured in RPMI-1640 (Wako Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 μg/ml streptomycin (Meiji Seika Pharma Co., Ltd., Tokyo, Japan) and 100 U/ml penicillin (Meiji-Seika Pharma) at 37˚C with 5% CO_2. Plat-E and 293T cells were cultured in DMEM (Wako Pure Chemical Industries) supplemented with 10% heat-inactivated FBS, 100 μg/ml streptomycin and 100 U/ml penicillin at 37˚C with 5% CO_2. MDA-MB-231-EcoR cells were generated by infection of MDA-MB-231 cells with recombinant lentivirus (pLenti6-P_{Ubc}-mSlc7a1-Hyg^R; Addgene, Cambridge, MA, USA) and selection by 800 μg/ml hygromycin (Wako Pure Chemical Industries). MDA-MB-231-EcoR-luc2 cells were generated by retroviral infection (pMXd3-P_{EF1}-luc2-IRES-Bla^R) and selection by 2.0 μg/ml blastcidin (Invitrogen/Thermo Fisher Scientific, Waltham, MA, USA). Plat-E cells for retroviral packaging were kindly provided by T. Kitamura (Institute of Medical Science, The University of Tokyo).

Viral infection. Lentiviral packaging was performed by co-transfection of pLenti6-P_{Ubc}-mSlc7a1-Hyg^R (Addgene), HIV-based vectors including a cDNA expression cassette and the pPACK packaging plasmids (System Biosciences, Palo Alto, CA, USA). Plasmid DNAs were transfected into 293T cells by the calcium phosphate transfection method. The culture supernatant was harvested the following day and stored as virus stock. MDA-MB-231 cells were plated at a density of $2.5x10^5$ cells/well in 12-well plates, followed by infection with 5-fold diluted lentiviral stock solution. After 18 h, virus solution was replaced with fresh culture media or passaged to new dishes. Plat-E cells were transfected with pMXd3-P_{EF1}-luc2-IRES-Bla^R by calcium phosphate transfection method. MDA-MB-231-EcoR cells ($2.5x10^5$ cells) plated in 12-well plates were infected with 5-fold retroviral stock solution for 24 h. After infection, the mixture was replaced with culture media or cells were passaged to a new dish.

Mouse xenograft metastasis model and bioluminescence imaging. NOD.CB-17-$Prkdc^{scid}$/J mice (NOD-SCID; Charles River Laboratories Japan, Inc., Kanagawa, Japan) were used in xenograft models. For the TVI model, $5.0x10^5$ cancer cells suspended in 100 μl D-PBS(-) (Wako Pure Chemical Industries) were injected using a 27-gauge needle into the tail vein. For the orthotopic xenograft model, $1.0x10^6$ cancer cells in 10 μl D-PBS(-) were implanted orthotopically using a 28-gauge needle into the fourth fat pads. Eight weeks after the injection, primary tumors were removed under anesthetized conditions with 2.5% isoflurane (Wako Pure Chemical Industries). After the primary tumors were removed, mice were periodically monitored for metastasis formation using an *in vivo* imaging system for 2-4 weeks.

Mice were intraperitoneally injected with 200 μl D-Luciferin (15 mg/ml; Gold Biotechnology, Inc., St. Louis, MO, USA) and 20-25 min later, luminescence and X-ray imaging were taken under anesthesia using an *in vivo* imaging system (IVIS Lumina XR3; Perkin-Elmer, Waltham, MA, USA). Animal experiments in this study were conducted under the approval of the Animal Committee of Waseda University.

Tumor cell isolation. Lung metastases removed from xenograft model mice were finely cut by scissors for dissection in 5 ml of culture medium in a 6-cm dish and incubated at 37˚C with 5% CO_2. After 2-3 days, cells invaded from sliced organs were sufficiently grown at the bottom of the dish and tumor-derived cells were selected by 2-3 passages in the presence of 2.0 μg/ml blastcidin to remove primary lung cells.

DNA microarray analysis. Total RNA extraction, RT-PCR and DNA microarray analysis were performed as described (12).

Statistical data analysis and network analysis. Statistical analyses of microarray data were performed using software packages obtained from Bioconductor (http://www.bioconductor.org/) and CRAN (https://cran.r-project.org/) with the R statistical programming language. Heatmap analyses were executed by 'ggplot2' and 'gplots' packages and graphical outputs in the form of Venn diagram were produced by 'GeneVenn' (http://genevenn.sourceforge.net/). DAVID (version 6.7; https://david.ncifcrf.gov/) (13) was used for GO (Gene Ontology) enrichment analysis. Gene regulatory network analyses were performed using 'igraph' package. Network visualization and topological analysis was generated by Cytoscape ver.3.2.0. (http://www.cytoscape.org/) (14-16).

Results

Establishment of highly metastatic cell lines and gene expression analysis. MDA-MB-231-luc2 cells were transplanted into NOD-SCID mice using either OX or TVI methods. Lung metastasis (LM) cells were recovered and established from lung metastatic lesions. LM cells were then re-injected into NOD-SCID mice using the same method to concentrate cells with higher metastatic activity (Fig. 1A). Established cell lines exhibited higher metastatic activities to lung than the parental cell line (data not shown).

We next analyzed gene expression profiles of LM cell lines using cDNA microarrays and calculated the fold change (FC) ratio of gene expression for each concentrated LM cell line vs. parental cell line. We converted the FC ratios to z-scores and selected genes that showed z-score >2.0 as metastasis signature genes. As a result, 189 genes were upregulated and 286 genes downregulated in the signature of OXconc (OX concentration), while 223 genes were upregulated and 344 genes

Table I. Top 10 genes regulated in OXconc and TVIconc.

ID	Gene symbol	OXconc z-socre ave.	TVIconc z-score ave.
Upregulated in OXconc			
NM_002421	MMP1	10.85713119	10.38631079
NM_032744	C6ORF105	7.353197074	0.519698021
X97966	CAPS	7.102005496	6.482245813
NM_021939	FKBP10	7.01711925	5.597510346
NM_000825	GNRH1	5.906347541	6.171646277
NM_005424	TIE1	5.468619563	0.95133873
NM_144650	ADHFE1	5.464548106	4.515672606
X84003	TAF13	5.40888736	4.538398605
NM_000079	CHRNA1	5.184489038	5.029493896
NM_003739	AKR1C3	5.141155382	2.306068
NM_000584	IL8	5.064377448	1.340679004
Upregulated in TVIconc			
NM_002421	MMP1	10.85713119	10.38631079
NM_021912	GABRB3	8.488617576	7.398710432
AF192259	LOC100294275	-1.154418998	6.716756187
X97966	CAPS	7.102005496	6.482245813
NM_000825	GNRH1	5.906347541	6.171646277
NM_001849	COL6A2	3.041595714	6.055789712
NM_021939	FKBP10	7.01711925	5.597510346
BC002831	MGC4294	5.049699232	5.25073357
NM_130467	PAGE5	1.474724337	5.094903228
NM_000079	CHRNA1	5.184489038	5.029493896
X84003	TAF13	5.40888736	4.538398605
Downregulated in OXconc			
NM_000782	CYP24A1	-7.08697021	-3.3821009
NM_004105	EFEMP1	-5.823406185	-1.667764645
NM_002832	PTPN7	-5.635932207	-5.722217371
NM_004852	ONECUT2	-5.162895035	-4.953350067
NM_000820	GAS6	-4.823851904	-4.770537217
NM_000093	COL5A1	-4.564581733	-0.116120033
NM_000541	SAG	-4.499544857	-4.04299429
NM_006663	PPP1R13L	-4.499225213	-4.060105507
NM_024682	TBC1D17	-4.46688297	-4.275238798
NM_015234	GPR116	-4.431324672	-1.856370815
Downregulated in TVIconc			
NM_002832	PTPN7	-5.635932207	-5.722217371
NM_145664	SPANXB2	0.820012286	-5.238287217
AK055209	ANKRD17	-4.028084478	-5.137378579
NM_004852	ONECUT2	-5.162895035	-4.953350067
NM_000820	GAS6	-4.823851904	-4.770537217
NM_198449	EMB	0.696756501	-4.296909426
NM_024682	TBC1D17	-4.46688297	-4.275238798
NM_006663	PPP1R13L	-4.499225213	-4.060105507
NM_000541	SAG	-4.499544857	-4.04299429
AK002183	MIER3	-2.513486213	-3.960036141

downregulated in the signature of TVIconc (TVI concentration). Analysis of these genes by hierarchical clustering and Venn plot demonstrated that gene expression profiles were different between OXconc and TVIconc, even though both LM cell lines are derived from an identical cell line (Fig. 1B and C and Table I).

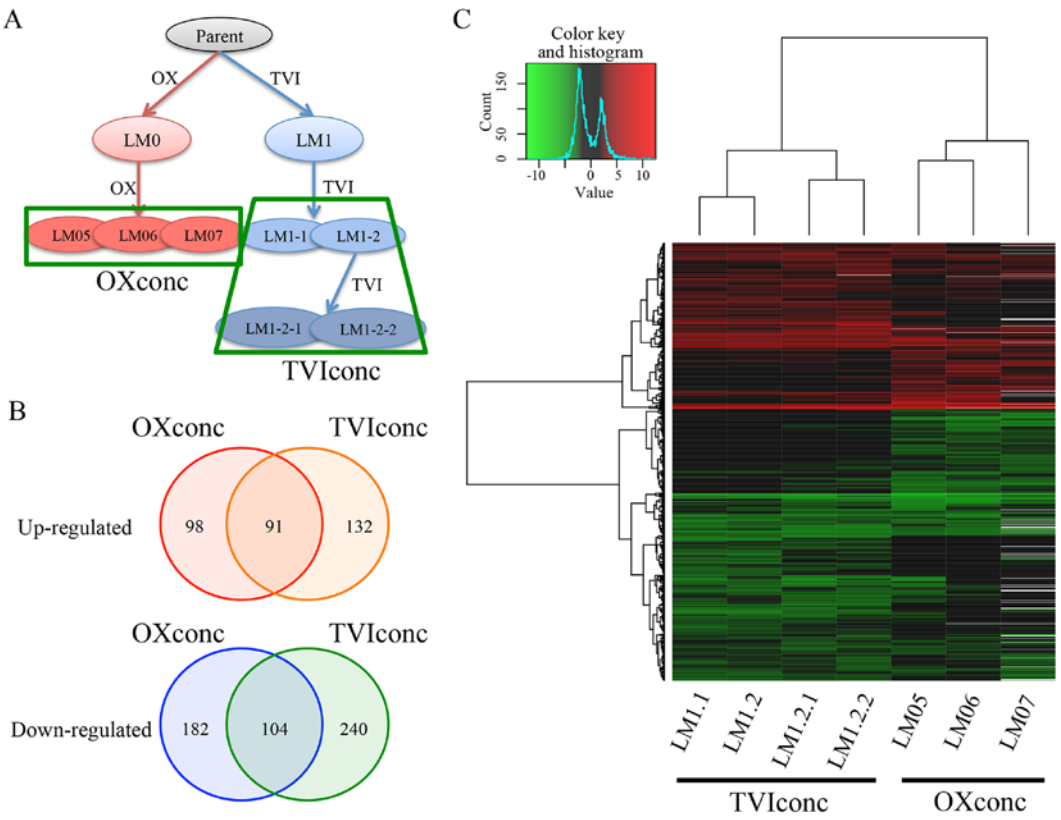

Figure 1. (A) Schematic of metastatic concentration strategy. Metastatic activities to lung were concentrated using OX or TVI. The green box indicates the stage of transcriptome analysis. (B) Venn plot of metastasis signature genes using GeneVenn. (C) Hierarchical clustering and heatmap using group average method.

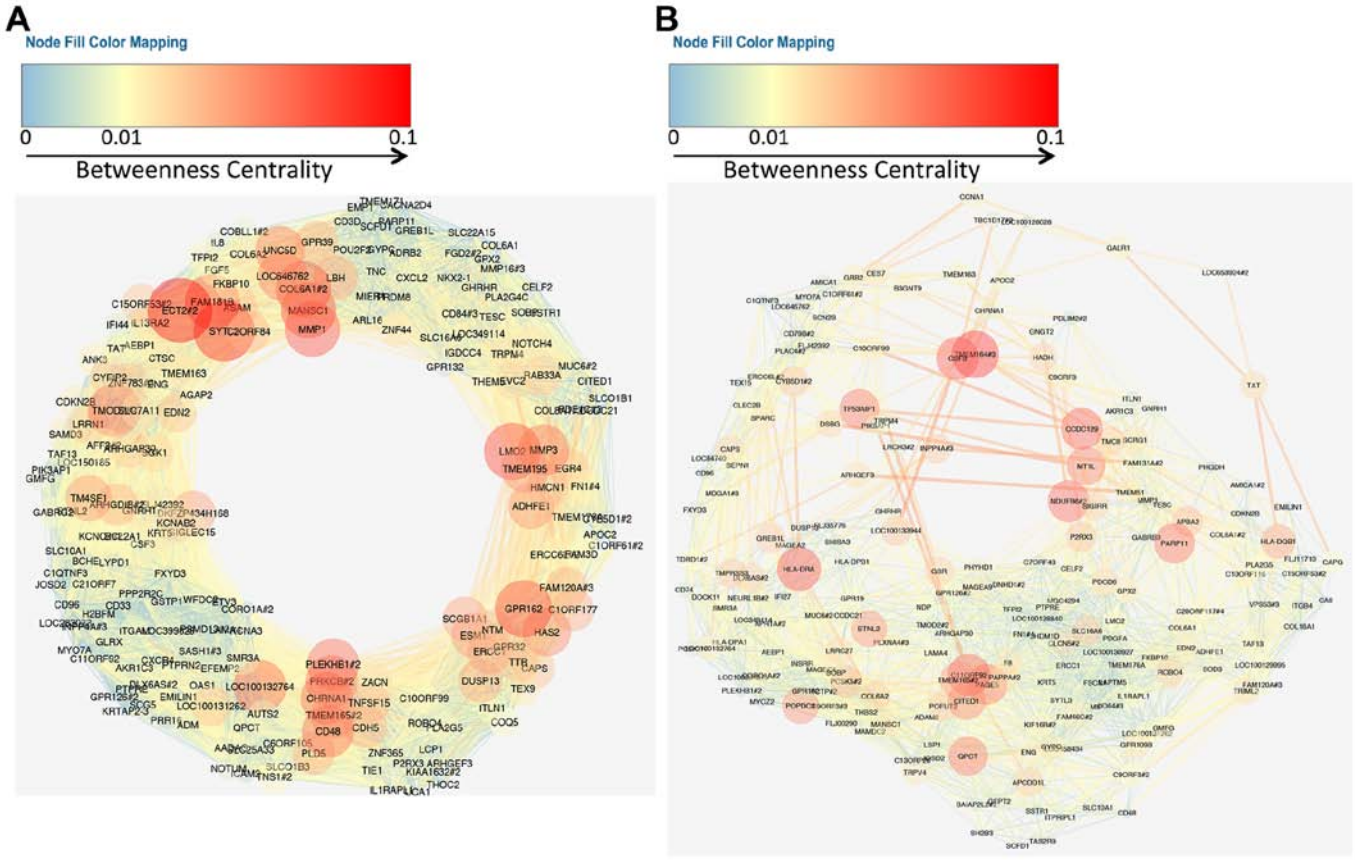

Figure 2. Upregulated gene regulatory network. Betweenness centralities are displayed in blue-red scale color in organic layout using Cytoscape 3.2.0. Red indicates high centrality and potential of network hub. (A) OXconc upregulated network. (B) TVIconc upregulated network.

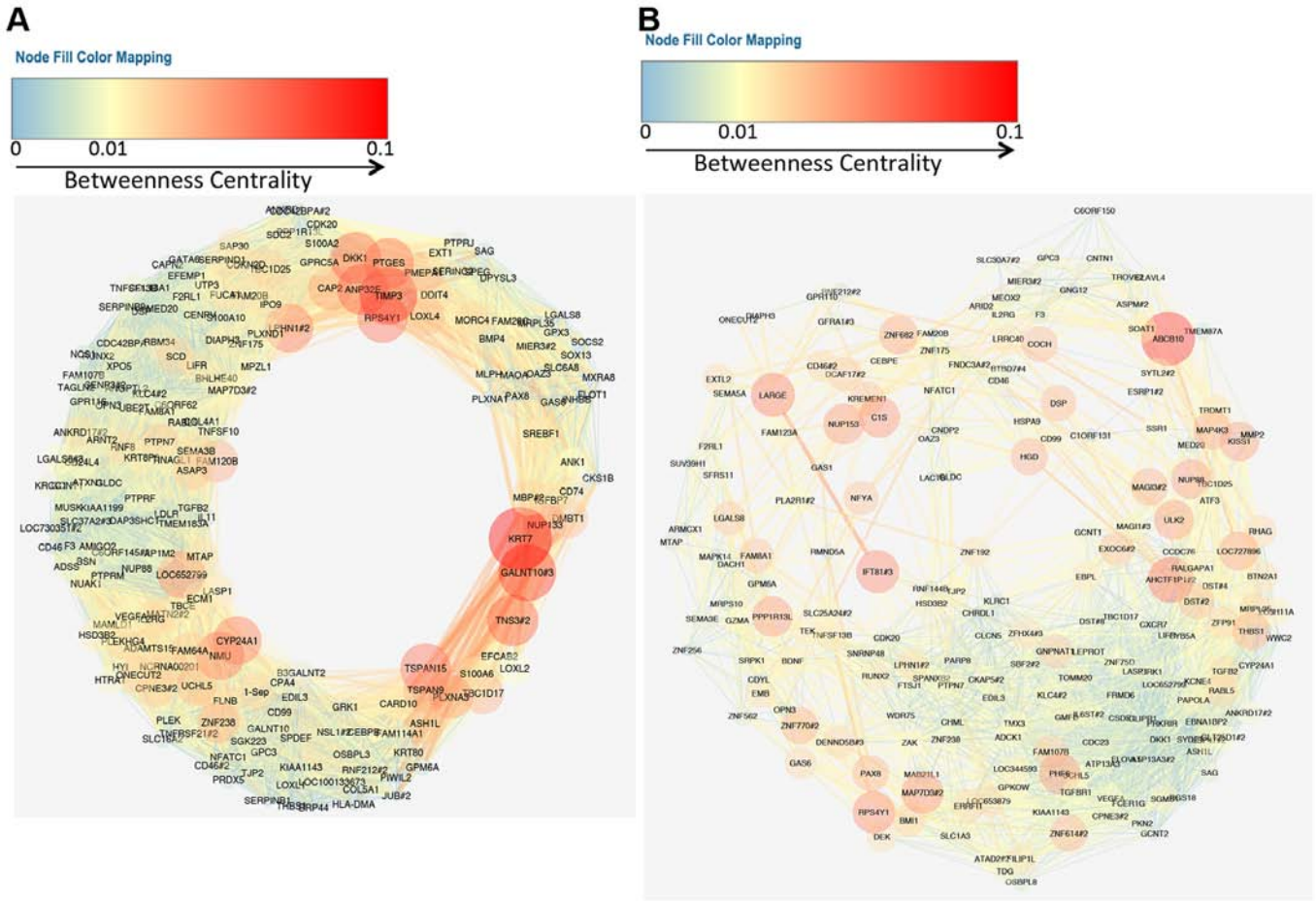

Figure 3. Downregulated gene regulatory network. Betweenness centralities are displayed in blue-red scale color in organic layout using Cytoscape 3.2.0. Red indicates high centrality and potential of network hub. (A) OXconc downregulated network. (B) TVIconc downregulated network.

GO analysis and gene regulatory network analysis. To evaluate the signature genes expressed in each metastatic cell line, we next examined enrichment terms of the biological process categories in GO by DAVID annotation tools (Table II). In the OXconc signature, genes associated with cell proliferation were repressed, while genes associated with cell adhesion and chemotaxis were upregulated. In the TVIconc signature, genes associated with antigen recognition were enriched in addition to those with cell adhesion.

For gene regulatory network analysis, we focused on correlations of gene expression patterns in each concentrated metastatic cell line. We calculated each gene-gene Pearson's correlation coefficient in each methodological metastatic cell line using gene expression data. We performed network analysis using Cytoscape for network visualization and topological analysis. To display the gene regulatory network, we focused on the top 200 genes of the metastasis signature genes. We selected gene-gene correlations in which the correlation coefficient value is >0.9 and displayed network with betweenness centrality using yFiles organic layout (Figs. 2 and 3). Intriguingly, both the OXconc gene regulatory network of upregulated genes and that of downregulated genes formed circle network topologies.

Among the common genes observed in the upregulated gene regulatory network, a well-known metastasis gene, matrix metalloproteinase 1 (MMP1), showed the highest z-score. We extracted the sub-network related to MMP1 from each network (Fig. 4A). The sub-network of MMP1 in the OXconc network had more edges and nodes than that in the TVIconc network. Sub-networks of protein tyrosine phosphatase non-receptor type 7 (PTPN7), which showed the lowest z-score in common downregulated genes, also showed the same feature (Fig. 4B).

To evaluate the significance of genes encoding transcription factors such as hub genes, we extracted genes categorized as 'GO:0003700~ transcription factor activity, sequence-specific DNA binding' in the molecular function (Table III). Contrary to expectations, betweenness centrality of transcriptional factors had low values in these gene regulatory networks (data not shown).

Discussion

Discovery of novel metastasis genes can help to understand the metastatic mechanisms and establishment of prognostic markers (17). Lung metastasis genes in breast carcinoma were previously identified by transcriptome analysis of metastatic cancer cells and patient metastases using DNA microarray and by gene knockdown experiments in highly metastatic cancer cells. However, the roles of some metastasis genes have not been analyzed in detail.

MMP1 was most highly expressed gene in OXconc and TVIconc networks. Previous research suggested that *MMP1*

Table II. Top 5 terms of biological process in GO.

Term	P-value	Bonferroni	FDR
OXconc upregulated			
GO:0007155~cell adhesion	4.18E-04	0.421154729	0.681110881
GO:0022610~biological adhesion	4.25E-04	0.426351205	0.692306461
GO:0060326~cell chemotaxis	5.31E-04	0.500690093	0.864454327
GO:0007610~behavior	6.21E-04	0.555853784	1.009433266
GO:0008285~negative regulation of cell proliferation	7.64E-04	0.631767293	1.241108958
OXconc downregulated			
GO:0042127~regulation of cell proliferation	3.33E-05	0.061001348	0.056809783
GO:0008284~positive regulation of cell proliferation	4.58E-04	0.579599391	0.779301878
GO:0000902~cell morphogenesis	7.66E-04	0.765190332	1.299678793
GO:0007010~cytoskeleton organization	8.25E-04	0.790008941	1.399174442
GO:0010647~positive regulation of cell communication	0.001039447	0.860215882	1.760794114
TVIconc upregulated			
GO:0002504~antigen processing and presentation of peptide or polysaccharide antigen via MHC class II	1.94E-05	0.024628491	0.031592274
GO:0007155~cell adhesion	7.67E-04	0.627271392	1.242712326
GO:0022610~biological adhesion	7.79E-04	0.633111739	1.262473127
GO:0006690~icosanoid metabolic process	0.001316141	0.816398686	2.124806716
GO:0019882~antigen processing and presentation	0.001577084	0.868836337	2.541013583
TVIconc downregulated			
GO:0032103~positive regulation of response to external stimulus	4.58E-05	0.083059484	0.078184221
GO:0070120~ciliary neurotrophic factor-mediated signaling pathway	8.24E-05	0.144470972	0.140644741
GO:0051272~positive regulation of cell motion	1.78E-04	0.286274159	0.303742875
GO:0048584~positive regulation of response to stimulus	3.66E-04	0.500037902	0.623336012
GO:0030335~positive regulation of cell migration	4.70E-04	0.589711307	0.800366129

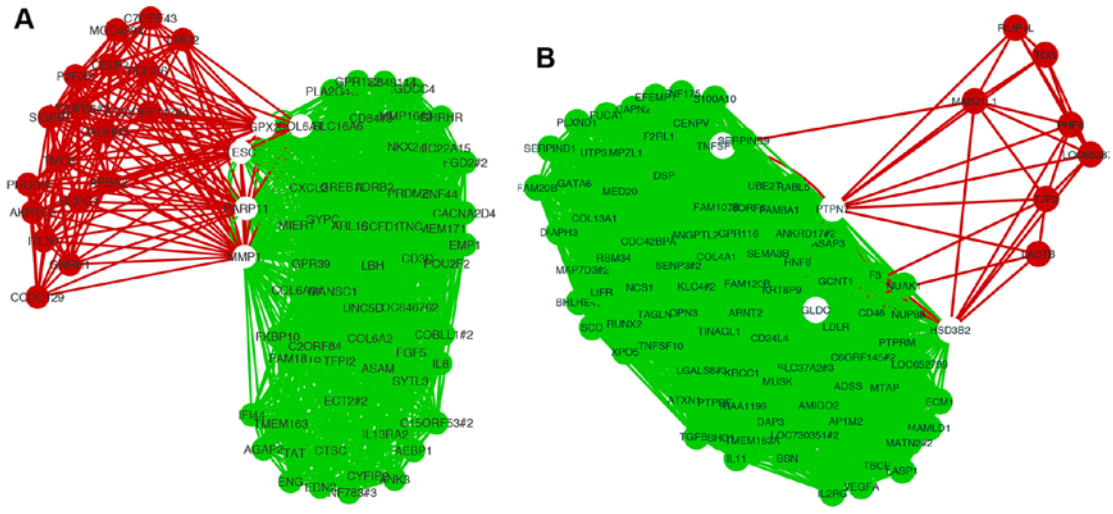

Figure 4. Subnetwork analyses of MMP1 and PTPN7. Green, OXconc; red; TVIconc. White node; common genes. (A) Differences of MMP1 subnetwork between OXconc upregulated network and TVIconc upregulated network using DyNet ver.0.9.1 in Cytoscape 3.2.0. (B) Differences of PTPN7 subnetwork between OXconc downregulated network and TVIconc downregulated network.

activity alone was not enough for metastasis to lung and *MMP1* requires other metastasis genes to promote metastases in the lung (6). In our studies, the OXconc network revealed a group of genes whose expressions highly correlated with *MMP1* expression. These genes may cooperate with *MMP1* to contribute to lung metastasis. The *CXCR4* (C-X-C chemokine receptor type 4) gene was expressed in only the OXconc signature. Expression of *CXCR4* in metastatic cancer cells

Table III. Transcriptional factors in gene regulatory network.

ID	Gene name
OXconc upregulated	
NM_001129	AEBP1
NM_004143	CITED1
NM_003317	NKX2-1
NM_002698	POU2F2
X84003	TAF13
NM_001965	EGR4
NM_005240	ETV3
OXconc downregulated	
NM_001805	CEBPE
NM_005257	GATA6
NM_012391	SPDEF
NM_005686	SOX13
NM_014862	ARNT2
NM_003670	BHLHE40
NM_006162	NFATC1
NM_004852	ONECUT2
NM_013951	PAX8
NM_004176	SREBF1
NM_007147	ZNF175
TVIconc upregulated	
NM_001129	AEBP1
NM_004143	CITED1
X84003	TAF13
TVIconc downregulated	
AK097133	ATAD2
NM_001805	CEBPE
NM_004024	ATF3
NM_080759	DACH1
NM_005924	MEOX2
NM_006162	NFATC1
NM_002505	NFYA
NM_021705	NFYA
NM_004852	ONECUT2
NM_013951	PAX8
NM_004348	RUNX2
AK095274	ZFHX4
NM_007147	ZNF175
NM_006298	ZNF192
NM_006352	ZNF238
NM_005773	ZNF256
NM_007131	ZNF75D

highly expressing *CXCL12* (C-X-C chemokine motif ligand 12) enhanced metastasis to distant organs, such as lung, liver and bone marrow. Thus, *CXCR4* in breast cancer is believed to decide the metastatic organ tropism (18,19). In our studies, expression of *CXCR4* may not be necessary to form a pre-metastatic niche and metastatic colonization. Instead, *CXCR4* may especially regulate the early metastatic phase, namely invasion from primary tumor. Expression of interleukin 8 (*IL8*) promotes angiogenesis and metastasis to distant organs in an orthotopic xenograft model (20). Consistently, upregulation of *IL8* was observed in the OXconc signature. However, in this and others studies, *IL8* was not found in metastasis signature genes by the intra-circulation model using MDA-MB-231 cells (3,5). Our findings suggest that some important metastasis signature genes may have been missed in the previous analysis using intracirculation injection methods. Thus, establishing highly metastatic cell lines by different methods will lead to novel insights into invasion-metastasis mechanisms and help identify novel metastatic genes.

Shedding of tumor cells into the circulation occurs at the early stage of metastasis. Circulating tumor cells (CTCs) that survive in the circulation and spread in the whole body are likely necessary for distant organ metastasis (21). In fact, the number of CTCs in breast cancer patients is associated with prognosis (22). In GO analysis, the terms 'cell adhesion' (23) and 'cell chemotaxis' were enriched in OXconc, while 'chemotaxis' was not enriched in TVIconc. CTCs require the abilities of chemotaxis and cell adhesion with vascular endothelial cells for homing in distant organs (22). Thus, our results confirm the role of genes involved in chemotaxis and cell adhesion for metastasis to distant organs.

A gene regulatory network analysis method using gene expression data is most commonly used for comparative genomic data. Analysis to examine the relationship between regulatory network and phenotype is important to discover novel markers and understand disease mechanisms (24,25). In previous studies, interference of hub genes in networks showed that a hub gene and its neighbor genes regulate the biological phenotype. For example, the hub genes *MYOG* (myogenin) and *CTNNA2* [catenin (cadherin-associated protein) alpha 2] in a transcriptional network of muscle satellite cells promote myoblast differentiation (26). The metastasis gene *BACH1* (BTB and CNC homology 1) was found by transcriptional network analysis of cancer cell lines using DNA microarrays (27). Although these genes do not have transcriptional activity, they contributed to the biological phenotype as hub genes of the network. We used betweenness centrality to extract hub genes. In our network, most genes of core nodes do not encode transcription factors. Thus, the molecular links and their biological importance between hub genes and neighbor genes remain to be revealed.

In network analysis, the focus on similarities and differences of correlation is an important strategy (28). Common regulated genes in OXconc and TVIconc may be of importance in the invasion-metastasis cascade and could be cancer prognosis markers. Among the common genes observed in upregulated gene regulatory networks, *CITED1* (Cbp/p300-interacting transactivator) may also be a metastasis marker of breast cancer, because it is a known marker of lymph node metastasis in colorectal carcinoma (29). Differential network analysis is now becoming prevalent as a tool to more comprehensively interrogate biological systems in a variety of organisms (30).

Comparative analysis of the sub-network of common genes in OXconc and TVIconc showed that the OXconc sub-network had a closer relationship than the TVIconc. Previous research

in lung cancer showed that the *MMP1-MMP3-MMP12* gene cluster on chromosome 11q22 plays important roles in prognosis and metastasis (31). In our studies, the sub-network of *MMP1* revealed *MMP1-MMP3* correlation in the OXconc network. This finding suggests that some relationships of metastasis genes have been missed in previous analyses using intra-circulation injection methods.

To display gene regulatory networks, we used the yFiles organic layout algorithm on the basis of the force-directed paradigm. These structurally different regions of a organic network can be identified by looking at the produced drawing (32). Our findings demonstrated that the OXconc networks showed a circle structure. Circle topologies suggest that genes involved in metastasis may have complementary relationships at the gene community level and have the potential for strong robustness.

In the present study, we performed transcriptional profiling of highly metastatic cell lines established by two different methods: the orthotopic transplantation method and intra-circulation method. Although these metastatic lines are derived from an identical cell line, gene expression profiles and gene regulatory networks were different in OXconc and TVIconc signatures, suggesting that those cell lines were activated at different steps of the invasion-metastasis cascade. Our findings will contribute to the understanding of the detailed metastatic mechanisms and development of therapeutics or diagnostic tools.

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