

MET-dependent cancer invasion may be preprogrammed by early alterations of p53-regulated feedforward loop and triggered by stromal cell-derived HGF

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MET, a receptor protein tyrosine kinase activated by hepatocyte growth factor (HGF), is a crucial determinant of metastatic progression. Recently, we have identified p53 as an important regulator of MET-dependent cell motility and invasion. This regulation occurs via feedforward loop suppressing MET expression by miR-34-dependent and -independent mechanisms. Here, by using Dicer conditional knockout, we provide further evidence for microRNA-independent MET regulation by p53. Furthermore, we show that while MET levels increase immediately after p53 inactivation, mutant cells do not contain active phosphorylated MET and remain non-invasive for a long latency period at contrary to cell culture observations. Evaluation of mouse models of ovarian and prostate carcinogenesis indicates that formation of desmoplastic stroma, associated production of HGF by stromal cells and coinciding MET phosphorylation precede cancer invasion. Thus, initiation mutation of p53 is sufficient for preprogramming motile and invasive properties of epithelial cells, but the stromal reaction may represent a critical step for their manifestation during cancer progression.

Introduction

MET receptor protein tyrosine kinase is a key regulator of cell motility and invasion.^{1,2} Not surprisingly, aberrant activation of MET has been associated with increased metastatic propensity of cancer

cells as well as poor prognosis of cancer patients.³ Stimulation of MET is largely accomplished by its ligand, hepatocyte growth factor (HGF), also known as scatter factor.^{4,5} Upon HGF binding to the extracellular domain of MET, tyrosine residues Y1234 and 1235 are phosphorylated, leading to activation of the autocatalytic domain. Subsequently, the MET C-terminal binding domain (Y1349/1356) is phosphorylated, further stimulating downstream targets, such as metalloproteases, osteopontin, plasminogen activator and integrins.⁶ HGF has been originally identified as a fibroblast-derived epithelial morphogen,^{5,7} and HGF-MET pathway is regarded to be a prototypical example for stromal-epithelial interactions during developmental morphogenesis, wound healing, organ regeneration and cancer progression.^{1,2,8}

Recently we have reported that MET-dependent cell motility and invasion are controlled by tumor suppressor p53.⁹ p53 executes its control by two mechanisms: (1) transactivation of miR-34 genes that target MET 3'UTR¹⁰⁻¹² and (2) inhibition of SP1-activating binding to MET promoter.⁹ Since both mechanisms lead to suppression of MET expression, this type of regulation can be classified as a coherent type 3 feedforward loop or type II circuit based on previously proposed classifications of signaling pathways.^{13,14}

Since genes encoding miR-34 family were first identified as direct targets of p53 transcriptional activation,^{10,15-19} a number of other microRNAs were additionally reported to be regulated by p53.²⁰

Key words: feedforward loop, HGF, invasion, MET, metastasis, motility, p53 mutation

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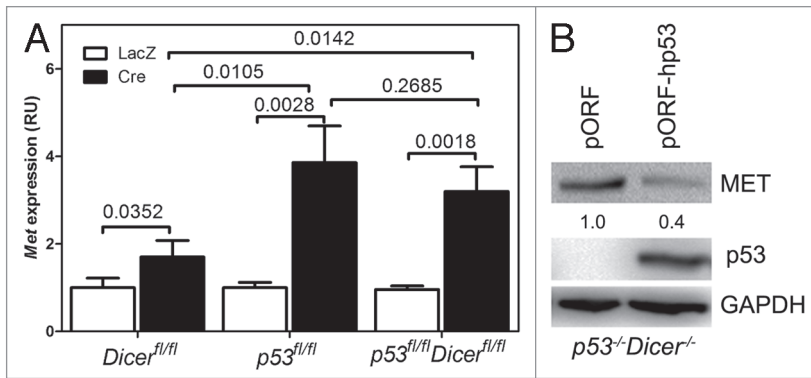


Figure 1. miR-34-independent regulation of MET in microRNA-free environment. (A) *Met* expression in primary OSE cells isolated from *Dicer^{fl/fl}*, *p53^{fl/fl}* and *Dicer^{fl/fl}p53^{fl/fl}* mice. *Met* mRNA expression (mean \pm SD, n = 3) was measured by qRT-PCR 48 h after infection with Ad-Cre or Ad-LacZ. (B) MET expression in *p53^{-/-}Dicer^{-/-}* OSE cells 48 h after transfection with either empty vector (pORF) or pORF-hp53.

Thus, it remains uncertain if p53-dependent expression of other microRNAs in addition to miR-34 may have additional impact on MET regulation.

We have reported that elevated levels of MET and associated increases in cell motility and invasion were observed in cell culture within first 24–72 h after Cre-*loxP* mediated *p53* inactivation.⁹ Addition of HGF further stimulated motility and invasion of p53-deficient cells ex vivo.⁹ Elevated levels of MET were also observed in the ovarian surface epithelium (OSE) in vivo 72 h after *p53* inactivation. However, in the mouse model of epithelial ovarian cancer (EOC) based on conditional inactivation of *p53* and *Rb* in OSE, invasion by mutant cells is rarely observed before 100–120 d after the initiation, and EOC develops after a long latency period, with median survival time of 227 d.^{21,22} These results indicate that immediate implementation of the phenotypical traits associated with alterations in p53 and Rb pathways is either prevented by some compensatory mechanisms or requires some additional exogenous stimuli similar to those present in cell culture medium.

Here, we explore the feedforward loop regulation of MET in the microRNA-free cell system. Furthermore, by using autochthonous mouse models of high-grade serous EOC and prostate carcinoma, we provide evidence that stromal-epithelial interactions may play a crucial role in cancer pathogenesis by activating HGF-MET signaling and thereby facilitating motility and invasion of *p53* mutant cells.

MicroRNA-Dependent Regulation of MET Mainly Depends on miR-34 Family but is Insufficient for Complete MET Control

Previously, we have shown that p53 has a miR-34-independent regulation of MET expression in miR-34-deficient background.⁹ To test whether there are other microRNAs targeting MET, in addition to miR-34 family, we performed a bioinformatics search using microRNA data sets obtained from OSE cells within two passages after *p53* inactivation.¹⁸ Besides miR-34 family, none of the microRNAs downregulated after *p53* inactivation had a seed sequence predicted to target MET. While the mechanism of microRNA action is believed to be either mRNA degradation or destabilization by 3'UTR binding, some small dsRNAs and microRNAs have been identified to activate promoter activities through complementary sequence binding.^{23,24} However, none of microRNAs upregulated after *p53* inactivation¹⁸ had a seed sequence predicted to bind *MET* promoter region or 5'UTR. These results further indicate that the microRNA-dependent component of MET regulation by p53 is mainly represented by members of miR-34 family, at least in OSE cells.

To further confirm microRNA-independent MET regulation by p53, primary OSE cells were derived from *Dicer^{fl/fl}*, *p53^{fl/fl}* and *Dicer^{fl/fl}p53^{fl/fl}* mice. Consistent with the lack of mature microRNAs, including miR-34, in *Dicer*-null cells,²⁵

Dicer inactivation led to an increase of *Met* expression by 1.7-fold (Fig. 1A). However, concomitant inactivation of *p53* and *Dicer* resulted in further significant elevation of *Met* levels, similar to levels after *p53* inactivation alone. At the same time, reconstitution of p53 expression in *p53*- and *Dicer*-null OSE cells resulted in down-regulation of MET expression (Fig. 1B). Thus, our findings support our earlier observations of existence of miR-34-independent regulation of MET by p53.

MET Activation Coincides with Accumulation of Desmoplastic Stroma and Expression of HGF by Connective Tissue Cells

To determine the time of MET activation, we have performed immunohistochemical detection of functionally active phosphorylated MET (P-MET) at various time after initiation of carcinogenesis in the mouse model of high-grade serous EOC associated with p53 and Rb deficiency.²¹ In this model cancer is induced by a single administration of the adenovirus expressing Cre into the ovarian bursa of adult mouse, and tumors develop relatively synchronously.²¹ Thus, this model is well-suited for stage-by-stage studies of carcinogenesis. Importantly, *p53* mutations are detected in 96% cases of high-grade serous EOC²⁶ and reported to be the early or initiating event in this EOC type.^{27,28} Taken together with the fact that alterations of RB pathways occur in 67% of cases of high-grade serous EOC,^{26,29} this mouse model is highly relevant to the pathogenesis of human EOC.

Consistent with our previous reports in references 22 and 30, early atypical cells have been consistently detected beginning 60 d after *p53* and *Rb* inactivation. Desmoplastic stroma accumulates under dysplastic lesions within 160–220 d following by development of highly invasive and metastatic carcinomas. Expression of functionally active P-MET was below detectable level in normal OSE, while it was easily detectable in invasive adenocarcinomas (Fig. 2). Interestingly, P-MET was barely detectable in early atypical cells at 60 d after EOC induction, even so that inactivation of both *p53* and *Rb* in over 80% of OSE has been confirmed

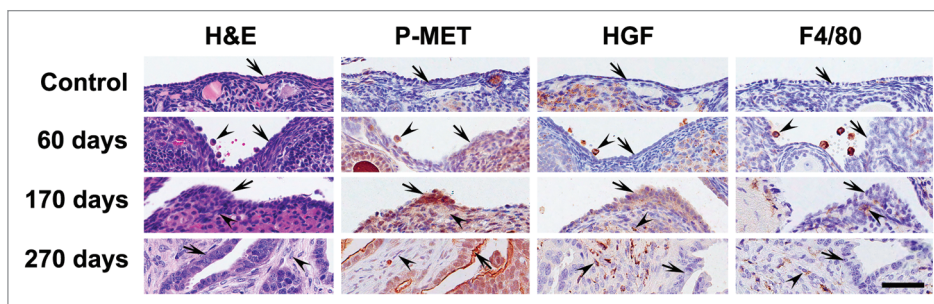


Figure 2. Expression of phosphorylated MET (P-MET) during OSE carcinogenesis. Normal OSE (Control, arrow) and preinvasive neoplastic cells (60 d, arrow) above the regular stroma express negligible levels of P-MET. Preinvasive OSE (170 d, arrow) above desmoplastic stroma (arrowhead) and invasive neoplastic OSE cells (270 d, arrow) contain detectable amount of P-MET. Note expression of HGF α (HGF) and F4/80 in macrophages (60 d, arrowheads) and desmoplastic cells and macrophages/histiocytes (170 and 270 d, arrowheads). Hematoxylin-eosin (H&E) and ABC Elite method, hematoxylin counterstaining (P-MET, HGF and F4/80). Calibration bar, all images, 50 μ m.

by microdissection-PCR genotyping as well as EGF and LacZ reporters.^{9,21,22} At the same time, P-MET was easily detected in dysplastic OSE above desmoplastic stroma. Appearance of P-MET coincided with elevated expression of HGF in stromal cells forming desmoplastic response. In an agreement with this observation, HGF from fibroblasts has been shown to activate MET in epithelial cancer cells.³¹ Interestingly, while diffuse expression of HGF was observed in the majority of desmoplastic cells, its particularly high levels were associated with cells of macrophage/histiocyte morphology. Together with our recent cell culture studies showing that addition of HGF stimulates motility and invasion of p53-deficient OSE cells,⁹ these results suggest that migratory and invasive features of p53 mutant cells are facilitated by desmoplastic stroma during cancer progression.

p53 and/or Rb Inactivation do not Upregulate HGF Expression in OSE Cells

Previously it has been reported that human, rat and bovine OSE also expresses HGF.^{32,33} At the same time, expression of HGF was rarely observed in the OSE cells from women with no family histories of ovarian cancer.³⁴ Notably, even in the OSE of women with familial history of ovarian cancer or ovarian cancer cell lines, the levels of HGF were far lower than those in fibroblasts. By using our previous microarray data,^{9,35} we have found that mouse OSE have only negligible levels of *Hgf*, which were significantly lower

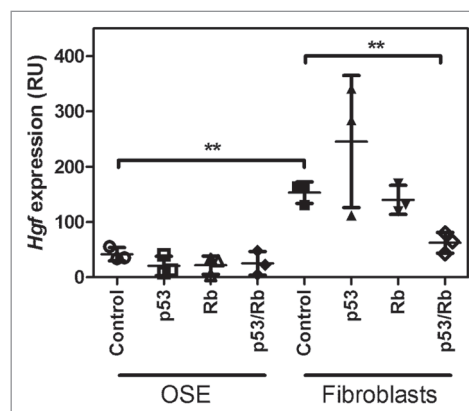


Figure 3. *Hgf* expression in OSE cells and fibroblasts after p53, Rb or p53/Rb inactivation. Signal intensity of *Hgf* expression (mean \pm SD, n = 3) was measured by Affymetrix GCOS software and normalized by scaling each GeneChip to a target signal of 500. **p < 0.01.

than those in primary mouse fibroblasts (Fig. 3). Importantly, no significant elevation of *Hgf* expression was observed in OSE cells after p53, Rb or p53/Rb inactivation (Fig. 3), while a modest decrease of *Hgf* expression was observed in fibroblasts lacking both p53 and Rb.

P-MET is Associated with Advanced Stages of Prostate Cancer

To test if our observations can be applicable to other types of cancer, we evaluated a mouse model of prostate cancer previously established by us. In this model, conditional inactivation of p53 and Rb in the prostate epithelium results in rapidly progressing metastatic carcinomas.³⁶ Notably, while atypical lesions were present in both proximal and distal regions of prostatic ducts, only the

former progressed to overt cancers.³⁷ Our present immunohistochemical studies indicate that only proximal neoplastic lesions exhibit elevated MET expression (Fig. 4), which may explain why progression rate of proximal vs. distal region-derived neoplasms greatly depends on microenvironment.³⁷ Consistent with these results, particularly high levels of P-MET are observed in the neoplastic cells positioned in proximity to the desmoplastic stromal cells expressing HGF (Fig. 4).

Discussion

The biological meaning of coherent feed-forward loop regulation remains insufficiently understood.^{38,39} It has been suggested that such a type of regulation contributes to filtering out fluctuations in input stimuli, while allowing rapid biological responses and surveillance

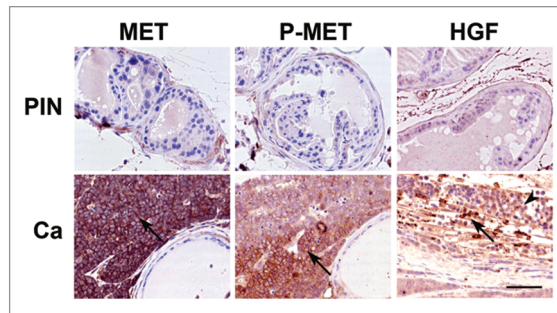


Figure 4. Expression (arrows) of total (MET) and phosphorylated (P-MET) MET and HGF α (HGF) in prostate neoplastic lesions. Carcinomas (Ca) and prostate intraepithelial neoplasms (PIN), are located, respectively, in the proximal and distal regions of prostatic ducts of mice with prostate epithelium-specific inactivation of *p53* and *Rb*. Note absence of MET expression in PIN and HGF α location in stromal (arrow) but not epithelial (arrowhead) cells. ABC Elite method, hematoxylin counterstaining. Calibration bar, 50 μ m.

mechanisms necessary to regulate a leaky transcription of target genes.¹³ The functional redundancy of coherent feedforward loops might also facilitate signal transduction or provide selective advantages during the process of evolution.⁴⁰

Recently we have shown that the degree of *p53*/miR-34/MET signaling network's impact on cell motility and invasion depends on the extent of alterations in its individual components.⁹ Mutant *p53* proteins commonly contain point mutations in DNA binding domain, which renders them unable to transactivate miR-34-encoding genes. However, such mutants are still able to bind SP1 and thereby repress *MET* promoter activity, albeit less efficiently than wild-type *p53* (Fig. 5). Complete lack of *p53* abolishes both mechanisms of MET regulation, leading to its maximal expression and associated metastasis-related cancer traits, such as cell motility and invasion. Consistent with these findings, it has been reported that patients with *p53*-null ovarian cancer have worse prognosis than those with other *p53* status in their cancers.⁴¹⁻⁴³ Furthermore, the poorest prognosis was also reported for patients with *p53*-null cancers of the lung⁴⁴ and breast.⁴⁵⁻⁴⁷ Notably, MET overexpression has been reported to be associated with poor prognosis of patients with these types of cancer as well.⁴⁸⁻⁵⁰ Therefore, our observations that *p53* has a feedforward loop regulation of MET expression may provide a mechanistic link between two independently reported prognostic factors, *p53*-null status and MET overexpression.

Detailed understanding of molecular mechanisms of feedforward loop regulation of MET may reshape our approach on cancer therapeutics. While inhibition of MET has been considered as an important drug target and has been under clinical trials,⁵¹ restoration of wild-type *p53* in ovarian cancer patients has failed.⁵² Our recent results⁹ suggest that in the case of patients with *p53* mutations retaining *MET* promoter repressive role, removal of mutant *p53* should be avoided. In addition to miR-34 reintroduction, considered as a promising therapeutic approach, intervention of SP1 function might be beneficial for cancer treatment. Recently, tolfenamic acid, a small-molecule SP1 inhibitor that is able to induce degradation of proteins of SP1, has been identified.^{53,54} As expected, this inhibitor downregulates MET expression in cancer cells, including ovarian cancer cells.^{55,56}

Carcinogenesis, including its metastatic stages, is usually described as a multistage process driven by sequential accumulation of genetic alterations responsible for expression of phenotypical traits beneficial for selection of the most autonomous and, by extension, most malignant cell clones, assuring further progression.⁵⁷⁻⁶¹ However, there are multiple evidences that the initiating mutation is continuously required for the maintenance of malignant phenotype (reviewed in ref. 62 and 63). Reconsolidation of the concept of cancer as a multigenic disease and tumor suppression by re-introduction or elimination of a single gene is complicated, because many tumors are genomically unstable,

and separation of critical alterations from "genetic noise" may be a daunting task in advanced stages of the disease. However growing evidence points to the possibility that some neoplastic cells may already have metastatic ability during the early stages of carcinogenesis.⁶⁴⁻⁶⁷ Furthermore, interactions between neoplastic cells and stroma may further affect carcinogenesis (reviewed in refs. 68 and 69). Our findings support this recent shift in paradigm of sequential cancer progression, indicating that an initiating mutation, such as *p53* inactivation, may preprogram advanced cancer traits, such as cell motility and invasion. At the same time, microenvironmental cues, as opposed to genetic or epigenetic alterations within the mutant cells, are critical for triggering preprogrammed invasive traits (Fig. 5). These observations are of particular interest, because the role of stroma in cancer pathogenesis receives increasing attention.⁷⁰ In the ovary, inflammatory stromal reaction is regarded as an essential part of the ovulatory process in the normal ovary⁷¹ and, thereby, may provide a missing link between inflammation and EOC pathogenesis.⁷² Importantly, our results in the prostate cancer model suggest a common critical role of stromal-epithelial interactions in carcinogenesis associated with alterations in *p53*/miR-34/MET signaling network.

Taken together, our recent and current studies have shed additional light on the role of feedforward loop regulation and stromal-epithelial interactions in cancer pathogenesis. Further elucidation of these mechanisms should facilitate development of personalized therapeutic approaches.

Materials and Methods

Experimental animals. Mice with floxed copies of *p53*, *Rb1* (*Rb*) and *Dicer1* allele genes and information for their genotyping were described in references 21, 25, 73 and 74. Modeling of EOC and prostate carcinoma associated with *p53* and *Rb* deficiency has been described by us in references 21 and 36. All mice were maintained identically following recommendations of the Cornell Institutional Laboratory Animal Use and Care Committee.

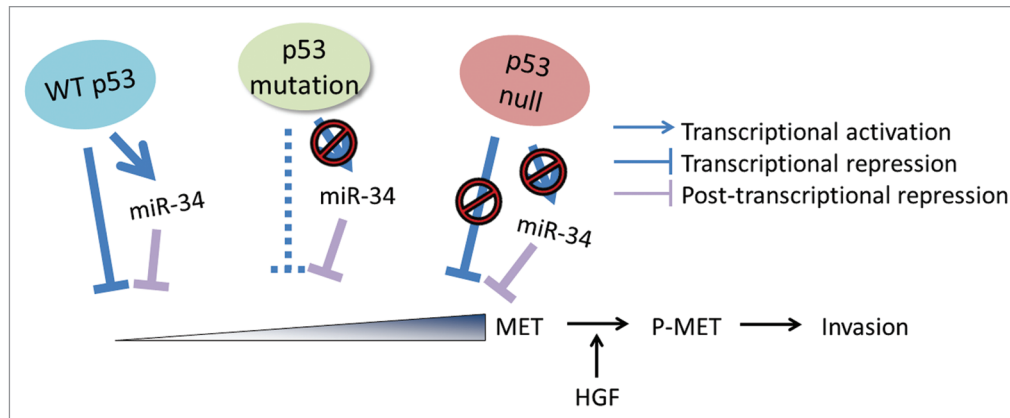


Figure 5. Proposed model of MET-dependent cancer invasion preprogrammed by early alterations of p53-regulated feedforward loop and triggered by stromal cell-derived HGF. Feedforward loop regulation of MET by wild-type p53 consists of miR-34-dependent and -independent mechanisms. p53 protein with point mutations in its p53 DNA binding domain is unable to transactivate MET targeting miR-34. However, it still binds SP1, thereby repressing *MET* promoter, albeit to a limited extent. In contrast, lack of p53 due to null mutations results in incapacitations of both miR-34 transactivation and *MET* promoter repression, thereby leading to highest levels of MET. Although p53 mutations result in increased expression of MET, cells become highly motile and invasive only after MET activation by phosphorylation (P-MET). This phosphorylation is triggered by MET ligand HGF produced by cells of newly forming desmoplastic stroma. Thus, while alterations in p53/miR-34/*MET* network preprogram cells for increased motility and invasion, microenvironment may play a crucial role in triggering those properties during cancer progression.

Immunohistochemical study. Paraffin sections of 4% paraformaldehyde fixed tissue were stained according to modified the avidin-biotin-peroxidase technique.^{35,75} The antibodies to MET (1:50; Santa Cruz Biotechnology), Phospho-MET Tyr1349 (1:50 Cell Signaling Technology), HGF α (1:150, H145, Santa Cruz Biotechnology), and F4/80 (1:200; Serotec), were incubated for 1 h at room temperature.

Cell culture. Primary mouse OSE cells were isolated and cultured as previously described in references 18, 21 and 30. In order to establish a *p53/Dicer*-deficient cell line, primary OSE cells were prepared from the ovary of *p53^{fl/fl}Dicer^{fl/fl}* mice, infected with Ad-*Cre* and passaged upon reaching confluence for 30 passages under the same conditions as other mouse OSE cell lines.

Quantitative real-time RT-PCR. Total RNA was isolated using Qiazol (Qiagen) according to manufacturer's protocol. Total RNA was reverse transcribed using SuperScript III (Invitrogen) and oligo-dT primers. The PCR reactions were done in a 30- μ l volume in a 96-well plate using pre-developed FAM TaqMan probes (Applied Biosystems). Mouse *Gapdh* was used as endogenous reference control (pre-developed TaqMan assay from Applied Biosystems). Cycling parameters were as follows: 95°C for 10 min. followed by 40 cycles of 95°C for 15 sec and 60°C for

1 min. Increase in real-time fluorescence was measured by an ABI7500 qPCR system and relative fold changes were calculated using the $2^{-\Delta\Delta C_t}$ method.⁷⁶

Protein gel blot analysis. Cell lysates were prepared using RIPA buffer (TRIS-HCl 50 mM, pH 7.4; NP-40 1%; Na-deoxycholate 0.25%; NaCl 150 mM; EDTA 1 mM; PMSF 1 mM; Aprotinin, leupeptin, pepstatin; 1 μ g/ml each; Na₃VO₄ 1 mM; NaF 1 mM), separated by 12% SDS-PAGE and transferred to PVDF membrane (Millipore). The membrane was incubated overnight at 4°C with antibodies to detect MET (SP 260, Santa Cruz Biotechnology, 1:1,000 dilution), p53 (FL 393, Santa Cruz Biotechnology, 1:1,000 dilution) and GAPDH (Advanced Immunohistochemical Inc.; 1:3,000 dilution) followed by incubation for 1 hr at room temperature with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) and developed using chemiluminescence substrate (SuperSignal West Pico from Pierce).

Bioinformatics. For prediction of microRNAs binding to 3'UTR of *MET*, Targetscan 5.2 (www.targetscan.org), Pictar (pictar.mdc-berlin.de/) and miRanda (www.microrna.org) database were searched. For prediction of microRNAs binding to 5'UTR and upstream promoter region of *MET*, miR-Walk (www.ma.uni-heidelberg.de) database was used.

Statistical analysis. Statistical tests used were two-sided Student's t-tests, using Prism 5.01 software (GraphPad, Inc.).

Acknowledgments

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