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Identification of a nuclear protein, LRRC42, involved in lung carcinogenesis

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DOI: 10.3892/ijo_xxxxxxxx

Abstract. On the basis of the gene expression profiles of 120 lung cancer cases using a cDNA microarray containing 27,648 genes or expressed sequence tags (ESTs), we identified *LRRC42* (Leucine-rich repeat containing 42) to be significantly upregulated in the majority of lung cancers. Northern blot analysis demonstrated that *LRRC42* was expressed only in testis among normal tissues examined. Knockdown of LRRC42 expression by siRNA against LRRC42 significantly suppressed the growth of lung cancer cells. On the other hand, stable induction of LRRC42 expression significantly promoted cell growth. LRRC42, which was found to localize in the nucleus of mammalian cells, is likely to interact with and stabilize GATAD2B (GATA zinc finger domain-containing 2B) and MBD3 (Methyl-CpG-binding domain protein 3) proteins that could contribute to lung cancer cell proliferation partly through the regulation of $p21^{Waf1/Cip1}$. Our findings suggest that LRRC42 overexpression as well as its interaction with LRRC42-GATAD2B might play essential roles in lung carcinogenesis, and be a promising molecular target for lung cancer therapy.

Introduction

Lung cancer is the leading cause of cancer-related death worldwide. Despite some advances in cancer diagnostics and recent improvements in its treatment, the overall 5-year survival rate is still only 15% (1). Several oncogenic alterations, such as *KRAS* and *EGFR* mutations, and *EML4-ALK* fusion genes as well as inactivation of tumor suppressor gene of *TP53* in lung cancer have been reported, however the precise molecular mechanisms of pulmonary carcinogenesis are still far from fully understood (2). Although several molecular targeted-drugs such as gefitinib, bevacizumab and crizotinib have been approved for lung cancer treatment, the portion of patients who are able to have the benefit of these drugs is still limited and several serious adverse reactions such as interstitial pneumonia by gefitinib and hemorrhage by bevacizumab have been reported (3,4). Hence, the development of molecular targeted agents providing better clinical benefits with less adverse events are eagerly required.

Systematic analysis of expression levels of thousands of genes using a cDNA microarray is an effective technique to identify molecules involved in carcinogenic pathways (5); some of such genes or their gene products may be good molecular targets for the development of novel therapies and/or cancer biomarkers. To isolate potential molecular targets for diagnosis, treatment, and/or prevention of lung carcinomas, we performed a genome-wide analysis of gene expression profiles of tumor tissues from 120 lung cancer cases by means of cDNA microarray consisting of 27,648 genes or expressed sequence tags (ESTs) (6-10). Among the transactivated genes, we identified *LRRC42* (Leucine-rich repeat containing 42) as a potential therapeutic target for lung cancer. LRRC42 protein contains two leucine-rich repeats (LRRs) which are widespread structural motifs comprising 20-30 amino acids with a characteristic repetitive sequence pattern rich in leucine residues. Leucine-rich repeat domains are built from tandems of two or more repeats and form curved solenoid structures that are particularly suitable for protein-protein interactions. LRR-containing proteins participate in many important biological processes, including plant and animal immunity, hormone-receptor interactions, cell adhesion, signal transduction, regulation of gene expression and apoptosis (11-14). However, the pathophysiological roles of LRRC42 in cancer cells have not been reported. Herein we report identification of LRRC42 as a potential therapeutic target and also provide evidence that LRRC42 could interact with GATAD2B (GATA zinc finger domain containing 2B) and MBD3 (Methyl-CpG-binding domain protein 3) proteins that are likely to play a significant role in human pulmonary carcinogenesis.

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Key words: oncogene, cancer-testis antigen, therapeutic target, lung cancer, novel molecular target

Materials and methods

Lung cancer cell lines and tissue samples. The human lung cancer cell lines used in this study were as follows: A549, NCI-H1373, NCI-H1781, SKMES-1, NCI-H520, NCI-H1703, NCI-H2170 and DMS114 were distributed from America Type Culture Collection (ATCC, Manassas, VA, USA). LC319 was kindly provided from Aichi Cancer Center (Aichi, Japan). PC14 was obtained from RIKEN BioRsource Center (Ibaraki, Japan). LU61 and LX1 were obtained from Central Institute for Experimental Animals (Kanagawa, Japan). DMS273 was obtained from European Collection of Animal Cell Cultures (ECACC, Salisbury, UK). SBC-3 and SBC-5 were obtained from Japanese Collection of Research Bioresources (JCRB, Osaka, Japan). All cells were grown in monolayers in appropriate medium supplemented with 10% FCS and were maintained at 37°C in atmospheres of humidified air with 5% CO_2. Human small airway epithelial cells (SAEC) were grown in optimized medium purchased from Cambrex Bio Science, Inc. (Walkersville, MD, USA). Primary lung cancer tissue samples were obtained with informed consent as previously described (6,10). This study was approved by individual institutional ethical committees.

Quantitative real-time PCR. Total RNA was extracted from cultured cells using the QIAshredder (Qiagen, Valencia, CA, USA) and RNeasy® Plus mini kit (Qiagen) according to the manufacturer's protocol. Extracted RNAs were reverse transcribed using oligo (dT) primer (Life Technologies, Carlsbad, CA, USA) and SuperScript III (Life Technologies). Quantitative real-time PCR was conducted with the SYBR Green I Master kit on a LightCycler 480 (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's recommendations. Each experiment was done in duplicate. *GAPDH* was used for normalization of expression levels. For quantitative RT-PCR reactions, specific primers for all human *LRRC42, GATAD2B, MBD3, p21$^{Waf1/Cip1}$* and *GAPDH* were designed as follows: *LRRC42*, 5'-TTGATCATAGTAACTGCAAGACAGAG-3' and 5'-ACGCTCCCACTGCAGAAC-3'; *GARAD2B*, 5'-CACCAACCGGCTGAAAAAT-3' and 5'-GCTGCT GTAATCGCTGTTCA-3', *MBD3*, 5'-ACCATGGACC TCCCCAAG-3' and 5'-CGACAGCAGCGTCTCATC-3'; *p21$^{Waf1/Cip1}$*, 5'-GACCTGTCACTGTCTTGTACCC-3' and 5'-AAGATCAGCCGGCGTTTG-3'; *GAPDH*, 5'-ACCATG GGGAAGGTGAAG-3' and 5'-AATGAAGGGGTCATT GATGG-3'.

Immunofluorescence analysis. Cells were plated onto glass coverslips (Becton Dickinson, Mountain View, CA, USA), fixed with 4% paraformaldehyde, and permeablilized with 0.1% Triton X-100 in PBS for 5 min at room temperature. Non-specific binding was blocked by 5% skim milk for 10 min at room temperature. Cells were then incubated overnight at 4°C with primary antibodies for mouse monoclonal anti-Flag antibody (Catalog no. F3165, Sigma, St. Louis, MO, USA) and anti-GATAD2B antibody (Catalog no. HPA017015, ATLAS, Stockholm, Sweden) diluted in PBS containing 1% BSA. After being washed with PBS, the cells were stained with Alexa Fluor 488-conjugated secondary antibody (Molecular Probes, Eugene, OR, USA) (Fig. 1C), or with Alexa Fluor 488-conjugated secondary antibody and Alexa Fluor 594-conjugated secondary antibody (Molecular Probes) (Fig. 3C) for 60 min at room temperature. After another wash with PBS, each specimen was mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA) containing 4', 6'-diamidine-2'-phenylindolendihydrochrolide (DAPI) and visualized with Spectral Confocal Scanning Systems (TSC SP2 AOBS: Leica Microsystems, Wetzlar, Germany).

Northern blot analysis. Human multiple-tissue blots (Clontech, Carlsbad, CA, USA) were hybridized with ^{32}P-labeled PCR products of *LRRC42*. The cDNA probe of *LRRC42* was prepared by RT-PCR using following primers: *LRRC42*, 5'-GACCAGATCGTTCTGCAGTG-3' and 5'-CCTCCCA CACCACAAAAGTA-3'. Prehybridization, hybridization, and washing were performed according to the supplier's recommendations. The blots were autoradiographed at -80°C for 14 days.

RNA interference assay. To evaluate the biological functions of LRRC42 in lung cancer cells, we used small interfering RNA (siRNA) duplexes against the target genes (Sigma). The target sequences of the synthetic oligonucleotides for RNA interference were as follows: control-1: [EGFP, enhanced green fluorescence protein (GFP) gene, a mutant of *Aequorea gictoria* GFP], 5'-GAAGCAGCACGACUUCUUC-3'; control-2 (LUC, luciferase gene from *Photinus pyralis*), 5'-CGUACG CGGAAUACUUCGA-3'; si-LRRC42-#1, 5'-CUUACUA CCUCAGCUCAGA-3'; si-LRRC42-#2, 5'-GACUUGUUA AAUUCCUAUU-3'; si-GATAD2B, 5'-GCCAAUAGCG AGUUCAUCU-3'; si-MBD3, 5'-CACAGUCGAGGCACG UCAU-3'. Lung cancer cell lines, LC319 and SBC-3, were plated onto 10-cm dishes ($5.0x10^5$ per dish), and transfected with either of the siRNA oligonucleotides (50 μM) using 30 μl of Lipofectamine® RNAiMAX (Life Technologies) according to the manufacturers' instructions. After seven days of incubation, the cells were stained by Giemsa solution to assess colony formation, and cell numbers were assessed by Cell Counting Kit-8 (Dojindo Co., Kumamoto, Japan); briefly, Cell Counting Kit-8 solution was added to each dish at concentration of 1/10 volume, and the plates were incubated at 37°C for additional 1 h. Absorbance was then measured at 490 nm, and at 630 nm as a reference, with a 2030 ARVO™ X3 (PerkinElmer, Courtaboeuf, France).

Anti-LRRC42 antibody. Plasmids expressing partial LRRC42 that contained His-tagged epitopes at their NH_2 termini were prepared using pET28 vector (Novagen, Darmstadt, Germany) and primers LRRC42-F (5'-GGAATTCTGGGCTGA CCAGATCGTTCTGC-3') and LRRC42-R (5'-ATAGTTT AGCGGCCGCTTAGTTATTCTGTTCTGTCTCTGACT-3'. Recombinant proteins were expressed in *Escherichia coli* BL21 codon-plus strain (Stratagene, San Diego, CA, USA) and purified using Ni-NTA (Qiagen) according to the supplier's protocol. The protein was inoculated into rabbits; immune sera were purified on affinity columns according to standard methods. Affinity-purified anti-LRRC42 antibodies were used for western blotting. We confirmed that the antibody was specific to LRRC42 on western blots using lysates from cell lines that had been transfected with LRRC42 expression vector.

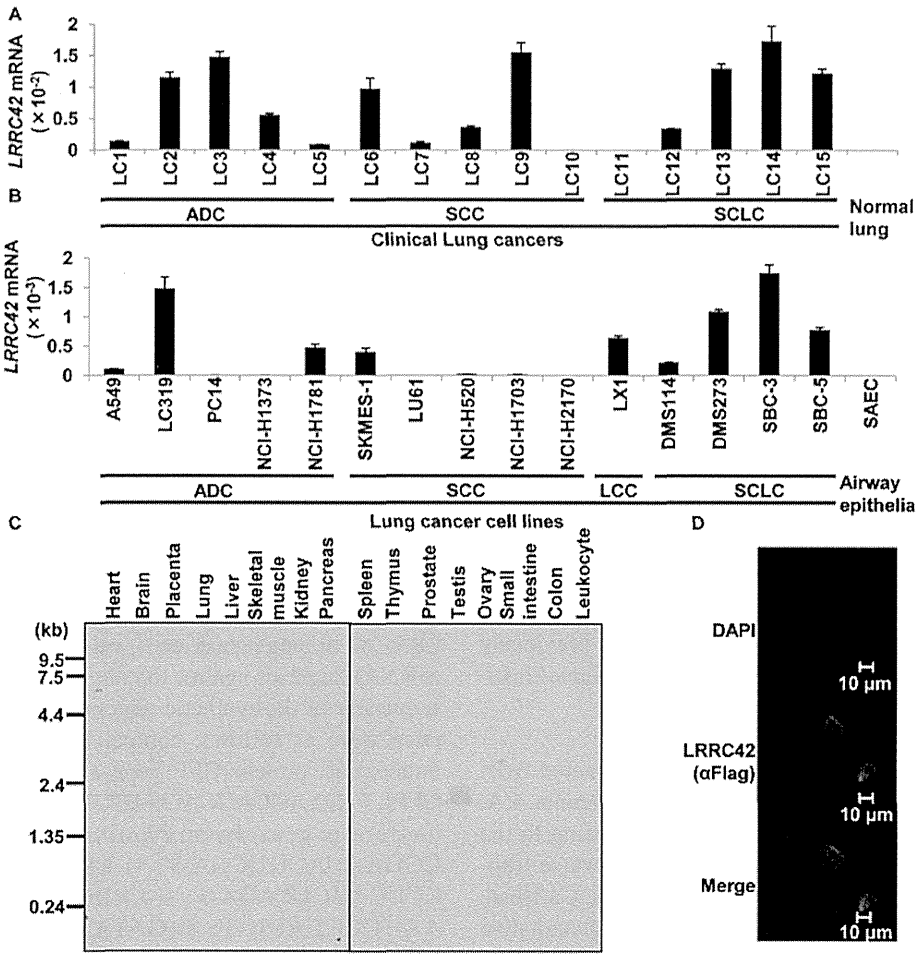

Figure 1. LRRC42 expression in lung cancers and normal tissues. (A) Expression of *LRRC42* in clinical NSCLC (non-small cell lung cancer) and SCLC (small cell clung cancer) samples, and normal lung tissues, analyzed by quantitative real-time PCR. mRNA expression levels were normalized by *GAPDH* expression. The columns and bars represent the mean and SE, respectively. (B) mRNA expression analysis of *LRRC42* in lung cancer cell lines by quantitative real-time PCR. The columns and bars represent the mean and SE, respectively. (C) Subcellular localization of exogenous LRRC42 protein in COS-7 cells detected by anti-Flag (green), which were co-stained with DAPI (blue). (D) mRNA expression analysis of *LRRC42* in normal human tissues by northern blot analysis.

Western blotting. Cells were lysed with immunoprecipitation assay buffer [50 mmol/l Tris-HCl (pH 8.0), 150 mmol/l NaCl, 1% NP40, 0.5% deoxychorate-Na, 0.1% SDS] containing Phosphatase Inhibitor Cocktail Set II and Protease Inhibitor Cocktail Set III EDTA-Free (Calbiochem, San Diego, CA, USA). Protein samples were separated by SDS-polyacrylamide gels and electroblotted onto Hybond-P PVDF membranes (GE Healthcare Bio-Sciences, Piscataway, NJ, USA). Blots were incubated with a mouse monoclonal anti-Flag antibody, a rabbit anti-Flag antibody (Catalog no. F7425, Sigma), an anti-GATAD2B antibody, an anti-MBD3 antibody (Catalog no. sc-9402, Santa Cruz, Santa Cruz, CA, USA), an anti-$p21^{Waf1/Cip1}$ antibody (Catalog no. 2947S, Cell Signaling, Danvers, MA, USA) and an anti-p53 antibody (Catalog no. sc-126, Santa Cruz, Santa Cruz, CA, USA). Antigen-antibody complexes were detected using secondary antibodies conjugated to horseradish peroxidase (GE Healthcare Bio-Sciences). Protein bands were visualized by enhanced chemiluminescence western blot detection reagents (GE Healthcare Bio-Sciences).

Establishment of HEK293 cells expressing exogenous LRRC42. T-REx HEK293 cells that could induce the expression of exogenous LRRC42 (LRRC42-HEK293) cells were generated by Flp-In expression system, where LRRC42 expression is under control of the tetracycline-regulated cytomegalovirus/$tetO_2$ hybrid promoter, and the commercially available Flp-In T-REx 293 host cell line, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA).

Cell growth assay. LRRC42-HEK293 cells were grown for four days in DMEM containing 10% FBS, Blasticidin S HCl (Life Techonologies; 15 μg/ml) and Hygromycin B (Invitrogen; 100 μg/ml) supplemented with or without doxycycline (Sigma; 100 ng/ml). Viability of cells was evaluated by Cell Counting Kit-8.

Coimmunnoprecipitation and matrix-assisted laser desorption/ionizing-time of flight mass spectrometry mapping of LRRC42-associated proteins. Cell extracts from lung cancer cell line SBC-3 which was transfected with LRRC42 expression (carboxyl-terminal Flag-tagged pCAGGS plasmid vector) or mock vector were precleared by incubation at 4°C for 1 h with 80 μl of protein G-agarose beads in a final volume of 200 μl of immunoprecipitation buffer (0.5% NP40, 50 mmol/l

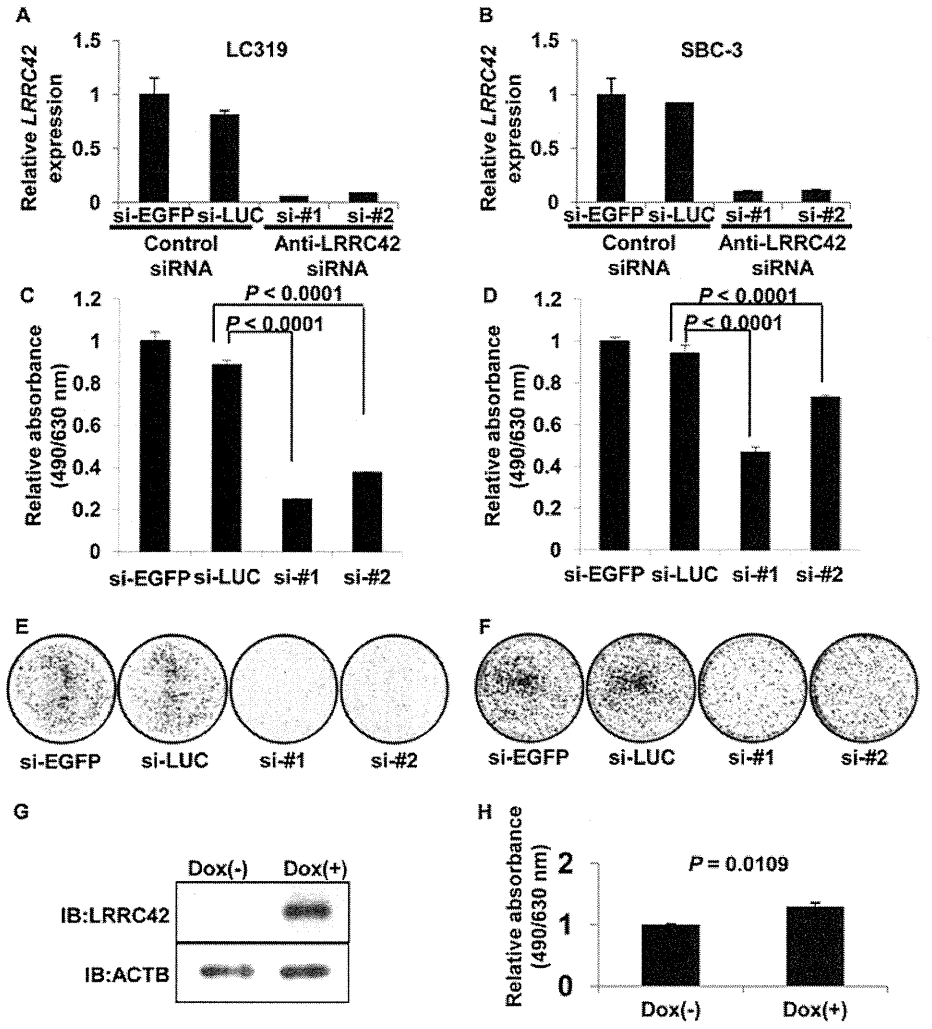

Figure 2. Involvement of LRRC42 in growth and/or survival of lung cancer cells. (A and B) Expression levels of *LRRC42* in response to treatment with si-*LRRC42* (si-#1 or si-#2) or control siRNAs (EGFP or LUC) in LC319 and SBC-3 cells, analyzed by quantitative real-time PCR. The columns and bars represent the mean and SE, respectively. (C and D) Viability of LC319 and SBC-3 cells evaluated by MTT assay in response to treatment with si-LRRC42 (si-#1 or si-#2), si-EGFP, or si-LUC. All assays were performed in triplicate using triplicate wells. The columns and bars represent the mean and SE, respectively. (E and F) Colony formation assays of LC319 and SBC-3 cells transfected with specific siRNAs or control siRNAs. (G) Induction of LRRC42 expression in T-REx™ 293 cells with Dox (Doxycycline) by western blot analysis. (H) Assays demonstrating the growth promoting effect of induced *LRRC42* expression in T-REx 293 cells. Assays were performed in triplicate using triplicate wells. The columns and bars represent the mean and SE, respectively.

Tris-HCl, 150 mmol/l NaCl) in the presence of phosphatase and proteinase inhibitors. After centrifugation at 1,000 rpm for 5 min at 4˚C, the supernatants were incubated at 4˚C with anti-Flag M2 agarose for 3 h. The beads were then collected by centrifugation at 1,000 rpm for 1 min and washed six times with 1 ml of each immunoprecipitation buffer. The washed beads were resuspended in 30 μl of Laemmli sample buffer and boiled for 5 min, and the proteins were separated using 5% to 20% SDS PAGE gels (Bio-Rad Laboratories, Marnes-la-Coquette, France). After electrophoresis, the gels were stained with SilverQuest (Invitrogen). Protein bands specifically found in extracts which was transfected with LRRC42 vector were excised and served for matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF-MS) analysis (AXIMA-CFR, Shimadzu-Biotech, Kyoto, Japan).

Flow cytometry. After transfection of each siRNAs, cells were treated with Aphidicolin (Sigma) at 1 μg/ml for 24 h. Then, the cells were washed with PBS four times and growth medium was added into the dish. The cells were collected in PBS every 3 h and fixed in 70% cold ethanol for 30 min. After treatment with 100 μg/ml of RNase (Sigma), the cells were stained with 50 μg/ml propidium iodide (Sigma) in PBS. Flow cytometry was analyzed by using FACScan (Becknman Coulter, Brea, CA, USA). The cells selected from at least 20,000 ungated cells were analyzed for DNA content.

Results

LRRC42 expression in lung cancers and normal tissues. To identify novel target molecules for the development of therapeutic agents and/or diagnostic biomarkers for lung cancer, we previously performed gene expression profile analysis of 120 lung carcinoma samples using cDNA microarray containing 27,648 genes or expressed sequence tags (6-10), and identified that LRRC42 was significantly transactivated (more than 3 times higher than in their corresponding normal tissues) in

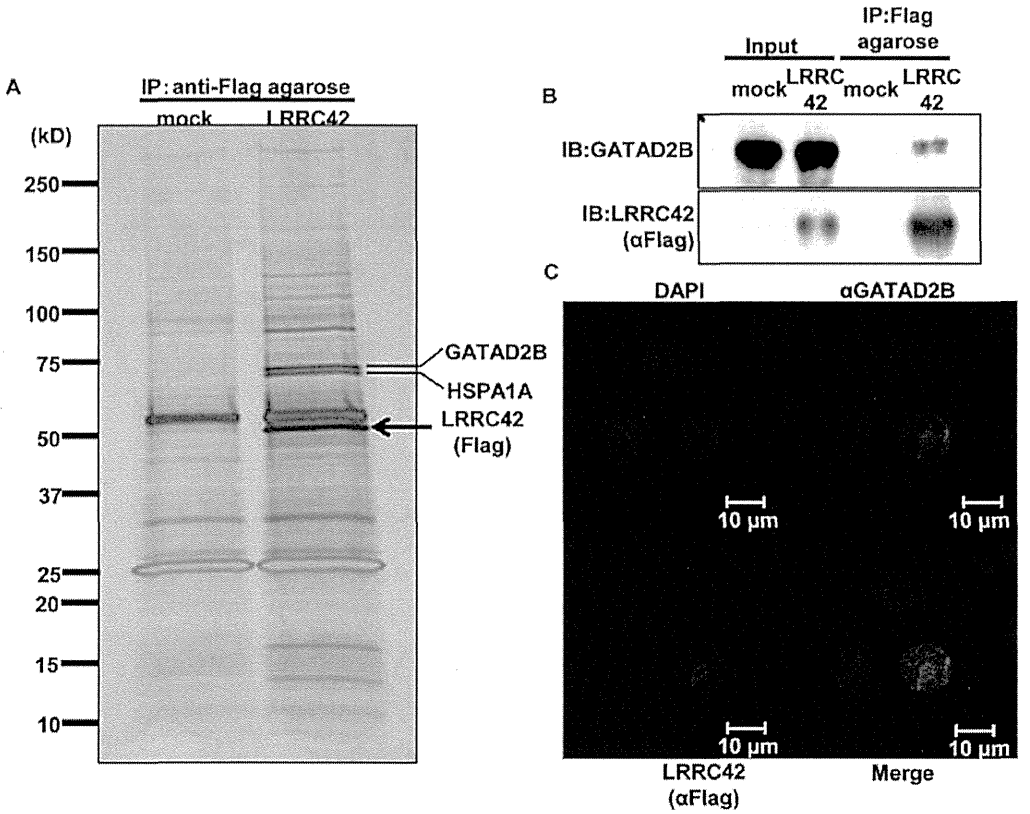

Figure 3. Interaction of LRRC42 with GATAD2B. (A) Silver staining of SDS-PAGE gels that contained immunoprecipitated lysates of lung cancer SBC-3 cells, which were transfected with Flag-tagged LRRC42 expression vector or mock vector, using anti-Flag M2 agarose. (B) Interaction between exogenous LRRC42 and endogenous GATAD2B in SBC-3 cells transfected with LRRC42 expression vector by immunoprecipitation and western blot analysis. (C) Colocalization of LRRC42 and GATAD2B in the nucleus of SBC-3 cells transfected with LRRC42 expression vector, detected by immunocytochemical staining.

>50% of 120 lung cancer samples examined. We subsequently confirmed its transactivation by quantitative real-time PCR experiments in lung cancer tissues as well as lung cancer cell lines (Fig. 1A and B). Northern blot analysis with the *LRRC42*-specific probe identified a 1.7-kb transcript only in testis among 16 normal human tissues examined (Fig. 1C). To determine the subcellular localization of LRRC42 protein, we constructed a plasmid expressing LRRC42 (carboxyl-terminal Flag-tagged pCAGGS plasmid vector), transfected it into COS-7 cells and detected exogenous LRRC42 protein in the nucleus of the cells using an anti-flag antibody (Fig. 1D).

Effect of LRRC42 on cell growth. To assess whether LRRC42 is essential for growth or survival of lung cancer cells, we transfected synthetic oligonucleotide siRNAs against LRRC42 into lung adenocarcinoma LC319 and small cell lung cancer SBC-3 cells in which *LRRC42* was highly expressed. The mRNA levels of *LRRC42* in the cells transfected with si-LRRC42-#1 or -#2 were significantly decreased in comparison with those transfected with either of the control siRNAs (Fig. 2A and B). MTT and colony formation assays revealed a significant reduction of cell viability as well as the number of colonies in si-LRRC42-transfected cells (Fig. 2C-F).

To further clarify a potential role of LRRC42 in carcinogenesis, we established HEK293 cells using the Flp-In T-Rex expression system, where LRRC42 expression was under the control of the tetracycline-regulated cytomegalovirus/tetO_2 hybrid promoter. MTT assay demonstrated that the growth of the cells treated with doxycycline was enhanced compared with that without doxycycline, indicating the growth promoting activity of LRRC42 protein (Fig. 2G and H).

Interaction and colocalization of LRRC42 with GATAD2B. To elucidate the molecular mechanism of LRRC42 in lung carcinogenesis, we screened a protein(s) that could interact with LRRC42. Lysates of SBC-3 cells which was transfected with LRRC42 expression vector (carboxyl-terminal Flag-tagged pCAGGS plasmid vector) or mock vector were extracted, and immunoprecipitated with anti-Flag M2 agarose. The protein complex was separated by SDS-PAGE and visualized by silver staining (Fig. 3A). A 65-kDa band, which was detectable in lysates of cells transfected with LRRC42 vector, but not in those with mock vector, was extracted. The peptide sequence analysis determined by mass spectrometry indicated the protein to be GATAD2B (GATA zinc finger domain containing 2B) that is known to be a component of the MeCP1 complex that represses transcription through preferential binding, remodeling and deacetylation of methylated nucleosomes (15). We subsequently confirmed interaction between exogenous LRRC42 and endogenous GATAD2B in SBC-3 cells using anti-GATAD2B antibody by co-immunoprecipitation experiment (Fig. 3B). We also conducted immunofluorescence analysis and found colocalization of exogenous LRRC42 with endogenous GATAD2B in the nucleus of SBC-3 cells which were transfected with the LRRC42 expression vector (Fig. 3C).

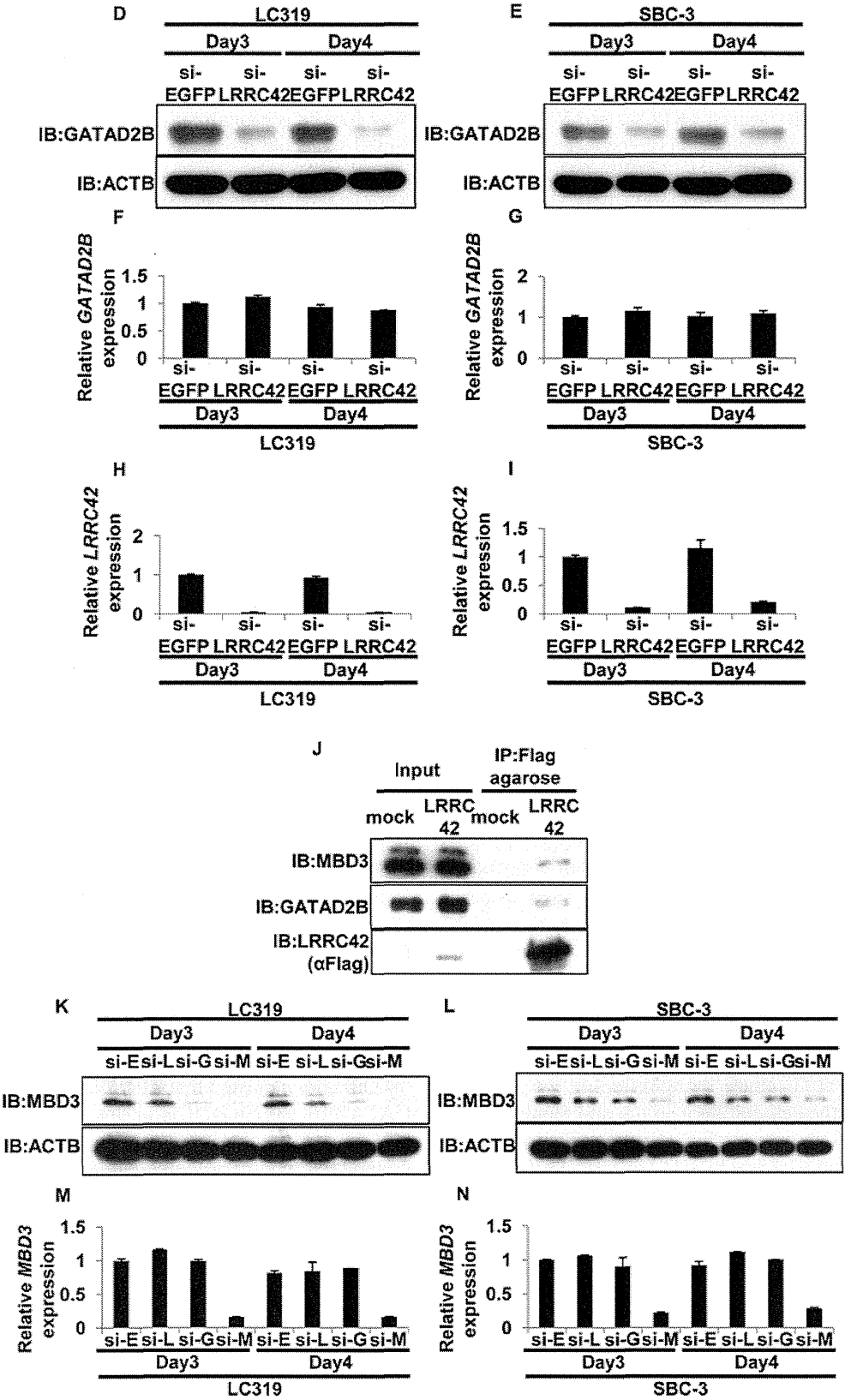

Figure 3 Continued. Interaction of LRRC42 with GATAD2B. (D and E) The level of GATAD2B proteins detected by western blot analysis in LC319 and SBC-3 cells transfected with si-EGFP or si-LRRC42. The columns and bars represent the mean and SE, respectively. (F and G) The level of GATAD2B mRNAs detected by quantitative real-time PCR analysis in LC319 and SBC-3 cells transfected with si-EGFP or si-LRRC42. The columns and bars represent the mean and SE, respectively. (H and I) The level of LRRC42 mRNAs detected by quantitative real-time PCR analysis in LC319 and SBC-3 cells transfected with si-EGFP or si-LRRC42. The columns and bars represent the mean and SE, respectively. (J) Interaction between exogenous LRRC42 and endogenous GATAD2B or MBD3 in SBC-3 cells transfected with LRRC42 expression vector by immunoprecipitation and western blot analysis. (K and L) The level of MBD3 proteins detected by western blot analysis in LC319 and SBC-3 cells transfected with si-EGFP, si-LRRC42, GATAD2B or MBD3. (M and N) The level of MBD3 mRNAs detected by quantitative real-time PCR analysis in LC319 and SBC-3 cells transfected with si-EGFP, si-LRRC42, si-GATAD2B or si-MBD3. The columns and bars represent the mean and SE, respectively.

To further investigate the biological significance of the interaction between LRRC42 and GATAD2B in cancer cells, we examined the protein level of GATAD2B after suppressing LRRC42 expression in LC319 and SBC-3 cells. Treatment of siRNA oligonucleotides against LRRC42 (si-LRRC42) effectively knocked down the expression of endogenous LRRC42, compared to the control siRNA (si-EGFP). Interestingly, the protein level of GATAD2B was also significantly decreased in cells transfected with si-LRRC42, while the transcript level of *GATAD2B* was unchanged (Fig. 3D-I). A previous study indicated GATAD2B as a key component of the MeCP1 complex that interacted with MBD3 (15). Furthermore, GATAD2B was shown to possess an ability to target MBD3 protein to specific nuclear loci (15,46). MBD3 was indicated to be recruited to a promoter region of the $21^{Waf1/Cip1}$ tumor suppressor gene and silence its expression (47). Hence, we have hypothesized that LRRC42 might have a very significant effect on the function of the MeCP1 complex through the interaction with GATAD2B as well as MBD3 (Fig. 3J). We found that knockdown of LRRC42 or GATAD2B reduced the amount of MBD3 protein while no change was observed in mRNA level of MBD3 (Fig. 3K-N).

LRRC42-GATAD2B-MBD3 axis regulates $p21^{Waf1/Cip1}$ expression. We then examined the downstream target of the LRRC42-GATAD2B-MBD3 complex in lung cancer cells. As described above, MBD3 might be recruited at the $p21^{Waf1/Cip1}$ promoter and silence its expression (47). Therefore, we firstly assessed the knockdown effect of either of LRRC42, GATAD2B or MBD3 on $p21^{Waf1/Cip1}$ expression by quantitative real-time PCR and western blot analysis. Suppression of either of LRRC42, GATAD2B or MBD3 by siRNA appeared to increase $p21^{Waf1/Cip1}$ at transcriptional and protein levels in LC319 (p53 null) and SBC-3 (p53 wild-type) cells (Fig. 4A-D). However, this effect on $p21^{Waf1/Cip1}$ was not clear in other lung cancer cell lines examined (NCI-H1781, NCI-H358, NCI-H1299 and DMS273; data not shown).

Since $p21^{Waf1/Cip1}$ expression was known to cause the G_0/G_1 arrest, we performed FACS (Fluorescence Activated Cell Sorter) analysis to evaluate the knockdown effect on the cell cycle in these cell lines. After synchronization of the cancer cells at G_1 phase by aphidicolin, we removed aphidicolin from the cell culture medium and monitored the cell cycle progression process (Fig. 4E). The cells transfected with si-EGFP progressed rapidly into the S and G_2/M phases. However, the cells treated with siRNA against *LRRC42, GATAD2B* or *MBD3* revealed significant delay in entering into S phase although the delay from the G_1 to S transition in the cells treated with si-MBD3 was less significant to those treated with siLRRC42 or si-GATAD2B. These data implied that the LRRC42-GATAD2B interaction may regulate MBD3 protein as well as the MeCP1 complex, and enhance the growth of cancer cells.

Discussion

Recent advances in understanding the molecular mechanisms underlying cancer development/progression have driven the design of new therapeutic approaches, termed 'molecular targeted therapies', that selectively interfere with molecules or pathways involved in tumor growth and progression. Inactivation of growth factors and/or their receptors on tumor cells as well as the inhibition of oncogenic tyrosine kinase pathways that play crucial roles in cancer cells constitute the main rationale of new cancer treatments and also lead to the way for the personalized treatment for individual patients. Small-molecule inhibitors and monoclonal antibodies are at present major components of these targeted approaches for various types of human caner (16). Molecular targeted cancer therapies hold the promise of being very selective to cancer cells, but not affecting normal cells. Hence, they are expected to be less harmful to normal cells, reduce severe side effects, and improve the quality of life of cancer patients. Toward identification of molecular targets for drug development, we had performed whole-genome expression profiles of 120 clinical lung cancer samples using cDNA microarray data and subsequent loss-of-function phenotype analyses by means of RNA interference systems (17-38). On the basis of this approach, we found LRRC42 to be highly over-expressed in the majority of clinical lung cancer cases as well as in most of the lung cancer cell lines examined, while its expression was absent in normal tissues except testis.

Furthermore, we demonstrated that knockdown of LRRC42 expression suppressed the growth of lung cancer cells. In addition, induction of LRRC42 expression by a T-REx system resulted in enhancement of cell growth, suggesting that LRRC42 is likely to be an important growth promoting factor in lung cancer cells and could serve as a valuable target for the development of an anticancer agent for lung cancer.

LRR family members were reported to participate in many biologically important processes such as hormone-receptor interactions, enzyme inhibition, cell adhesion and cellular trafficking. A number of studies clarified the involvement of LRR proteins in early mammalian development (39), development of neuron (40), cell polarization (41), regulation of gene expression (42) and apoptosis (43). It was also shown that LRR domains may be critical for the cell morphology as well as the cytoskeleton dynamics (44,45). In these processes, the LRR motifs are probably essential in mediating protein-protein interactions. However, there has been no report describing the involvement of LRRC42 in human carcinogenesis.

Our data indicated that LRRC42 was able to interact with GATAD2B. GATAD2B is an important component of the MeCP1 complex that represses transcription of genes through preferential binding to, remodeling, and deacetylating methylated nucleosomes. It has the ability to translocate MBD3 protein to specific nuclear foci (15,46). We demonstrated that LRRC42 could activate this transcription-repressive complex through interacting with and stabilizing GATAD2B and MBD3 proteins. We also revealed that the LRRC42-GATAD2B-MBD3 interaction is likely to play a significant role in transcriptional regulation of the cyclin-dependent kinase inhibitor $p21^{Waf1/Cip1}$ which is a well-known tumor suppressor and an inhibitor of cell cycle progression from G_1 to S phase. $p21^{Waf1/Cip1}$ negatively regulates DNA replication through the interaction with PCNA (Proliferative cell nuclear antigen), and also binds to the CDK (Cyclin-dependent kinase) complex and inhibits the G_1-S transition (48).

Several transcriptional regulators, activators or repressors of $p21^{Waf1/Cip1}$ have been reported. p53 is an activator of $p21^{Waf1/Cip1}$ and the activation of the p53-$p21^{Waf1/Cip1}$ pathway is

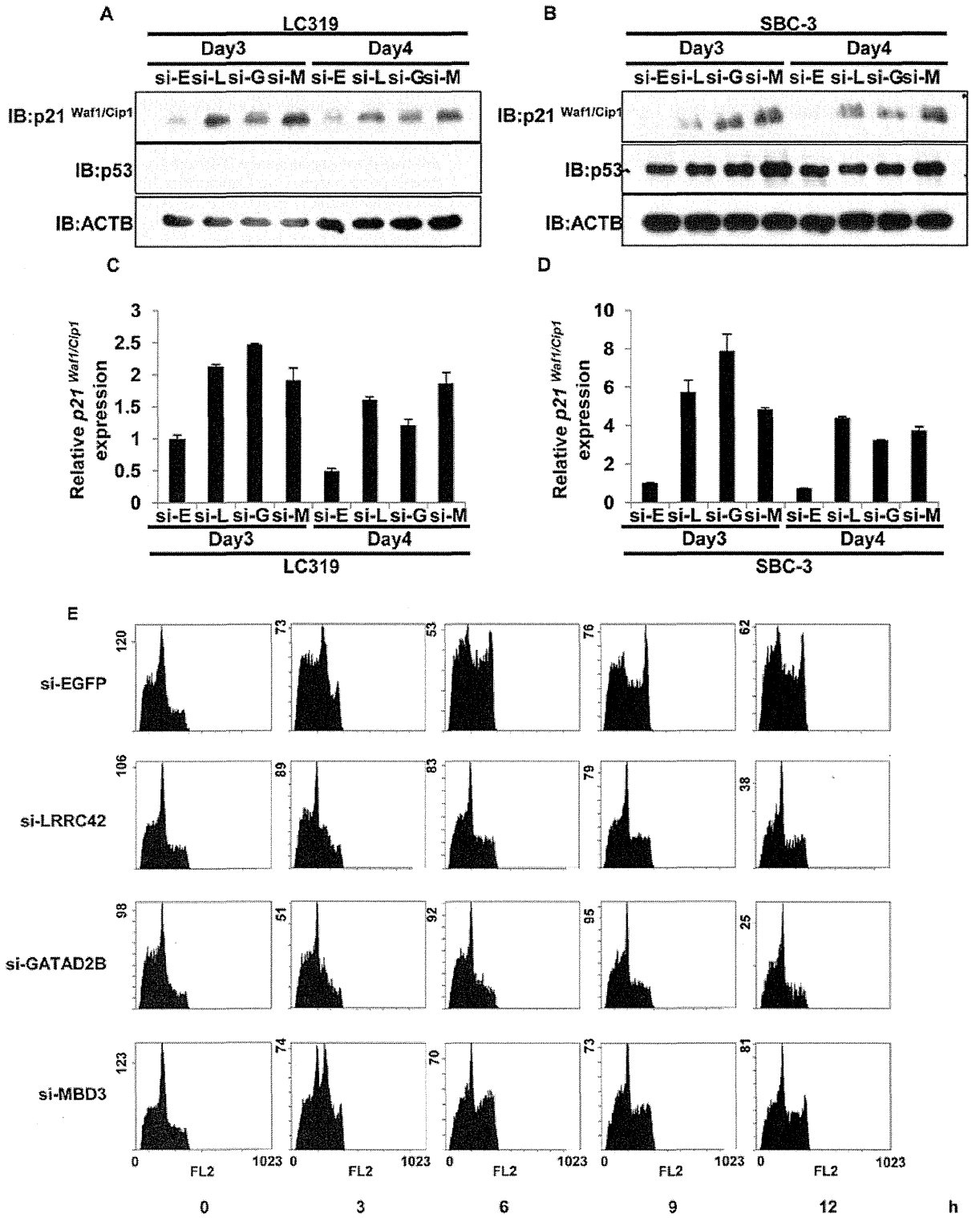

Figure 4. Induction of $p21^{Waf1/Cip1}$ and the effect on cell cycle progression by knockdown of LRRC42, GATAD2B or MBD3. (A and B) Expression levels of $p21^{Waf1/Cip1}$ protein after suppression of LRRC42, GATAD2B or MBD3 by siRNA in LC319 and SBC-3 cells. (C and D) Expression levels of $p21^{Waf1/Cip1}$ mRNA after suppression of LRRC42, GATAD2B or MBD3 by siRNA in LC319 and SBC-3 cells. The columns and bars represent the mean and SE, respectively. (E) Histogram of the cell cycle phases after treatment with si-EGFP, si-LRRC42, si-GATAD2B or MBD3 in SBC-3 cells.

critically important when cells need to arrest the cell cycle and repair the DNA damage. Myc, one of $p21^{Waf1/Cip1}$ repressors, was reported to induce the expression of AP4 (Transcription Factor AP-4) that has the ability to repress $p21^{Waf1/Cip1}$ expression. Myc may also repress $p21^{Waf1/Cip1}$ expression through the interaction with MIZ1 (ZBTB17) (49). MBD3 is known to be one of the repressors which directly regulate $p21^{Waf1/Cip1}$ expression and play important roles in oncogenic transformation and proliferation (50). FACS analysis clearly demonstrated that suppression of *MBD3* caused the delay of G_1-S transition

although its effect was not as significant as the knockdown of LRRC42 or GATAD2B.

In conclusion, human LRRC42 is essential for growth and survival of lung cancer cells. Our data imply that targeting LRRC42 and/or the LRRC42-GATAD2B interaction may be a good approach for development of new treatment of lung cancer with specific activity and minimum toxicity.

Acknowledgements

This study was supported in part by Grant-in-Aid for Scientific Research (B) and Grant-in-Aid for Scientific Research on Innovative Areas from The Japan Society for the Promotion of Science to Y.D. Y.D. is a member of Shiga Cancer Treatment Project supported by Shiga Prefecture (Japan).

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Cancer Science
Japanese Cancer Association JCA
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Review Article

Clinical significance of macrophage heterogeneity in human malignant tumors

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Keywords
Cancer, CD163, M2, macrophage, TAM

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Funding information
Ministry of Education, Culture, Sports, Science, and Technology of Japan.

Received July 8, 2013; Revised October 16, 2013; Accepted October 27, 2013

Cancer Sci **105** (2014) 1–8

doi: 10.1111/cas.12314

The fact that various immune cells, including macrophages, can be found in tumor tissue has long been known. With the recent introduction of the novel concept of macrophage differentiation into a classically activated phenotype (M1) and an alternatively activated phenotype (M2), the role of tumor-associated macrophages (TAMs) is gradually beginning to be elucidated. Specifically, in human malignant tumors, TAMs that have differentiated into M2 macrophages act as "protumoral macrophages" and contribute to the progression of disease. Based on recent basic and preclinical research, TAMs that have differentiated into protumoral or M2 macrophages are believed to be intimately involved in the angiogenesis, immunosuppression, and activation of tumor cells. In this paper, we specifically discuss both the role of TAMs in human malignant tumors and the cell–cell interactions between TAMs and tumor cells.

It has long been known that many leukocytes including macrophages are present in tumor tissues and that these cells, together with fibroblasts and vascular endothelial cells, form the tumor microenvironment (Fig. 1).[1–4] Previously, activated macrophages were believed to exhibit antitumor activity by directly attacking tumor cells in the tumor microenvironment.[5] However, many recent studies have indicated the protumoral functions of tumor-associated macrophages (TAMs), and thus, TAMs are believed to directly or indirectly promote tumor progression.[6–8] Great advances have been made in TAM research over the past dozen years or so, with one of the most significant breakthroughs being the development of immunohistochemical methods for identifying TAMs in tumor tissue. Numerous studies using human samples have been carried out using CD68 as a macrophage marker, whereas CD163 and CD204 have been used as markers of M2 macrophages in recent studies.[9,10] Although variability is observed according to tumor tissue type and location, over 80% of immunohistochemical studies using various human tumor tissues have shown that higher numbers of TAMs are associated with worse clinical prognosis.[9] Supporting these clinical observations, *in vitro* experiments using human tumor cells and experiments using animal models indicate that TAMs promote tumor cell growth by suppressing antitumor immunity and inducing angiogenesis.[11,12]

As the relationship between TAMs and malignant tumors becomes clearer, TAMs have begun to be seen as the target of new cancer treatments. Clarification of how TAMs are involved in tumor progression and metastasis is anticipated to lead to the development of novel treatments and drugs.

Intratumoral infiltration of TAMs

Intratumoral infiltration of monocytes/macrophages is induced by various chemokines including chemokine (C-C motif) ligand (CCL)2, CCL5, CCL7, and chemokine (C-X3-C motif) ligand (CX3CL)1, as well as cytokines such as macrophage colony-stimulating factor (M-CSF), granulocyte-macrophage colony-stimulating factor, and vascular endothelial growth factor (VEGF), which are produced by tumor cells.[13–15] Subsequent differentiation into TAMs is induced by various factors produced by tumor cells. While the tumor size is small, macrophages from the surrounding tissue accumulate in and around the tumor by tumor cell-derived chemotactic molecules described above, and TAMs derived from the surrounding tissue macrophages account for the majority of TAMs.[4,16] As the tumor subsequently increases in size and an intratumoral vascular network forms, monocyte-derived TAMs become the dominant source of TAMs.[4,16]

Although many macrophage chemotactic factors are secreted by tumor cells, CCL2 and M-CSF are considered to be impor-

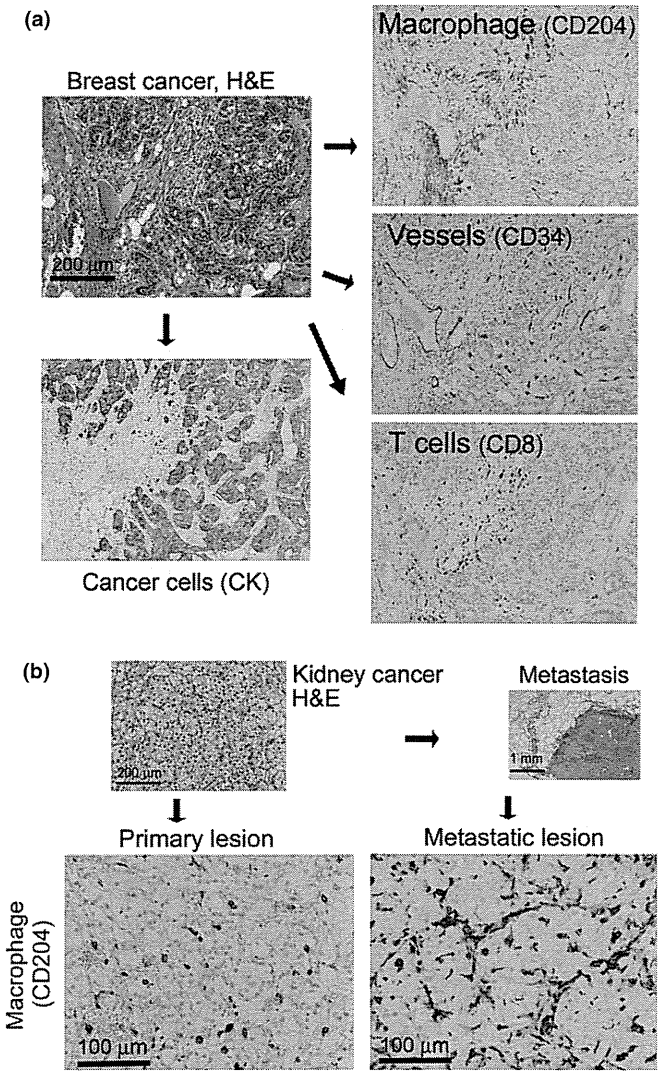

Fig. 1. Tumor microenvironment. (a) Tumor tissue contains not only tumor cells, but also large numbers of normal cells, including tumor-associated macrophages, lymphocytes, blood vessels, and fibroblasts, that affect tumor development in various ways. The photographs show an example of a clinical case of human breast cancer (invasive ductal carcinoma). The relative distributions of the above-mentioned cell types differ by organ and tissue type as well as individual case. CK, cytokeratin. (b) Metastatic tumors contain a larger number of tumor-associated macrophages. The photographs show an example of a clinical case of human kidney cancer (clear cell renal cell carcinoma). The primary tumor tissues and the metastatic (lung) tumors are shown.

tant molecules involved in macrophage infiltration. CCL2 is expressed in a wide variety of tumor cells, including gliomas, squamous cell carcinoma, ovarian cancer, prostate cancer, lung cancer, cervical cancer, and undifferentiated sarcoma, CCL2 also plays an important role in the intratumoral infiltration of monocytes.[13,17] In addition to inducing monocyte infiltration, M-CSF plays a critical role in the differentiation of monocytes into macrophages and, in particular, into M2 macrophages.[18–20]

Role of TAMs in tumor progression

Based on numerous studies using murine tumor models, activated TAMs were found to produce a variety of angiogenic, immunosuppressive, and growth-related factors.[7,8] However, few studies have been carried out using human materials, and thus the detailed mechanisms and molecular characterization of TAMs in human tumors have yet to be described. One method for studying the relationship between TAMs and tumor development is to carry out statistical analysis using clinical data related to survival rates or survival times. Studies comparing TAM infiltration into diseased tissue, using CD68 as a macrophage marker, are summarized in Table 1. The majority of studies in human malignant tumors have found that a higher level of TAM infiltration is associated with lower survival rates, and these observations indicate that TAMs may enhance tumor progression. However, other reports in certain types of cancer such as gastric, colon, and prostate cancer, have shown that a higher number of TAM infiltration results in a better outcome.

For a localized tumor a few millimeters in size to grow larger, intratumoral angiogenesis must occur. Genetic analysis has revealed that TAMs produce VEGF, interleukin (IL)-8 (CXCL8), basic fibroblast growth factor, thymidine phosphorylase, MMP, and other molecules that are involved in angiogenesis, indicating that TAMs promote the formation of intratumoral blood vessels. Furthermore, TAMs produce immunosuppressive factors, including prostaglandin E2 (PGE_2), indoleamine 2,3-dioxygenase, and IL-10, and thus contribute to the immunosuppressed state of cancer patients.[5–7] In fact, in studies using human tissue samples, the number of intratumoral TAM infiltration is positively correlated with formation of blood vessels and the number of regulatory T cells. Tumor-associated macrophage-derived PGE_2, indoleamine 2,3-dioxygenase, and IL-10 play important roles for induction of regulatory T cells and TAM-derived CCL17, CCL18, CCL22 are chemotactic factors for regulatory T cells.[5–7] These results indicate that TAMs create environments conducive to tumor progression through their effect on angiogenesis and immunosuppression. In addition, growth factors produced by TAMs, including basic fibroblast growth factor, hepatocyte growth factor, epidermal growth factor, platelet-derived growth factor, and transforming growth factor-β (TGF-β), are considered to directly promote tumor cell growth.[5–7]

Of further interest is the suggestion, based on the results of animal model analysis, that TAMs may play a role in forming premetastatic niches in organs to which the tumor will eventually metastasize.[21–23] Specifically, tumor necrosis factor-α, VEGF, and TGF-β (VEGF and TGF-β are also produced by cancer cells), which are secreted by TAMs in cancer tissues, are believed to be transported through the bloodstream to destination organs such as the lung, where they induce macrophages to produce S100A8 and serum amyloid A3.[23] Both S100A8 and serum amyloid A3 recruit macrophages and tumor cells to these organs and promote the formation of metastatic foci.[24,25] Thus, TAMs are believed to not only influence their local environment, but also to impact macrophages throughout the body and contribute to disease progression.

CD163 and CD204 as markers for protumoral or M2 macrophages

The heterogeneity of macrophage functions was suggested as early as the late 1990s.[26,27] Macrophage activation can be broadly divided into the following two types: classically activated macrophages (M1), which promote inflammation, and alternatively activated macrophages (M2), which inhibit

Table 1. High numbers of CD68+ tumor-associated macrophages are correlated with clinical prognosis in human malignant tumors

Tumor type	Favorable prognosis	Poor prognosis
Epithelial	Gastric cancer (adenocarcinoma)[68] Colorectal cancer (adenocarcinoma)[71] Prostate cancer (adenocarcinoma)[73]	Uterine cancer (endometrioid adenocarcinoma)[69,70] Esophageal cancer (squamous cell carcinoma)[72] Liver cancer (hepatocellular carcinoma)[74] Breast cancer (invasive ductal carcinoma)[75,76] Thyroid cancer (poorly differentiated)[77] Gastric cancer (adenocarcinoma, intestinal type)[78] Bladder cancer (urothelial carcinoma)[79]
Non-epithelial		Malignant mesothelioma (sarcomatous)[80] Malignant melanoma[81] Neuroblastoma[82] Ewing's sarcoma[83]
Hematopoietic		Hodgkin's lymphoma[84] Follicular lymphoma[85]

inflammation.[27,28] Those TAMs demonstrating enhanced expression of CD163 (hemoglobin scavenger receptor), CD204 (class A macrophage scavenger receptor), CD206 (mannose receptor, C type 1), stabilin-1, arginase-1, and accelerated production of IL-10, VEGF, PGE_2, and MMP9, generally show characteristics of M2 macrophages.[6–8] The proangiogenic and immunosuppressive activity in the tumor microenvironment mediated by TAMs can also be considered the result of M2 macrophage function.[6–8] Because CD163 and CD204 are specifically expressed on macrophages, and antibodies to these antigens that are suitable for immunohistochemical analysis are commercially available,[10,29,30] many researchers have used these molecules as markers of the M2 phenotype in both *in vitro* and *in vivo* studies. The details of the functions of these molecules remain unclear; however, a few studies have indicated that these molecules are involved either in regulating the inflammatory responses or in maintaining the protumoral functions of macrophages.[31–33] The clinicopathological studies using anti-CD163 or anti-CD204 antibodies are summarized in Table 2. In malignant lymphoma, glioma, and kidney cancer, higher CD163 expression on TAMs is associated with worse clinical prognosis, but no correlation exists between clinical prognosis and the number of CD204-expressing TAMs.[10,34–36] In esophageal cancer, a higher number of CD204-expressing TAMs is associated with poor clinical outcome, but the number of CD163-positive TAMs is not.[37] These observations suggest that CD163 and CD204 are not expressed in completely identical macrophage populations. In addition, the functional significance of CD163- or CD204-positive TAMs might be different among sites and histological types of cancer. We suggest that both CD163 and CD204 should be analyzed to evaluate the polarization of TAMs and that CD163- and/or CD204-positive TAMs are considered as "protumoral" macrophages/TAMs.

In a recent review, based on their location and function, Qian and Pollard[38] classified TAMs into the following six types: angiogenic; immunosuppressive; invasive; metastasis-associated; perivascular; and activated macrophages. Not all of these macrophage types of TAMs show the phenotype of M2 macrophages. Tumor-associated macrophages with M1 characteristics have also been observed in animal models of glioma and human pancreatic cancer.[39,40] Although the concept of "M1/M2 macrophages" is a convenient hypothesis simply dividing TAMs into two populations, we should note that TAMs contain various macrophage populations with a wide range of polarization statuses stimulated by complex signals in tumor microenvironment.

Significance of direct cell–cell interactions between TAMs and tumor cells

As shown in Figure 1, TAMs and tumor cells often directly contact each other, indicating that intimate cell–cell interactions exist between them. During the initial stages of tumor progression, monocyte migration factors produced by tumor cells induce infiltration of monocytes/macrophages, as described above. The macrophages that have infiltrated the tumor are

Table 2. Correlation between CD163+ or CD204+ tumor-associated macrophages and clinical prognosis in human malignant tumors

Tumor type	Favorable prognosis	Poor prognosis
Epithelial	Colorectal cancer (adenocarcinoma)[86]	Kidney cancer (clear cell type)[34] Liver cancer (hepatocellular carcinoma)[87,88] Liver cancer (cholangiocellular carcinoma)[89] Pancreatic cancer (invasive ductal carcinoma)[90,91] Lung cancer (adenocarcinoma)[92,93] Lung cancer (squamous cell carcinoma)[92,94] Oral cancer (squamous cell carcinoma)[95] Ovarian cancer (serous adenocarcinoma)[96] Esophageal cancer (squamous cell carcinoma)[37]
Non-epithelial	Osteosarcoma[97]	Leiomyosarcoma[98] Brain tumor (high-grade glioma)[10,42] Malignant melanoma[99,100]
Hematopoietic		Diffuse large B-cell lymphoma[101] Hodgkin's lymphoma[101–104] Follicular lymphoma[105] Angioimmunoblastic T-cell lymphoma[35] Adult T-cell leukemia/lymphoma[36] Multiple myeloma[106]

activated by tumor cell-derived molecules, including IL-6, M-CSF, PGE_2, and heat shock protein-27, and differentiate into protumoral/M2 macrophages.[6,20] Protumoral/M2 TAMs produce a variety of angiogenic and immunosuppressive factors, as described above, and create a microenvironment conducive to tumor progression. Signal transducer and activator of transcription 3 (Stat3) has received recent attention as an important transcription factor that mediates the interaction between TAMs and tumor cells.[12] Many angiogenic and immunosuppressive factors are transcriptionally regulated by Stat3. Therefore, activation of Stat3 not only plays an important role in the differentiation of macrophages into protumoral /M2 macrophages, it is also involved in tumor cell growth, metastasis, epithelial–mesenchymal transition, and the acquisition of resistance to anticancer drugs and radiation therapies.[12,41] Direct coculture of tumor cells and macrophages shows that Stat3 in macrophages is activated and that various factors secreted by activated macrophages, including EGF, IL-6, and IL-10, activate Stat3 in tumor cells.[18,42] Activation of the M-CSF receptor (CD115) and sphingosine-1-phosphate receptor 1 (S1PR1) on the cell surface is believed to contribute to the cell–cell interaction mediated by Stat3.[42,43] Membrane-type M-CSF on the surface of tumor cells serves as a ligand for CD115, and sphingosine-1-phosphate derived from tumor cells serves as a ligand for S1PR1. Stimulation of these receptors activates a variety of signal transduction pathways, including that of Stat3, causing TAMs to differentiate into the protumoral/M2 phenotype.[44] The activation of Stat3 through cell–cell interactions between tumor cells and macrophages contributes to the formation of the microenvironment necessary for development of primary and metastatic lesions (Fig. 2).

Recent studies using a murine cancer model showed that Stat3 is also an important molecule in the maintenance and anticancer drug responses of cancer stem-like cells (CSCs).[45–47] The TAM-derived milk fat globule-EGF factor VIII, which is a glycoprotein belonging to an epidermal growth factor superfamily, contributes to Stat3 activation in cooperation with proinflammatory cytokines such as IL-6. And Stat3 activation is preferentially associated with tumorigenesis and drug resistance in CSCs.[46] In human colorectal cancer, overexpression of stem cell markers in tumor cells is reported to be associated with a high number of TAMs.[48] Further studies are expected to clarify the details of the relationships between TAMs and CSCs.

Tumor-associated macrophages and myeloid-derived suppressor cells

Regarding the functional analysis of TAMs, tumor xenograft mouse or rat models are more useful than human tumors. The majority of myeloid cells infiltrating tumor tissues are immature cells in some types of murine tumors.[49,50] A strong immunosuppressive response has long been known to be induced when cancer cells are transplanted into mice. In the 1980s, myeloid cells in the bone marrow of tumor-bearing mice were shown to inhibit the activation of lymphocytes.[51,52] Subsequently, the same types of cells were shown to exist in the spleen, and with the advancement of analysis resulting from the identification of myeloid markers CD11b and Gr1, these cells were also shown to exist in lymph nodes and tumor tissues.[51,52] Immature myeloid cells are derived from bone marrow myeloid cells and exhibit immunosuppressive activity; therefore, they are referred to as myeloid-derived suppressor cells (MDSCs).[51,52] Distinct from mature neutrophils and monocyte/macrophages, MDSCs have recently been divided into granulocytic MDSCs ($CD11b^+Ly6C^{int}Ly6G^{hi}$), showing characteristics similar to neutrophils, and monocytic MDSCs ($CD11b^+Ly6C^{hi}Ly6G^{neg}$), showing characteristics similar to monocytes/macrophages.[53] Despite the observed differences among tumor histopathological types, mature macrophages (TAMs, $Gr1^-$) and MDSCs ($Gr1^+$) appear to coexist in the tumor tissues of mice. As MDSCs from tumor tissues differentiate into mature macrophages in *ex vivo* assays, MDSCs are considered to be the immature phenotype of TAMs.[52,53] However, which cell type plays a greater role in angiogenesis and the activation of tumor cells remains unclear.

Systemic immunosuppression is also observed in human patients with advanced malignant tumors, suggesting the existence of cells similar in nature to the MDSCs that are found in mice. A significant increase in the number of $CD14^+HLA\text{-}DR^{low}$, $CD11b^+CD14^-CD15^+$, or $Lin^-HLA\text{-}DR^-CD33^+$ cells is observed in the peripheral blood of patients with malignant tumors.[49,53] In an *ex vivo* study using human blood or tumor samples of melanoma patients, MDSCs were shown to contribute more substantially to immunosuppression than TAMs.[54] Given that these cell types indicate immunosuppressive activity, they may correspond to the MDSCs that are found in mice. As differences in gene expression and cell markers exist between mice and humans, sufficient care must be taken when attempting to apply the results of mouse studies to humans.

Dendritic cells in human tumor tissues

Dendritic cells (DCs) serve as other myeloid lineage cells in the tumor microenvironment, and play a critical role in integrating both innate and adaptive arms of immune responses. Myeloid DCs (mDCs) and plasmacytoid DCs constitute two major subsets of the DC population, and are distinguished from macrophages according to their unique surface marker expressions. In human DCs, mDCs are further classified as blood dendritic cell antigen (BDCA)1(CD1c)$^+$CD11b$^+$ and BDCA3(CD141)$^+$ C-type lectin(CLEC)9$^+$ populations, which are equivalent to $CD11b^+CD4^{+/-}$ and $CD8\alpha^+$ or $CD103^+$ tissue-resident mDCs, respectively.[55,56] The BDCA3$^+$ mDCs are specialized for cross-presentation of antigens from necrotic cells, whereas BDCA1$^+$ mDCs have pleiotropic functions to prime diverse repertories of T cell subsets, in particular, dermal and mucosa-associated T cells.[56–58] Human plasmacytoid DCs are characterized for their expression of BDCA2(CD303) and CD123 (IL3Rα), and produce large amounts of type-I interferon in response to viral or self-nucleic acids.[54] As it is difficult to identify these molecules in paraffin-embedded pathological specimens, there are few articles describing DCs in human tumor samples. However, these phenotypic differences should help clarify the distinct functions and molecular pathways of TAMs and DCs in tumor tissues.

Targeting TAMs: a novel concept of anticancer therapy

As previously explained, TAMs promote tumor progression through induction of angiogenesis and suppression of antitumor immunity. In particular, in humans, protumoral TAMs are believed to exhibit characteristics similar to M2 macrophages, and are intimately involved in the progression of malignant tumors. As such, treatment strategies aimed at local inhibition of macrophage differentiation into the M2 phenotype are anticipated to be effective. Signal transduction path-

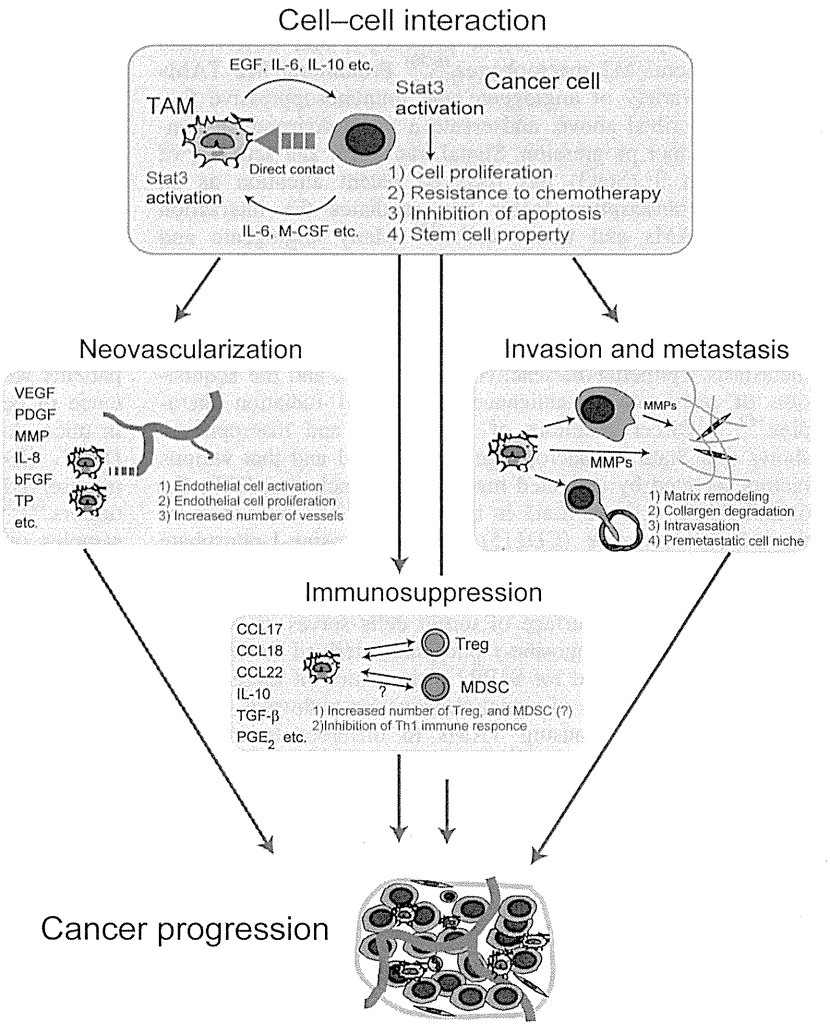

Fig. 2. Schema of the functional role of tumor-associated macrophages (TAMs). Tumor-associated macrophages are activated by macrophage colony-stimulating factor (M-CSF), interleukin (IL)-6, and other compounds secreted by tumor cells both to induce angiogenesis by producing angiogenic factors such as VEGF and platelet-derived growth factor, and to create immunosuppressive conditions by producing immunosuppressive factors such as IL-10 and prostaglandin E2 (PGE_2). At the same time, growth factors that are secreted by TAMs, such as epidermal growth factor (EGF), directly promote cancer cell growth, whereas MMP and other compounds responsible for stroma remodeling promote tumor cell infiltration and metastasis. Activation of tumor cells and TAMs induced by direct cell–cell interactions may represent an extremely important event in relation to the development of malignant tumors. bFGF, basic fibroblast growth factor; CCL, chemokine (C-C motif) ligand; MDSC, myeloid-derived suppressor cell; PDGF, platelet-derived growth factor; Stat3, signal transducer and activator of transcription 3; TGF-β, transforming growth factor-β; TP, thymidine phosphorylase; Treg, regulatory T cell; VEGF, vascular endothelial growth factor.

ways, including nuclear factor (NF)-κB, Stat3, Stat6, c-Myc, and interferon regulatory factor 4, are involved in differentiation into the M2 phenotype.[44,59–61] Nuclear factor-κB and Stat3 are also strongly involved in tumor cell growth, and drugs targeting these molecules are currently being developed. Among such molecule-specific drugs, synergistic efficacy due to direct effects on tumor cells, as well as inhibition of the differentiation of TAMs into the M2 phenotype, is expected. Among drugs currently in use, some are active against TAMs. Cyclosporin A and trabectedin not only directly inhibit tumor cell growth, they also suppress activation of TAMs.[16,62] Bisphosphonates not only suppress bone resorption by osteoclasts, they also inhibit the differentiation of TAMs into the M2 phenotype.[63] The angiogenic inhibitor bevacizumab (a VEGF-inhibiting antibody) has recently been used to treat solid tumors such as colorectal adenocarcinoma, and this drug also exhibits antitumor activity by suppressing TAM migration.[64,65]

We developed a screening system of chemical compounds that suppress macrophage polarization toward the M2 phenotype. By screening a library of naturally occurring compounds, we have identified several compounds, including corosolic acid, that suppress M2 polarization of macrophages.[66] These compounds suppress Stat3 activation and NF-κB activation both in macrophages and tumor cells *in vitro*.[66] However, as the blocking effect of these compounds on Stat3 and NF-κB was not adequate in tumor cells, the direct effect on tumor cells was weaker than that of other anticancer drugs.[66] In an *in vivo* study, corosolic acid appeared not to directly suppress tumor cells, but rather to stimulate the antitumor immunity of lymphocytes by inhibiting the activation of TAMs and MDSCs.[67] Corosolic acid was therefore considered to show antitumor activity by means of indirect effects to myeloid cells.

Conclusion

With the recent introduction of the concept of macrophage differentiation into M1 and M2 macrophages, and clarification of the function of each of these cell types, the role of TAMs in malignant tumors is gradually emerging. Specifically, in human tumors, TAMs that have differentiated into the M2 phenotype act as "protumoral macrophages" and contribute to the progression of disease. Based on current basic research, TAMs that have differentiated into the M2 phenotype are believed to be intimately involved in angiogenesis, immunosuppression, and activation of tumor cells. Clarification of the mechanisms of TAM activation and the process of differentiation into the protumoral/M2 phenotype is anticipated to lead to new strategies for treating malignant tumors.

Acknowledgments

This study was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

Disclosure Statement

The authors have no conflict of interest.

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Cancer Metastasis Rev (2014) 33:737–745
DOI 10.1007/s10555-014-9501-9

NON-THEMATIC REVIEW

Immune regulation of therapy-resistant niches: emerging targets for improving anticancer drug responses

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Published online: 23 April 2014

Abstract Emerging evidence has unveiled a critical role for immunological parameters in predicting tumor prognosis and clinical responses to anticancer therapeutics. On the other hand, responsiveness to anticancer drugs greatly modifies the repertoires, phenotypes, and immunogenicity of tumor-infiltrating immune cells, serving as a critical factor to regulate tumorigenic activities and the emergence of therapy-resistant phenotypes. Tumor-associated immune functions are influenced by distinct or overlapping sets of therapeutic modalities, such as cytotoxic chemotherapy, radiotherapy, or molecular-targeted therapy, and various anticancer modalities have unique properties to influence the mode of cross-talk between tumor cells and immune cells in tumor microenvironments. Thus, it is critical to understand precise molecular machineries whereby each anticancer strategy has a distinct or overlapping role in regulating the dynamism of reciprocal communication between tumor and immune cells in tumor microenvironments. Such an understanding will open new therapeutic opportunities by harnessing the immune system to overcome resistance to conventional anticancer drugs.

Keywords Anticancer drug resistance · Intratumor immune response · Chemotherapy · Radiotherapy · Molecular targeting

1 Overview of therapy-resistant tumor phenotypes

Resistance to anticancer therapies serves as a major barrier that greatly contributes to tumor progression and dismal clinical prognoses. Thus, a deeper understanding of how resistance to anticancer therapies affects the biological and functional aspects of tumorigenicity is necessary to improve clinical remission and survival in patients with cancer. It has been established that multiple arrays of intrinsic genetic and epigenetic alternations imposed by stimulation with anticancer drugs have a major role in regulating the responses to anticancer therapeutics [1–3]. For example, chronic exposure to cytotoxic chemotherapy promotes drug efflux systems in tumor cells *via* activation of ATP-binding cassette transporter-G1 (ABCG1) [1, 4]. In addition, specific epigenetic modification by H3K4 demethylase KDM5B contributes to slow cell cycling or cellular quiescence of tumor cells thereby causing resistance to most cytotoxic agents, which target rapidly proliferating tumor cells [5, 6]. Furthermore, cancer stem cells, small subsets of tumor cells that share with hematopoietic stem cells the properties of high self-renewal activity and cellular quiescence, constitute a specific population that tends to be refractory to conventional cytotoxic chemotherapy [7, 8]. Treatment with molecular-targeted therapies such as EGFR-TKI, anti-HER2 antibodies, and BRAF-KI, *etc.* poses selective pressure on tumor cells triggering genetic mutations of the targeted sites that suppress antitumor efficacy and block the specific targeting strategies [9–11].

On the other hand, accumulating evidence has unveiled the contribution of tumor microenvironments (TMEs) to the regulation of therapeutic outcomes [12–14]. TMEs include stromal cells, endothelial cells, and inflammatory cells. Therapy-resistant tumor cells may modify the biologic properties of non-transformed cells in the TMEs thus amplifying tumorigenic signals to worsen the clinical prognoses of cancer patients [15, 16]. Therefore, resistance to anticancer therapeutics has a profound impact on the tumorigenic, invasive, and metastatic activities of tumor cells by broadly modifying the biological properties of TMEs.

In this review, we focus on the role and relative contribution of therapy-resistant variants of tumor cells in the

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