Depletion of FOXM1 via MET targeting underlies establishment of a DNA damage-induced senescence program in gastric cancer

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Running Title:

MET, FOXM1 and DNA damage-induced senescence

Keywords:

MET, FOXM1, senescence, DNA damage, gastric cancer

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Disclosure of Potential Conflicts of Interest: A. Blaukat is employee of Merck KGaA. There are no other conflicts of interest to disclose.

Financial support: This study was supported by the Hedy und Werner Berger-Janser Stiftung grant (to M.M.) and by the Swiss National Science Foundation (grant. no. 31003A_156816 to Y.Z. and M.M.).
Statement of Translational Relevance

It is widely accepted that cellular senescence may function as a fundamental barrier of tumor progression. Therefore, the significance of senescence and underlying mechanisms for particular clinical interventions, including tyrosine kinase targeting-based modalities, needs to be well defined. Interestingly, despite numerous studies evaluating efficacies of MET targeting strategies in both preclinical and clinical settings, the relevance of MET inhibition-associated senescence in MET-positive tumors remains still largely uncharacterized. Here we demonstrate that induction of a senescence program is the major consequence in MET-driven models of gastric cancer following exposure to a combined regimen of the MET inhibitor tepotinib and irradiation. Mechanistically, the work identifies FOXM1 as a critical negative regulator of senescence that directly associates to MET targeting-related DNA damage. Furthermore, the work demonstrates co-aberrant expression of MET and FOXM1 in gastric cancer patients samples and suggests FOXM1 expression level as a novel potential marker for patients stratification for MET-based therapies.
Abstract

Purpose: Deregulated signaling via the MET receptor tyrosine kinase is abundant in gastric tumors, with up to 80% of cases displaying aberrant MET expression. A growing body of evidence suggests MET as a potential target for tumor radiosensitization.

Methods: Cellular proliferation and DNA damage-induced senescence were studied in a panel of MET-overexpressing human gastric cancer cell lines as well as in xenograft models following MET inhibition and/or ionizing radiation. Pathways activation and protein expression were assessed by immunoblotting and immunohistochemistry. Tumor tissue microarrays (91 gastric cancer patients) were generated and copy number alteration (178 patients) and gene expression (373 patients) data available at The Cancer Genome Atlas were analyzed to assess co-alterations of MET and FOXM1.

Results: MET targeting administered prior ionizing radiation instigates DNA damage-induced senescence (~80%, P<0.001) rather than cell death. MET inhibition-associated senescence is linked to blockade of the MAPK pathway, correlates with downregulation of FOXM1 and can be abrogated (11.8% vs. 95.3%, P<0.001) by ectopic expression of FOXM1 in the corresponding gastric tumor cells. Cells with ectopic FOXM1 expression demonstrate considerable (~20%, P<0.001) growth advantage despite MET targeting, suggesting a novel clinically-relevant resistance mechanism to MET inhibition as the co-presence of both MET and FOXM1 protein (33%) and mRNA (30%) overexpression as well as gene amplification (24.7%) is common in patients with gastric cancer.

Conclusions: FOXM1, a negative regulator of senescence, has been identified as a key downstream effector and potential clinical biomarker that mediates MET signaling following infliction of DNA damage in gastric tumors.
Introduction

Signaling networks downstream of MET that drive normal physiological processes are exploited in cancer cells to promote key features such as tumor uncontrolled growth, survival, angiogenesis, local invasion and systemic dissemination (1). Dysregulation of the MET/hepatocyte growth factor (HGF) axis via establishment of ligand-receptor autocrine loops, presence of activating point mutations and most frequently via receptor overexpression have emerged as common events of many human malignancies (1, 2). In that respect MET overexpression has been described in various solid tumors including colorectal, hepatocellular, ovarian, pancreatic, prostate and gastric carcinomas (2).

Gastric cancer remains the fifth leading cause of cancer-related death worldwide (3). Most patients are diagnosed with locoregionally advanced or metastatic disease, and despite improvements in surgical and multimodal approaches combining chemotherapy and radiation, patients with non-resectable tumors have a median survival of only 10 months (4). In this respect, approaches targeting oncogenic drivers of gastric tumors such as HER2 (overexpressed in 10-15% of cases) have emerged as promising treatment strategies (5).

MET overexpression, which is reported in 18–82% of gastric cancer cases (6), correlates with advanced-stage tumors and poor prognosis (6, 7). In a subset of patients, MET overexpression is associated with increased MET copy number due to gene amplification (8, 9). Additionally, about 20% of human gastric cancer cell lines exhibit MET amplification with a corresponding MET-addiction phenotype, rendering these tumor cells extremely sensitive to MET inhibition (10). Accordingly, MET is considered to be a prognostic marker and at the same time an important therapeutic target for locally advanced or metastatic gastric cancers (11, 12). Currently, several anti-MET antibodies and small molecule MET inhibitors are undergoing Phase I and Phase II clinical trials in different types of solid tumors, including gastric cancer (e.g. NCT02435108, NCT01468922, NCT00725712, NCT02002416), with only a modest benefit in otherwise unselected patient populations (13).

A growing body of evidence suggests MET as a potential target for tumor sensitization to DNA damaging agents (DDAs), such as ionizing radiation (IR), which are commonly used in
cancer therapy (14-16). In 2011 Medová et al. (17) reported that inhibition of the MET receptor tyrosine kinase (RTK) impairs the ability of irradiated MET-overexpressing gastric cancer cell lines to properly execute DNA double strand breaks (DSBs) repair. It is well established that persisting DNA damage may trigger a broad range of different outcomes on affected cells including apoptosis, necrosis, mitotic catastrophe or senescence (18, 19). Cellular senescence was first described in a seminal study by Hayflick and Moorhead in 1961 (20) as a limit to the replicative lifespan of somatic cells after serial cultivation in vitro. More recently it was shown that senescence can be triggered also by other stimuli such as oncogene expression, oxidative stress and DNA damage (18, 21). This premature senescence seems to play a critical role in tumor suppression by preventing cancer initiation and progression (22) and often accompanies apoptotic response elicited by chemotherapy or IR (23, 24).

FOXM1, a member of the Forkhead box (FOX) superfamily of transcription factors (25), is ubiquitously expressed in actively proliferating tissues and plays an essential role in the regulation of a wide spectrum of biological processes (26). Elevated expression of FOXM1 has been detected in a broad range of malignancies including gastric cancers and has been shown to correlate with tumor progression and poor prognosis (27). In recent years, numerous studies have reported FOXM1 playing a crucial role in both homologous recombination repair of DNA DSBs (28, 29) and negative regulation of the senescence program (29, 30), significantly turning research interests towards FOXM1 as a potential target in cancer therapy.

In this study, we investigated the impact of MET inhibition on irradiated gastric cancer models both in vitro and in vivo. Our data show that MET inhibition sensitizes human gastric cancer cells to IR by promoting DNA damage-induced senescence, conceivably via interfering with the MAPK pathway. We report that impairment of the MET signaling cascade leads to accumulation of unrepaired DNA DSBs in parallel to a down-regulation of FOXM1 protein levels, providing thereby for the first time evidence for a role for FOXM1 downstream of MET signaling. Although the regulatory mechanism of FOXM1 with respect to cancer
development and progression are still poorly understood, our current findings provide important mechanistic insights into the role of FOXM1 in DNA damage-induced senescence following MET inhibition. Furthermore, assessment of MET and FOXM1 protein levels in gastric cancer tissue microarrays (TMAs) and analysis of the TCGA database show that co-alteration of MET and FOXM1 is common in patients with gastric cancer. These observations may have important therapeutic translational implications for the treatment of gastric cancer through MET targeting.
Material and Methods

Cell lines

Human gastric carcinoma cells GTL-16, MKN-45 and SNU-5 were provided by the laboratory of Dr. Paolo Comoglio (Medical School University of Torino, Italy). GTL-16 cells were grown in RPMI medium (GIBCO, Invitrogen) supplemented with 5% FCS (Sigma) and antibiotic-antimycotic (penicillin 100 U/ml, streptomycin sulfate 100 U/ml, amphotericin B as Fungizone 0.25 mg/ml; GIBCO). MKN-45 cells were maintained in DMEM (GIBCO, Invitrogen) with 10% FCS and antibiotic-antimycotic, while SNU-5 were grown in IMDM (GIBCO, Invitrogen) with 20% FCS and antibiotic-antimycotic. SNU-638 and KATO-II gastric carcinoma cells ((Korean Cell Line Bank) and Dr. Morag Park (McGill University, Montreal, QC, Canada), respectively) were maintained in RPMI with 10% FCS. All cell lines were obtained within the last 5 years. No cell lines authentication has been performed by the authors for this study. The SNU638_FOXM1 and GTL16_FOXM1 cell lines were established by transfecting SNU638 and GTL16 cells with a Flag-FoxM1-NT2 expression vector (kindly provided by Prof. René Medema, Netherlands Cancer Institute, Amsterdam) that contains a geneticin selection marker. Cells were then selected and maintained in presence of 500 μg/mL of geneticin (Fisher Scientific).

shRNA Lentiviral Transduction

Stable transduction with lentiviral vectors for scrambled control and p53 short-hairpin RNA (shRNA) were described previously (31).

Treatments

Tepotinib (EMD-1214063; Merck), AZD6244 (Selleckchem) and LY294002 (Sigma) were dissolved in DMSO. Cells were irradiated using Gammacell 40 (MDS Nordion, Ottawa, Canada), animals using XStrahl 150 (Xstrahl Limited, Surrey, UK).

Western blotting and antibodies
Cells were lysed (20 mM HEPES (pH 8.0), 9.0 M urea, 1 mM sodium orthovanadate, 2.5 mM sodium pyrophosphate, 1mM β-glycerol-phosphate) and sonicated. Protein concentration was determined (Bio-Rad Laboratories, Inc.), proteins were resolved by SDS–PAGE, transferred onto PVDF membranes and followed by incubation with primary (rabbit phospho-MET (Tyr1234/1235), MET, Ki67, phospho-Akt (Ser473), phospho-Erk1/2 (Thr202/Tyr204), phospho-p53 (Ser15), p53, BRIP1 and BMI1 from Cell Signaling Technology (CST; Danvers, MA, USA); phospho-Histone H2AX, p21, Histone H3 (tri methyl K9) and β-Actin from Millipore Corporation; FOXM1 and p16 from Santa Cruz Biotechnology (CA, USA)) and secondary antibodies.

**Cell Viability/Toxicity Assay**

Cell death and viability were assessed by the Live/Dead assay kit (Molecular Probes). Briefly, cells were stained with green-fluorescent calcein-AM (viable cells) and red-fluorescent ethidium homodimer-1 (dead cells). Images were analyzed with a fluorescence microscope (Leica) at 10x magnification.

**Senescence-associated-β-galactosidase assay**

Cells were seeded in 6-well plates and treated after 24 hours. Staining for senescence-associated β-galactosidase was done as previously described (32) following 7 days of treatment. Images were obtained with a Leica inverted microscope at 40X magnification. For the in vivo study, frozen sections of xenograft tumors (5 µm) were fixed in 2% glutaraldehyde and stained as the in vitro experiments and then counterstained with haematoxylin for 1 minute.

**BrdU incorporation assay**

BrdU incorporation assay was performed in vitro using the BD Pharmingen BrdU Flow Kit (BD Biosciences). Briefly, cells were seeded in 6-well plates and treated after 24 hours as
indicated for 3 days. BrdU at a final concentration of 10 μM was added overnight to the cells before performing the staining protocol. Samples were acquired on LSRII flow cytometer (Beckton Dickinson™) and analyzed using the FlowJo software.

**In vivo model**

GTL-16 and SNU-638 xenografts were grown in 12-week-old female Rag2 common gamma-null mice. A total of 10⁶ cells were injected subcutaneously on the right flank of each mouse and the growth of the tumors was regularly monitored by caliper measurements. Once tumors had reached a size of 300mm³ (day 0), mice were randomly divided in the following treatment groups: vehicle control (Solutol HS 15, BASF Chem-Trade GmbH), tepotinib only (15mg/kg/d per os, days 1-6), radiation only (one single 6 Gy radiation dose, on day 3) and tepotinib with radiation. Tumors were harvested 24 hours after the last tepotinib treatment (on day 7), frozen in Tissue-Tek optimum cutting temperature medium (OCT; Sakura Finetek Europe B.V.) and stored at -20°C.

**Immunohistochemical analysis**

Tissue samples were cut into 5 μm sections with a Leica Microtome and fixed in 4% paraformaldehyde (PFA) for 10 minutes. Sections were washed with 1x phosphate buffered saline (PBS)-Tween 0.1% (PBST) and antigen retrieval was performed in 1xPBS-Triton-X 0.1% (FLUKA) for 10 minutes. Endogenous peroxidase was blocked with H₂O₂ 0.1% in H₂O for 10 minutes. Sections were blocked with 1% normal goat serum in PBS for 1 hour. Primary rabbit antibodies were incubated at room temperature (RT) in blocking solution overnight. Sections were washed in PBST and incubated with secondary antibody at RT for 1 hour. Signal was detected using the Vectastain ABC Kit (Vector Laboratories) and 3,3’-Diaminobenzidine (DAB) (Sigma-Aldrich). DAB incubation varied between 3-10 minutes and was rinsed with distilled H₂O before counterstaining with haematoxylin for 1 minute. Dehydrated sections were mounted using Eukitt (Kindler, GmbH). Images were obtained with a Leica DMRB microscope (20X and 40X magnifications).
**XTT assay**

Cells were plated into a 96-well plate in 50μl of the corresponding medium (six wells per treatment condition) and the next day treated with tepotinib. Seventy-two hours later, cellular proliferation was measured using the commercially available kit (based on the cleavage of the yellow tetrazolium salt XTT to form an orange formazan dye by metabolic active cells; Roche). Briefly, at the end of treatment cells were incubated with the XTT reagent at 37°C, 5% CO2 for 2 hrs; the resulting formazan was quantified by measuring the absorbance at 490 nm (reference wavelength: 655nm) using a Tecan Platereader.

**Tumor tissue microarrays (TMAs) and immunohistochemistry**

TMA was built using a next-generation tissue microarray, H&E slides of all samples were scanned for morphologic assessment by a board-certified pathologist (R.L.). In addition, a commercially-available TMA of gastric cancer patients was purchased from Lucerna Chem. TMA sections were deparaffinized and rehydrated. Antigen retrieval was performed by heating the slides for 20-30 in a microwave oven (600-700 W) in either 0.01 M sodium citrate or Tris-EDTA (pH 9.0). Sections were incubated overnight at 4°C with primary antibodies (all CST): Ki67 (1:400; D2H10), MET (1:150; D26), and FOXM1 (1:600; D1205).

Stained TMAs were scored semi-quantitatively by three independent observers blinded to each other’s results (grading: 1=negative/low; 2=intermediate; 3=high).

**Extraction and analysis of data from The Cancer Genome Atlas (TCGA) database**

RNASeq data (297 patients sequenced in Illumina HiSeq 2000 (HiSeq) platform) was normalized according to the trimmed mean of M-values normalization method using the edgeR package (33).

CNV data was available from two different platforms, SNP arrays and Low Pass DNASeq, with 443 and 107 patients profiled on each technology respectively. In order to avoid platform
biases, we only selected data from SNP arrays for subsequent analysis. We extracted CNVs that overlapped with MET or FOXM1. Genes start and end positions were from the UCSC Known Genes dataset, GRCh37/hg19-Feb 2009 genome reference. Transcription start and end positions were augmented by 5 kb to account for promoter and enhancers. If two or more CNVs were present in a gene in the same sample, we only considered the CNV with highest absolute value.

After preprocessing, RNASeq, CNV and protein levels data were z-transformed,

\[ z = \frac{(x - mean(x))}{std(x)} \]

We used a threshold of ±1.5 on the z-scores to consider a sample overexpressed or downregulated with respect to the population, or respectively amplified or deleted.

**Statistical analysis**

Statistical analysis was performed with GraphPad Prism Software using the One-Way Analysis of Variance or Student t test as indicated in legends to the figures.
Results

*MET inhibition along with IR inflicts DNA damage-induced senescence rather than cell death in MET-overexpressing human gastric cancer cell lines.*

As activation of the MET receptor was reported to protect cancer cells from IR-induced cell death by enhancing DNA damage repair activity (14, 17), here we examined the effect of MET inhibition by the small molecule tyrosine kinase inhibitor tepotinib (MSC2156119; Merck) alone or along with IR in MET-overexpressing human gastric cancer cell lines GTL-16, MKN-45, SNU-638, SNU-5 and KATO-II. Importantly, we observed that exposure of cells to low doses of tepotinib alone or in combination with IR resulted in a significant decrease in cell proliferation as indicated by loss of incorporation of BrdU (Fig. 1A and Suppl. fig. 1A, 1B). Surprisingly, the treatment did not induce massive cell death (Fig. 1B and Suppl. fig. 1C and 1D). Accumulating evidence suggests that tumor cell response to traditional cytotoxic chemotherapy, which aim at inducing extensive DNA damage, is not strictly confined to cell death but may also lead to the induction of cell senescence (24, 34, 35).

As irreversible cell-cycle arrest is a feature of cellular senescence, the panel of human gastric cancer cell lines was examined for senescence-associated β-galactosidase (SA-βGal) activity, the most widely used senescence biomarker. In all the studied cell lines, MET inhibition enhanced SA-βGal staining particularly in combination with IR and notably, this was accompanied by characteristic morphologic features of cellular senescence, e.g., enlarged and flattened cells (Fig. 1C, Suppl. fig. 1E).

The execution of senescence programs is also associated with formation of senescence-associated heterochromatic foci (SAHF) enriched in trimethylated histone H3 (H3K9me3) and with increased levels of DNA damage markers such as Ser139 phosphorylated histone variant H2AX (γH2AX). We observed that tepotinib treatment led to increased expression of both H3K9me3 and γH2AX especially in combination with IR (Fig. 1D, Suppl. fig. 1F).
**MET inhibition promotes DNA damage-induced senescence in vivo**

The significance of the *in vitro* observations was further validated *in vivo* by establishing two independent xenograft tumor models with mice bearing subcutaneous GTL-16 or SNU-638 tumors. As shown in Fig. 1E, 1F and Suppl. fig. 2A, 2B, MET inhibition (15mg/kg/day) caused a substantial decrease in tumor cell proliferation, as indicated by reduction in Ki67 staining and tumor size in both GTL-16 and SNU-638 xenografts treated with tepotinib. Moreover our data demonstrate the ability of MET inhibition to massively induce DNA damage *in vivo*: tissues derived from mice treated with tepotinib alone and particularly along with IR displayed enhanced γH2AX staining compared to untreated controls (Fig. 1E and 1F). Decrease in cell proliferation and elevated γH2AX levels coincided also with an increase in SA-βGal activity when tepotinib was combined with IR (Fig. 1E and 1F), further indicating that inhibition of the MET RTK along with IR leads to DNA damage-induced senescence *in vivo*.

*Characterization of pathways involved in the execution of the senescence program*

The senescence program is predominantly established and maintained by the p53 and the p16\(^{INK4A}\)-RB signal transduction pathways (36). Importantly, the relative preponderance of each pathway in the execution of senescence is variable and dependent on cell type and species (36). While some cells need both pathways to be intact in order to undergo senescence, others may rely on a single pathway (36). Furthermore, some studies indicate that cells may also undergo senescence in a p53- and p16-independent manner through alternative pathways which are currently under investigation (36, 37).

We determined the levels of p53 as well as its downstream target p21, and of p16 following induction of senescence by tepotinib treatment combined with IR in our panel of cell lines harboring wild-type (GTL-16 (38), MKN-45 (39) and KATO-II (data not shown)), mutated (SNU-638 (40)) or deleted (SNU-5 (40)) p53. Even though there were no detectable changes in p53 levels and its phosphorylation on Ser15 when IR was combined with MET inhibitor as compared to IR alone, an increase in p21 protein levels was observed in GTL-16, MNK-45
and SNU-638 cells upon the combined treatment when compared to untreated cells or cells treated by MET inhibition or IR (Fig. 2A). In KATO-II cells, this increase correlated with IR only (Suppl. fig. 3), while as expected, in SNU-5 cells p53 and p21 were not detected (Fig. 2A). Conversely, p16 expression was down-regulated following MET inhibition alone or in combination with IR in all the cell lines apart of SNU-638 cells, where increased protein levels were observed (Fig. 2A). Such distinct expression profiles of p21 and p16 proteins indicate alternative pathways regulating the senescence program in the human gastric cell lines used in the present study.

To determine the p53 dependence of the observed senescence phenotype in GTL-16 cells (wild-type p53), a p53-depleted isogenic cell line (GTL-16 shp53) has been used (31). As shown in Figure 2B and 2C, p53 knock-down surprisingly did not abrogate induction of senescence and exposure of GTL-16 shp53 cells to the combined treatment resulted rather in enhanced SA-βGal staining and increased H3K9me3 and γH2AX protein levels as compared to control (GTL-16 shc) cells. Elevated expression of p16 in shp53 cells suggests that METi-associated senescence is in the absence of p53 executed by the p16 pathway.

Involvement of the MAPK pathway in senescence induction following MET inhibition

We next aimed at investigating which pathway downstream of MET may enforce cellular senescence following MET inhibition. To that end, two key MET-downstream networks, the MAPK and the PI3K/AKT pathways (1), were inhibited using AZD6244 and LY294002, respectively. As Fig. 3A and Suppl. fig. 4A show, treatment with the MEK1/2 inhibitor AZD6244 significantly enhanced SA-βGal staining and led to characteristic morphologic features of cellular senescence in irradiated GTL-16, MKN-45, KATO-II, SNU-638 and SNU-5 cells. On the other hand, inhibition of the PI3K pathway alone or in combination with IR did not reproduce the effects of tepotinib and AZD6244 treatment on SA-βGal staining in any of the cell lines (Fig. 3A and Suppl. Fig 4A). We further evaluated the effects of AZD6244 and LY294002 on additional senescence markers in GTL-16 and SNU-638 cells (Fig. 3B). Western blot analysis revealed that AZD6244, but not LY294002, had a comparable effect to
Tepotinib on γH2AX, H3K9me3 and p21 protein levels, particularly in combination with IR. To further confirm these findings, AZD6244 treatment was also investigated in MKN-45, KATO-II and SNU-5 cells, where it was shown to recapitulate the effect of tepotinib on γH2AX, H3K9me3 and p21 levels (data not shown).

**Downregulation of Forkhead box protein M1 (FOXM1) following MET inhibition is crucial to induce MET inhibition-dependent senescence**

Within a phosphoproteomics discovery survey, we observed that MET inhibition results in a nearly 10-fold decrease in phosphorylation of the transcription factor FOXM1 (Ser 605, Ser 620) in MET-addicted human non-small cell lung cancer cell line EBC-1 (data not shown). The fundamental role of FOXM1 in both the DNA damage response and senescence (28-30) prompted us to investigate whether the same was relevant in gastric cancer cells and if the aforementioned diminished phosphorylation of FOXM1 might in fact reflect lower levels of the total protein.

Indeed, FOXM1 expression levels were downregulated in all the studied human gastric cancer cell lines following MET inhibition independently of IR (Fig. 4A, Suppl. fig. 5). This observation was also confirmed *in vivo* in both GTL-16 and SNU-638 xenografts, where no FOXM1 staining could be detected in tumor tissues of mice treated with tepotinib (Fig. 4B).

FOXM1 is required for an efficient repair of DNA DSBs through direct up-regulation of BRIP1 as well as indirect targeting of BMI-1, one of the major negative regulators of the senescence program (41). As Fig. 4C shows, tepotinib treatment decreased the expression of BRIP1 and BMI-1 in GTL-16 as well as SNU-638 cells *in vivo*, suggesting that down-regulation of FOXM1 protein levels followed by MET inhibition could affect the ability of these cells to repair DNA damage induced by IR, leading to senescence.

As FOXM1 activity depends on MAPK pathway activation (following phosphorylation by phospho-ERK1/2, FOXM1 translocates to the nucleus and activates the transcription of several targets involved in cell proliferation, survival, invasion and metastasis (26)), we analyzed FOXM1 levels following treatment with inhibitors of both ERK/MAPK and PI3K/AKT.
pathways. Indeed, while AZD6244 treatment recapitulated the effect of tepotinib on FOXM1 protein level, LY294002 did not affect FOXM1 expression (Suppl. fig. 5).

To directly assess the impact of FOXM1 on MET inhibition-dependent DNA damage-induced senescence, we stably expressed FOXM1 in SNU-638 (SNU638_FOXM1) and in GTL16 cells (GTL16_FOXM1). Figure 5A and Suppl. fig. 6A and 6B show that in FOXM1-overexpressing cells MET inhibition does not lead to a complete down-regulation of FOXM1 expression when compared to the empty vector control cells. Interestingly, SNU-638_FOXM1 cells displayed less H3K9me3 and p21 accumulation and significantly less SA-βGal activity (Figure 5A and 5B, respectively) following MET inhibition alone or in combination with IR when compared to the empty vector control cells (SNU638_pcDNA3). We also investigated the effect of FOXM1 overexpression on the ability of MET inhibition to induce DNA damage by evaluating γH2AX levels in SNU638_FOXM1 (Fig. 5A and Suppl. fig. 6B) and GTL16_FOXM1 cells (Suppl. fig. 6A) with or without the irradiation treatment. The results showed that FOXM1 ectopic expression abrogated MET inhibition-dependent increase of γH2AX independently of irradiation, highlighting an important role of FOXM1 in the execution of DNA DSBs repair by tumor cells following MET inhibition. To further demonstrate that the lack of accumulation of γH2AX is possibly a result of a more efficient repair of DNA DSBs in SNU-638_FOXM1 and GTL-16_FOXM1 cells, we evaluated BRIP1 expression by western blot analysis and we showed that FOXM1 overexpression leads to higher levels of BRIP1 protein, which could enable SNU-638 and GTL-16 cells to repair DNA DSBs more effectively (Fig. 5A and Suppl. fig. 6A and 6B). Furthermore, cells ectopically expressing FOXM1 seem to gain a significant growth advantage over their parental counterparts upon MET inhibition (Fig. 5C, 5D and Suppl. fig. 6C), avoiding cell cycle arrest and subsequent senescence.

Co-alterations of MET and FOXM1 are common in patients with gastric cancer

In order to gain further translational insights into the potential relevance of the MET-FOXM1 proposed network in patients with gastric cancer, we assessed MET and FOXM1 protein levels in two TMAs containing gastric cancer tissues from 91 patients. High protein levels of
MET and FOXM1 were detected in 33% of the samples, and 80.3% of the tumors displayed high levels of MET and/or FOXM1 (Fig. 6A). We further used Ki67 as a marker of tumor proliferation and could observe a higher prevalence of MET/FOXM1 high tumors in samples with intermediate or high proliferation index (Fig. 6B). Tumor- and patient-related data were available for 72 patients. We found that high FOXM1 and especially high FOXM1/MET expression was significantly more prevalent in patients with lymph node metastases (p-values 0.046 and 0.008, respectively; Suppl. Table 1).

We next extracted gene copy number (CNVs from SNP arrays) and gene expression (RNA sequencing (RNA seq)) data from the TCGA database. We selected cases with full clinical and follow-up data (178 in total). Amplification of both oncogenes was found in 24.7% of patients, representing the largest subgroup of patients followed closely only by the group of patients with MET amplification/FOXM1 deletion, which represented 21.9% of the cohort (Fig. 6C). RNA seq data revealed that all cases featuring FOXM1 mRNA overexpression (30%) also displayed MET mRNA overexpression (Fig. 6D).

Neither gene copy number variation nor mRNA levels correlated with clinico-pathological features or survival in the TCGA cohort, consisting of patients treated with modalities other than anti-MET targeted therapy (data not shown).

Given the lack of data from patients treated with anti-MET therapies, the potential relevance of these findings in terms of outcomes or treatment selection criteria needs further elucidation. While the main interest of the present results is descriptive, these data show that our proposed MET-FOXM1 axis is commonly deregulated in human gastric cancer, at the genomic, transcriptomic, and protein levels. Consequently, upcoming clinical trials should allow specifying the role of the MET-FOXM1 axis in responses to therapy in gastric cancer.

**Discussion**

Gastric cancer is one of the most common malignant tumors worldwide accounting for more than 700’000 cancer-related deaths annually (42). Complete resection of localized tumors is
the mainstay of treatment, but patients newly diagnosed with gastric cancer tend to present with advanced and often incurable disease (42). The MET RTK is frequently dysregulated and strongly implicated in the malignant transformation and progression of this disease (43). Here we examined the impact of inhibiting MET signaling by the highly selective and potent anti-MET small molecule tepotinib (44) in MET-addicted gastric cancer models and report for the first time that tepotinib sensitized cancer cells to IR by promoting DNA damage-induced senescence both \textit{in vitro} and \textit{in vivo}.

It is not yet fully understood what determines whether cells undergo senescence or cell death, but the nature and intensity of insult/damage as well as cell type and inactivation of apoptosis pathways during tumorigenesis (e.g., Bcl2 overexpression (45)) may play a role [12, 14, 15]. Interestingly, we observed different expression profiles of p21 and p16 in our panel of cell lines following the combined treatment. Surprisingly, p53-null cells underwent senescence without increased p16 levels, suggesting that senescence can be activated also independently of p53/p16\textsuperscript{INK4A}-RB. These data are in contrast with some other studies where inactivation of these two pathways prevents senescence induction (46). However, according to the study of \textit{Wang et al.} (47) where induction of p16 expression in cell lines with endogenous mutant p53 following senescence onset has been reported, we similarly observed an up-regulation of p16 protein levels in GTL-16 shp53 cells compared to control cells. This suggests that in cancers harboring mutations of either p53 or p16, the presence of a wild-type version of the other gene could mediate/execute the senescence program.

Even though cell death is the favored outcome of anticancer treatments, a current parallel perception advocates that activation of non-apoptotic mechanisms should be considered a potential endpoint following chemo- and radiosensitization approaches as well (23). Using the E\textsubscript{µ}-myc model of lymphomagenesis, the pioneer work of \textit{Schmitt et al.} in 2002 provided direct evidence that cellular senescence can be induced following chemotherapy \textit{in vivo} and thus be a potential desirable outcome of cancer treatment (35). Since then, several studies have described senescence induction by chemo- or radiotherapy in a variety of cancer types as well as in cancer cells grown \textit{in vitro} (19, 22, 24, 47). Similarly, we report that proliferation...
of cells treated with tepotinib together with IR was dramatically impaired \textit{in vitro} and this effect was also observed \textit{in vivo}, where tumor volumes were 4-6-fold smaller compared to untreated tumors (data not shown). The fact that senescence can be achieved by applying lower dosages of DDAs than those required to drive short-term proliferative arrest or cell death may effectively result in reduced toxic side-effects inherent to many therapies (48). Consistently, in the current report we have observed the induction of senescence using the MET inhibitor tepotinib in concentrations as low as 10-15nM for \textit{in vitro} and 15mg/kg for \textit{in vivo} studies.

In recent years, many studies have focused on the importance of FOXM1 in both DDR and negative senescence regulation (28-30). It has become evident that FOXM1 plays a fundamental role in DNA DSBs repair through up-regulation of key DDR proteins such as RAD51, NBS1 and BRIP11 (28, 30). Likewise, various studies described a role for FOXM1 in the regulation of the senescence program, mainly through its downstream target BMI-1 (41).

As Rovillain et al. reported, FOXM1 overexpression bypassed senescence in human fibroblasts following activation of the p53 and the p16\textsuperscript{INK4A}-RB pathways, while Zeng and colleagues (49) showed that inhibition of FOXM1 in gastric cancer-derived cell lines led to senescence in a p53- and the p16\textsuperscript{INK4A}-RB-independent manner. Interestingly, our results suggest that FOXM1 levels are downregulated following MET inhibition, thus compromising the ability of irradiated cells to repair their DNA.

In order to elucidate the mechanism by which MET inhibition leads to down-regulation of FOXM1 protein levels/senescence, Ma et al. (50) reported that the nuclear translocation and activation of FOXM1 is regulated by phospho-ERK-dependent phosphorylation by the MAPK pathway. In accordance with this study, we similarly observed a decrease in FOXM1 protein levels following inhibition of the MAPK pathway by the MEK1/2 inhibitor AZD6244, which also induced senescence in irradiated GTL-16, MNK-45, KATO-II, SNU-638 and SNU-5 cells. Based on these findings, we propose a model to describe radiosensitization via MET inhibition in the context of gastric cancer: following MET inhibition, FOXM1 is no longer phosphorylated and activated by the MAPK pathway and therefore it does not translocate to
the nucleus. In this scenario, the ability of cells to repair the DNA damage inflicted by IR and potentially other genotoxic insults that inflict DNA damage, is impaired and the consequent accumulation of DSBs, together with the down-regulation of one of the major negative regulators of the senescence program, will lead to DNA damage-induced senescence. Importantly, our current data also suggests that MET targeting-dependent FOXM1 downregulation underlies the observed senescence phenotype, predominantly in combination with an additional required genotoxic stress such as extensive DNA damage elicited in this case by IR. These findings suggest a possible synergistic mechanism, which results from the downregulation of a critical senescence negative regulator, which also plays a positive role in DDR activation on one hand and concurrent generation of DNA damage on the other hand.

From a translational perspective, available data clearly indicates that alterations of MET and FOXM1 co-occur in a substantial subset of patients with gastric cancer, therefore underlining the potential relevance of our preclinical findings upon implementation of anti-MET targeted therapy. While copy number variation and mRNA levels of MET and FOXM1 did not correlate with tumoral features or survival, it is important to note that these patients did not receive anti-MET targeted therapy. Moreover, available data suggest that protein levels could be the most relevant readout in terms of prognosis. Relevant to this, Spigel et al. found that MET expression levels were predictive of responses to the anti-MET antibody Onartuzumab in patients with advanced non-small-cell lung cancer. Currently ongoing clinical trials aim at assessing the impact of MET expression and amplification in gastric carcinoma (NCT02002416).

Our findings highlight the potential benefit of using MET inhibitors in MET-overexpressing tumors to impair the ability of cancer cells to successfully repair damaged DNA and to enhance the effect of radiotherapy in gastric cancer, a devastating disease with an urgent need for improved treatment modalities. Molecular stratification seems essential in order to achieve the best possible outcomes. Importantly, based on our preclinical findings, FOXM1
overexpression may be a potential mechanism of resistance to MET inhibitors. This point should be explored in upcoming clinical trials.
Acknowledgements

We cordially thank Prof. R. Medema (Netherlands Cancer Institute, Amsterdam, The Netherlands) and Prof. M.P. Tschan (Institute of Pathology, University of Bern, Switzerland) for kindly providing us the Flag-FoxM1-NT2 expression vector and shp53 cells, respectively. Additionally, we thank Dr. A. Quintin and Mrs. M. Leuener-Tombolini for excellent technical assistance.
References

Figure legends

Figure 1. MET inhibition promotes DNA damage-induced senescence both in vitro and in vivo. A. percentage of BrdU positive cells. Data are mean ± SEM from 3 independent experiments. P values are calculated by one-way ANOVA. ***, P<0.001. B. representative images (10x magnification) of viable and dead cells, calcium AM (green) or ethidium homodimer (red) positive, respectively. Right, data quantification. C. representative images of SA-βGal activity (40x magnification). Right, percentage of SA-βGal-positive cells. Data are mean ± SEM from 3 independent experiments. P values are calculated by one-way ANOVA. ***, P<0.001. D. whole-cell lysates were subjected to western blotting using specific antibodies against p-MET, MET, H3K9me3 and yH2AX following the indicated treatments. E, F. representative images (40x magnification) of p-MET, ki67, yH2AX staining and SA-βGal activity in GTL-16 and SNU-638 xenografts, respectively, after treatment as indicated (day 7). Right, percentage of p-MET, Ki67, yH2AX and SA-βGal activity-positive cells. P values are calculated by one-way ANOVA. *, P<0.05, **, P<0.01 and ***, P<0.001.

Figure 2. Characterization of pathways involved in the execution of the senescence program upon MET inhibition. A. total cell proteins were subjected to western blotting with a specific antibody against p-p53, p53, p21 or p16 following the indicated treatments. B. top, representative images (40x magnification) of SA-βGal activity in GTL-16 p53 knocked down cells (GTL-16 shp53). Bottom, percentage of SA-βGal activity positive cells. P values are calculated by one-way ANOVA. ***, P<0.001. C. western blot analysis showing different expression of indicated senescence markers in GTL-16 cells harboring wild type (GTL-16 shc) or knocked down p53 (GTL-16 shp53).

Figure 3. The MAPK pathway links MET inhibition and senescence induction. A. representative images (40x magnification) of SA-βGal activity in GTL-16(top) and SNU-638 (bottom) cells, . Right, percentage of SA-βGal positive cells. Data are mean ± SEM from 3 independent experiments. P values are calculated by one-way ANOVA. ***, P<0.001. B.
western blot analysis showing H3K9me3, γH2AX and p21 levels in GTL-16 (left) and SNU-638 (right) cells after the indicated treatment.

**Figure 4.** MET inhibition-dependent radiosensitization through induction of DNA damage-induced senescence is mediated by FOXM1 down-regulation. A. Western blot analysis showing p-MET and FOXM1 levels following the indicated treatments. B. Representative images of FOXM1 staining in GTL-16 (top) and SNU-638 (bottom) xenografts. Right, percentage of FOXM1 positive cells. P values are calculated by one-way ANOVA. **, P<0.01 and ***, P<0.001. C. Representative images of BMI-1 and BRIP1 staining in GTL-16 (top) and SNU-638 (bottom) xenografts. Right, percentage of BMI-1 or BRIP1 positive cells. P values are calculated by one-way ANOVA. **, P<0.01 and ***, P<0.001.

**Figure 5.** Ectopic expression of FOXM1 abrogates MET inhibition-dependent DNA damage-induced senescence. A. Whole-cell lysates were subjected to western blotting using specific antibodies against FOXM1, BRIP1, γH2AX, H3K9me3 and p21 following the indicated treatments. B. Representative images of SA-βGal activity (40x magnification) in FOXM1-overexpressing SNU-638 cells (SNU-638_FOXM1), compared to empty vector control SNU-638 cells (SNU-638_pcDNA3). Right, percentage of SA-βGal-positive cells. Data are mean ± SEM from 3 independent experiments. P values are calculated by one-way ANOVA. ***, P<0.001. C. Representative images (4x magnification, 2% crystal violet staining) of SNU-638_FOXM1 and SNU-638_pcDNA3 cells following 3 days of MET inhibition treatment and stained 3 days after the removal of the MET inhibitor. D. Percentage of proliferating SNU-638_FOXM1 and SNU-638_pcDNA3 cells following 3 days of MET inhibition. Data are mean ± SEM from 3 independent experiments. Statistical significance was determined using the Student t test. ***, P<0.001.

**Figure 6.** Alterations of MET and FOXM1 commonly co-exist in patients with gastric cancer. A. Images of MET and FOXM1 staining in 4 representative gastric tumors included in the tumor TMA. B. Correlation of MET and FOXM1 expression in tumors included in the TMA with low, intermediate or high proliferation rate (Ki67 labeling index is used as a marker for proliferation).
Figure 1 (A-C)

A: Diagram showing the percentage of BrdU-positive cells in different conditions.

B: Images and graphs depicting the relative fluorescence signal for living and dead cells in G16, SNU-638, MKN-45, and SNU-5cells treated with 0 Gy and 4 Gy.

C: Images and graphs showing the percentage of β-Gal positive cells in different conditions.
Figure 1 (E, F)
Figure 2

Panel A: Western blot analysis showing the expression levels of p-p53, p53, p21, p16, and β-actin in different cell lines (GTL-16, SNU-638, MKN-45, SNU-5) treated with METi (15nM) and IR (Gy).

Panel B: Microscopic images of cell lines treated with different doses of IR and METi, showing morphological changes.

Panel C: Graphs showing the percentage of positive cells for H3K9me3, γH2AX, p16, p21, p-p53, and β-actin in GTL-16 shc and GTL-16 shp53 treated with METi (15nM) and IR (Gy).
Figure 3

A

C METi MEKi PI3Ki

0 Gy

4 Gy

GTL-16

B

C METi MEKi PI3Ki

0 Gy

4 Gy

SNU-638

H3K9me3 γH2AX p21 βactin

GTL-16

H3K9me3 γH2AX p21 βactin

SNU-638
Figure 4

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
Figure 5

A: Western blot analysis of FOXM1, BRIP1, γH2AX, H3K9me3, p21, and β-Actin in SNU638 pcDNA3 and SNU638 FOXM1 cells treated with METI (15 nM) compared to untreated cells.

B: Immunohistochemical staining of β-Gal positive cells in SNU638 pcDNA3 and SNU638 FOXM1 cells treated with C, IR, METI, and METI+IR.

C: Immunofluorescence staining of α-tubulin in SNU638 pcDNA3 and SNU638 FOXM1 cells treated with C and METI.

D: XTT assay showing the percentage of proliferating cells in SNU638 pcDNA3 and SNU638 FOXM1 cells treated with C and METI.
Figure 6

A

RNA expression levels

High MET
High FOXM1

High MET
Low FOXM1

Low MET
High FOXM1

Low MET
Low FOXM1

33%
3.3%
44%
18.7%

B

Copy number variation

Low MET
Low FOXM1

Low MET
High FOXM1

High MET
Low FOXM1

High MET
High FOXM1

C

RNA expression levels

Low MET/High FOXM1

Low MET/Low FOXM1

High MET/Low FOXM1

High MET/High FOXM1

D

Ki67

Number of tumors

0
20
40
60

High MET/High FOXM1

High MET/Low FOXM1

Low MET/High FOXM1

Low MET/Low FOXM1

Ki67

Number of tumors

0
20
40
60

High MET/High FOXM1

High MET/Low FOXM1

Low MET/High FOXM1

Low MET/Low FOXM1

Ki67

Number of tumors

0
20
40
60

High MET/High FOXM1

High MET/Low FOXM1

Low MET/High FOXM1

Low MET/Low FOXM1

Ki67

Number of tumors

0
20
40
60

High MET/High FOXM1

High MET/Low FOXM1

Low MET/High FOXM1

Low MET/Low FOXM1
Depletion of FOXM1 via MET targeting underlies establishment of a DNA damage-induced senescence program in gastric cancer


Clin Cancer Res  Published OnlineFirst May 16, 2016.

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