Genome-wide siRNA Screen Identifies the Radiosensitizing Effect of Downregulation of MASTL and FOXM1 in NSCLC

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Abstract

Lung cancer is the most common cancer worldwide and on top of that has a very poor prognosis, which is reflected by a 5-year survival rate of 5% to 15%. Radiotherapy is an integral part of most treatment regimens for this type of tumor, often combined with radiosensitizing cytotoxic drugs. In this study, we identified many genes that could potentially be exploited for targeted radiosensitization using a genome-wide siRNA screen in non–small cell lung cancer (NSCLC) cells. The screen identified 433 siRNAs that potentially sensitize lung cancer cells to radiation. Validation experiments showed that knockdown of expression of Forkhead box M1 (FOXM1) or microtubule-associated serine/threonine kinase-like (MASTL) indeed causes radiosensitization in a panel of NSCLC cells. Strikingly, this effect was not observed in primary human fibroblasts, suggesting that the observed radiosensitization is specific for cancer cells. Phosphoproteomics analyses with and without irradiation showed that a number of cell-cycle–related proteins were significantly less phosphorylated after MASTL knockdown in comparison to the control, while there were no changes in the levels of phosphorylation of DNA damage response proteins. Subsequent analyses showed that MASTL knockdown cells respond differently to radiation, with a significantly shortened G2/M phase arrest and defects in cytokinesis, which are followed by a cell-cycle arrest. In summary, we have identified many potential therapeutic targets that could be used for radiosensitization of NSCLC cells, with MASTL being a very promising and druggable target to combine with radiotherapy.

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Introduction

Lung cancer is the most frequently diagnosed cancer, representing 12% of all cancers worldwide (1). It is a heterogeneous disease and is divided into multiple classes. The major division is made between small cell lung cancer (SCLC) and non–small cell lung cancer (NSCLC), which make up 20% and 80% of the lung cancer cases, respectively (2). It has been shown that the different subtypes of lung cancer exhibit large variations in sensitivity to cytotoxic compounds and radiation, where SCLC was shown to be the most sensitive (3).

Materials and Methods

Cell culture

All cell lines used were cultured in DMEM supplemented with 5% FCS, 2 mmol/L l-glutamine, and 1% penicillin/streptomycin in a humidified atmosphere of 5% CO2 at 37°C. NSCLC cell lines SW1573, A549, H322, and H1299 were obtained from the American Type Culture Collection.
Department of Medical Oncology (VU University Medical Center, Amsterdam, the Netherlands), which were authenticated by T5PS sequencing. Hek293T cells were a kind gift from Peter Heutink (VU University Medical Center, Amsterdam). Human fibroblasts were isolated from uvulopalatopharyngoplasty specimen. Use of residual tissue from surgical specimen was according to the guidelines of the Dutch federation of Biomedical Sciences and Dutch legislation.

siRNA screens

The SW1573 NSCLC cell line was subjected to a high-throughput forward transfection in 96-well plates as described before (9). Cells were seeded at a density of 200 cells per 96-well and were transfected 24 hours later. In total, 25 nmol of each siRNA SMARTpool derived from the siARRAY Human Genome library (Catalog items G-003500 (Sept05), G-003600 (Sept05), G-004600 (Sept05) and G-005000 (Oct05); and 0.01% v/v DharmaFECT1 (Thermo Fisher Scientific) was delivered to the cells. The nontargeting siCONTROL#2, siPLK1, and siPRKDC (Thermo Fisher Scientific) were used as negative control, transfection control, and radiosensitization control, respectively. Plates were incubated for 48 hours and subsequently irradiated with a dose of 2 Gy of γ-irradiation using a 90Co source (Gammeccell 220; MDS Nordion). In total, 96 hours after irradiation, the cells were fixed and stained for 1 hour with a 3.7% formaldehyde solution in H2O containing 0.5 μg/ml Hoechst 33342. The number of stained nuclei was determined using the Acumen eX3 microplate cytometer (TTP LabTech). Data were normalized and robust Z-scores were calculated in Excel software, data were further analyzed using Ingenuity Pathway Analysis software (http://www.ingenuity.com/products/ipa).

Deconvolution of siRNA pools

To confirm the hits from the primary screen, SW1573 cells were transfected with the individual siRNAs from the siRNA pool used in the initial screen. Cells were transfected with 25 nmol siRNA and 0.015% v/v DharmaFECT1. Cells were irradiated with a dose of 2 Gy and cell viability was measured 96 hours after irradiation staining with Hoechst 33342 and Acumen analysis as described above.

Lentiviral transduction

Stable shRNA-expressing cell lines were generated by transduction of target cells (SW1573, A549, H1299, and H322) with lentiviral particles containing specific shRNAs targeting MASTL and FOXM1. Lentiviral particles were produced by Hek293T cells after transfection with the shRNA encoding pLKO.1 plasmids and the corresponding lentiviral helper constructs (Sigma-Aldrich). Cultured SW1573 cells were labeled with 2.5 μmol/L bromodeoxyuridine (BrdUrd) for 20 minutes at 37°C. Incorporation of BrdUrd was visualized by staining with mouse-anti-BrdUrd and FITC-conjugated Rabbit-anti-Mouse antibodies (Becton, Dickinson and Company).

Clonogenic assay

To measure clonogenic survival, cells were diluted to a single cell suspension and seeded in 25 cm² flasks as described in (11). Flasks were irradiated with a single dose of γ-irradiation in the range of 0 to 6 Gy. Cells were grown for a period of 7 to 21 days. Subsequently, cells were fixed with 100% EtOH and stained with Giemsa’s azur eosin methylene blue solution (Merck). Colonies of more than 50 cells were counted.

Western blotting

Western blot analyses were performed according to standard procedures (12). Proteins were separated by SDS-PAGE and blotted on PVDF membranes (Millipore). Antibodies used for detection were mouse anti-ATM (sc-23921), mouse anti-pATM (sc-47739), rabbit anti-FOXM1 (sc-502; Santa Cruz Biotechnology), rabbit anti-ENSA (DSZ111), rabbit anti-pENSA (G67), Rabbit anti-MASTL (D3J4Y; Cell Signaling Technology), and mouse anti-β-actin (AC-15; Sigma-Aldrich). Proteins were visualized using the secondary fluorescently-labeled antibodies goat-anti-mouse IRDye 680 RD and goat-anti-rabbit IRDye 800 CW (LI-COR Biosciences) and scanned on the Odyssey infrared imaging system (LI-COR Biosciences).

Fractionated irradiation

Cells were seeded at low density in 25 cm² flasks, after which they were irradiated on 5 sequential days with a dose of 2 Gy. After 2 days of recovery, the total number of cells was determined by cell counting and the cells were irradiated again using the same schedule. The schedule was repeated for 6 weeks in total, after which the cells had received a cumulative dose of 60 Gy.

Xenograft studies in mice

In total, 1.4 × 10⁶ A549 cells transduced with MASTL shRNA were injected in both flanks of immune-deficient athymic Nude-foxn1nu mice (Harlan). At days 6, 10, and 13, the mice received 2 Gy of total body irradiation on a linear accelerator. Tumor growth was measured twice a week by caliper measurement. After 4 weeks, the mice were euthanized and all tumors frozen. Experiments were performed in accordance to Dutch legislations, and the protocol was approved by the Institutional Review Board on animal experimentation.

Cell-cycle analysis

Cultured SW1573 cells were labeled with 2.5 μmol/L bromodeoxyuridine (BrdUrd) for 20 minutes at 37°C for FACS analysis. Cells were harvested by trypsinization and fixed in 70% EtOH. The samples were stored with PBS and taken up in 5 mol/L HCl with 0.5% TritonX100 (ICN Biomedicals INC.). Samples were incubated at room temperature for 30 minutes and subsequently neutralized by the addition of 0.1 mol/L di-Sodiumtetraborate (MERCK). Incorporated BrdUrd was visualized by staining with mouse-anti-BrdUrd and FITC-conjugated Rabbit-anti-Mouse antibodies (Dako). Next, cells were stained with propidium iodide (Sigma-Aldrich) and analyzed on a FACSCalibur using CellQuest Pro software (Becton, Dickinson and Company).

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Confocal imaging

Cells were grown in chamber slides and irradiated with 2 Gy of ionizing radiation. Twelve hours after irradiation, samples were fixed in 3.7% formaldehyde (Sigma) and stained with DAPI (Sigma). Slides were subsequently mounted using mowiol and analyzed on a confocal microscope (Leica) using Leica application suite software.

Results

Genome-wide loss-of-function screen for radiosensitizing genes

In our functional genetic screen, we tested siRNAs targeting over 21,000 human genes for their capacity to induce radiosensitization in the NSCLC cell line SW1573. This NSCLC cell line is relatively resistant to ionizing radiation in comparison with other cell lines tested (data not shown) and could therefore be sensitized to radiation. As a control, radiosensitization was achieved by siRNA-mediated knockdown of protein kinase, DNA activated, catalytic polypeptide (PRKDC), which is known for its role in the DNA damage response (Supplementary Fig. S1A; ref. 13).

To identify other unique siRNAs that could sensitize SW1573 cells to irradiation, cells were transfected in 96-wells plates with individual pools of four siRNAs targeting one unique gene per well. For all siRNAs, a duplicate transfection was performed: one of the duplicates was left untreated, whereas the other received 2 Gy of ionizing radiation 48 hours after transfection (Fig. 1A). Four
days later, all cells were fixed and stained with Hoechst 33342, and the total number of nuclei present in each well was determined. We previously tested the radiosensitivity of a panel of head and neck cancer cell lines and showed that this assay in 96-well plates reliably reflects results obtained in clonogenic assays at low doses of ionizing radiation (11). The whole screening procedure was performed in duplicate, after which statistical analyses were performed. An individual robust Z-score was computed on the average ratio of treated:untreated cells for each individual pool of siRNAs (Fig. 1B). The robust Z-score is a metric that indicates how strong each individual observation differs from the median, and is well applicable for the analysis of RNAi screens (14). As we included nontargeting siRNAs (siCON) and siPRKDC-positive controls on each individual plate in the screen, we first compared the effect of these individual controls (Supplementary Fig. S1B). This showed that siRNAs targeting PRKDC clearly have a lower robust Z-score than the controls and thus sensitized SW1573 to radiation in this screen (Supplementary Table S1). The cells were exposed to a single dose of 2 Gy of ionizing radiation (11). The whole screening procedure was performed in duplicate, after which statistical analyses were performed. An individual robust Z-score was computed on the average ratio of treated:untreated cells for each individual pool of siRNAs (Fig. 1B). The robust Z-score is a metric that indicates how strong each individual observation differs from the median, and is well applicable for the analysis of RNAi screens (14). As we included nontargeting siRNAs (siCON) and siPRKDC-positive controls on each individual plate in the screen, we first compared the effect of these individual controls (Supplementary Fig. S1B). This showed that siRNAs targeting PRKDC clearly have a lower robust Z-score than the controls and thus sensitized SW1573 to radiation in this screen (P < 1 × 10^-6, t test for independent samples), indicating that the screen was sensitive enough to identify relevant radiosensitizing siRNAs. To generate a list of putative hits in the screen, we set a cutoff at a robust Z-score of −2.75, at which more than 95% of the siPRKDC and less than 0.5% of the siCON controls were scored as hits. Using this cutoff value, a total of 433 siRNAs were identified that potentially sensitize SW1573 to radiation (Supplementary Table S1). Interestingly, several genes involved in the DNA damage response were amongst these hits, such as RAD51, TOPBP1, HHTATP, RNF168, PNKP, and TRIM28 (15–19). A more detailed pathway analysis of the hits using Ingenuity Pathway Analysis software (www.ingenuity.com) showed that proteins belonging to specific cellular functions, such as cell-cycle control as well as cell death and survival, were significantly overrepresented in the hit list (Table 1). As the function of cell-cycle proteins is frequently altered in cancer, we focused in more detail on this cluster of hits, especially since cell-cycle checkpoints are often activated after irradiation to allow DNA damage repair (Fig. 1C; ref. 20). The list of cell-cycle–related genes contains many novel targets that could possibly be inhibited by small molecules to obtain radiosensitivity, of which the kinase mitochondrial associated serine/threonine kinase-like (MASTL) was one of the top hits with a robust Z-score of −4.55. As MASTL is a protein kinase, it is potentially druggable and was therefore chosen for further follow-up. Next to MASTL, we also chose another cell-cycle–related gene for further validation. This gene, with a higher robust Z-score of −2.86, Forkhead box M1 (FOXM1) was chosen for validation experiments to show the validity of our obtained hit list. Inhibition of FOXM1 was recently shown to cause radiosensitization in a variety of cancer cells and therefore could prove that even the lower ranked hits in our hit list are potent radiosensitizers (21).

**Inhibition of FOXM1 or MASTL induces radiosensitivity**

To validate the radiosensitizing effect of two of the hits, MASTL and FOXM1, the separate siRNAs from the original pool were tested for their effect on the radiation response. All four individual siRNAs targeting either FOXM1 or MASTL were enhancing the effect of 2 Gy of radiation on SW1573 cells (Fig. 2A and B). To investigate possible effects of inhibition of FOXM1 and MASTL on the growth rate of untreated cells, the relative survival without radiation treatment was analyzed. This experiment showed that knockdown of these genes did not negatively affect the growth rate of untreated cells (Fig. 2C and D). To show that the siRNAs specifically decrease the RNA levels of their targeted genes, we performed a qPCR analysis on the siRNA-transfected cells. This analysis confirmed that all siRNAs decreased the mRNA levels of their target genes generally more than 80% in comparison with the controls (Supplementary Fig. S2A and S2B).

As our high-throughput assay to analyze radiosensitization is based on counting the nuclei of the total population of cells, we also validated our findings using the gold standard for analyzing radiosensitivity, the clonogenic assay. Also, this assay showed that knockdown of either FOXM1 or MASTL sensitizes SW1573 cells to radiation (Fig. 2E and F).

To independently validate our screening results, we decided to use shRNA-expressing vectors instead of siRNAs for stable knockdown of the target genes. For both target genes, we selected two shRNA vectors that gave a knockdown of over 70% at the RNA and protein level in SW1573 cells (Fig. 3A–D) and determined the sensitivity to radiation of SW1573 stably expressing these constructs. Indeed, also these shRNA-expressing cell lines were more sensitive to radiation compared with the controls in a clonogenic assay (Fig. 3E and F).

As treatment regimens for patients with lung cancer usually consist of fractionated schedules, we next determined the effect of a fractionated dose scheme on shMASTL-expressing SW1573 cells *in vitro*. The cells were exposed to a single dose of 2 Gy of ionizing radiation on 5 consecutive days and left untreated for 2 days, after which this cycle was repeated five times. The number of control cells rapidly increased in the first 2 weeks of treatment, after which a plateau was reached. Interestingly, the total number of shMASTL cells increased only in the first week of treatment after which a plateau was reached at a significantly lower number of cells in comparison with the control (Fig. 3G). Next, we checked the cell-cycle status of MASTL knockdown cells 2 weeks in the fractionated dose scheme. This analysis showed an almost complete absence of the G1-S-phase, without an increase in the sub-G1 population after 2 weeks of irradiation (Supplementary Fig. S2C). These results suggest that decreased levels of MASTL lead to an altered cell-cycle dynamics after radiation, without an induction of apoptosis.

To exclude a possible cell line-specific effect, we determined the effect of FOXM1 or MASTL knockdown on radiation response in three additional NSCLC cell lines. A knockdown of at least 60% of the mRNAs levels of FOXM1 or MASTL could be obtained after transduction of SW1573, A549, H1299, and H322 with an individual shRNA construct (Supplementary Fig. S2D and S2E). The knockdown of FOXM1 or MASTL gave a decreased survival in all four of these cell lines at a dose of 2 Gy of ionizing radiation, as determined by clonogenic survival (Fig. 4A and B). Strikingly, the reduction of FOXM1 or MASTL levels in primary human fibroblasts did not enhance radiosensitivity (Fig. 4C and D). Altogether, these results show that knockdown of FOXM1 or MASTL can induce a more radiosensitive phenotype in multiple NSCLC cell lines, but not in primary human cells.

**Table 1.** Overrepresented molecular and cellular functions as found in IPA.  

<table>
<thead>
<tr>
<th>Name</th>
<th>P</th>
<th># Molecules</th>
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<tr>
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<td>Molecular transport</td>
<td>3.15E-04-4.94E-02</td>
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Effect of MASTL on radiosensitivity in vivo

As indicated above, MASTL knockdown cells only showed an altered growth rate when they were treated with radiation. Therefore, we also tested the sensitivity of these cells to radiation in vivo. As SW1573 did not give rise to xenografts, we used A549 cells transduced with MASTL shRNA for these experiments. These experiments were hampered by the observation that only a 60% knockdown of MASTL expression was obtained in A549 cells (Supplementary Fig. S3). Nonetheless, these cells were injected in the flanks of 16 nude mice, of which 8 mice were left untreated and the other 8 mice were treated with 2 Gy of total body irradiation at days 6, 10, and 13 after injection of the cells. After 4 weeks of growth, no significant difference in tumor volume was observed between irradiated shMASTL-transduced xenografts or irradiated control transduced xenografts (data not shown). On the basis of the modest knockdown, we assumed that the cultures consisted of cell populations with variable levels of MASTL knockdown, and hypothesized that the populations with the most efficient knockdown would be preferentially eradicated by irradiation. We therefore isolated the RNA from the resulting tumors and analyzed MASTL expression by RT-qPCR. As can be seen in Fig. 4E, MASTL expression increased significantly after irradiation ($P < 0.001$) in comparison with the untreated tumors, which suggests that cells with a relatively efficient MASTL knockdown were preferentially eradicated by irradiation in vivo.

Altered phosphorylation upon MASTL knockdown

As MASTL is a kinase, we wondered which substrates would be causative for the observed effects. Endosulfine alpha (ENSA) has been shown to be a substrate for MASTL kinase in *Xenopus laevis*, where it appears to play a role in the mitosis by modifying cyclinB-CDK1 activity (22). As the G2–M checkpoint is critically important in the response to DNA damage, we wondered whether the regulation of ENSA activity by MASTL is involved in the observed phenotype after irradiation. Western blot analysis of shMASTL-transduced SW1573 cells did not show an altered level of pENSA compared with control cells despite a substantial reduction of MASTL protein (Fig. 5A and B). As the shMASTL-expressing cells only showed a phenotypic effect after irradiation, we also analyzed the pENSA levels after irradiation. Surprisingly, the levels of pENSA were not altered during the first 2 hours after irradiation (Fig. 5C). Together these data suggest that ENSA is not the key substrate of MASTL that is causative for the altered response to radiation in the cells used in our studies.

To identify other candidate proteins that have an altered phosphorylation level after MASTL knockdown, we performed...
a phosphoproteomics analysis on the MASTL knockdown cells in the absence and presence of irradiation. In total, 8,410 peptides were identified in the phosphopeptide enriched samples, of which 7,603 were validated to be phosphopeptides. In total, 8,263 phosphorylation sites were detected in the samples, of which 7,136 were pSerine (86.4%), 1,082 were pThreonine (13.1%), and 45 were pTyrosine (0.5%).

In comparison with the control, 392 peptides corresponding to 254 unique proteins were at least 2-fold less phosphorylated after MASTL knockdown, including MASTL itself (Supplementary Table S2). After irradiation, a total number of 363 peptides from 220 unique proteins were at least 2-fold less phosphorylated (Supplementary Table S3). A functional annotation clustering performed by DAVID software (http://david.abcc.ncifcrf.gov/home.jsp) showed that phosphorylation of DNA damage response and repair proteins was highly enriched after irradiation in both MASTL knockdown and control cells (Enrichment score: 6.84). This gene cluster consisted of 27 proteins, containing many established radiation response proteins, such as ATM, PRKDC, XPC, and XRCCC (Fig. 5D and Supplementary Table S4), and suggests that these initial phosphorylation steps in DNA damage signaling are not affected by MASTL knockdown. To confirm the finding that phosphorylation of DNA damage response proteins is not altered in MASTL knockdown cells, we checked the phosphorylation status of ATM (pATM) on Western blot analysis. Figure 5E shows that ATM phosphorylation is induced shortly after irradiation, regardless of MASTL knockdown, confirming that phosphorylation of this DNA damage response protein is not affected by MASTL.

In contrast with what had been observed in the phosphoproteomics data for DNA damage response proteins, we also identified a large number of proteins that had significantly lower phosphorylation levels after MASTL knockdown and irradiation (Supplementary Table S3). This suggests that MASTL plays a role in regulating the phosphorylation status of these proteins, which may be important for their function in DNA damage signaling. Further experiments are needed to determine the specific mechanisms by which MASTL regulates the phosphorylation of these proteins.

Figure 3. shRNA-mediated inhibition of FOXM1 and MASTL leads to radiosensitization. RT-qPCR analysis showing the relative mRNA levels of FOXM1 (A) and MASTL (B) after stable integration of the indicated shRNA constructs in SW1573 cells. Values represent the mean of triplicate experiments ± SD. C and D, Western blot analyses showing the protein levels of FOXM1 and MASTL of the cell lines harboring shFOXM1 or shMASTL constructs that induced a high level of mRNA knockdown. E and F, clonogenic survival of shFOXM1- or shMASTL-transduced SW1573 cells. All values represent the mean of triplicate experiments ± SD. G, growth assay of control and MASTL knockdown cells after treatment with a fractionated dose scheme.
phosphorylation levels in MASTL knockdown cells. DAVID software analysis of these proteins showed that a number of functional clusters were highly enriched in this list (Supplementary Table S5). A cluster involved in cell-cycle regulation was highly enriched in the list with lower phosphorylation levels after MASTL knockdown (Enrichment score: 5.1, Fig. 5F and Supplementary Tables S5 and S6), which suggests that after MASTL knockdown, a number of proteins involved in cell-cycle regulation seem to be significantly altered in their phosphorylation state. Of note, our data do not prove that these proteins are direct substrates of MASTL.

Inhibition of MASTL induces alterations in cell-cycle progression after irradiation

As the phosphoproteomics analyses suggested that MASTL inhibition directly or indirectly affects the phosphorylation of a large number of proteins involved in cell-cycle regulation, we determined the effect of MASTL knockdown on the growth of SW1573. SW1573 cells with lowered levels of MASTL seem to proliferate at the same rate as control cells (Supplementary Fig. S4A). However, when these cells are challenged with DNA damage caused by 2 Gy of irradiation, the total number of cells is decreased in comparison with the controls (Fig. 6A). The proliferation rates of both the control and the MASTL knockdown cell lines are identical between 24 and 72 hours after irradiation (Supplementary Fig. S4A), which suggests that the effect of MASTL knockdown takes place within the first 24 hours postirradiation. Therefore, we studied the cell-cycle distribution of MASTL knockdown cells in more detail during the first day after irradiation. MASTL knockdown by itself did not have a noticeable effect on the cell-cycle distribution without radiation (Fig. 6B). The cell-cycle profiles of MASTL knockdown and control cells did, however, differ substantially within the first 24 hours after irradiation. Interestingly, both cell lines seem to accumulate in G2-M phase.
within the first hours after irradiation (Fig. 6C). The shMASTL-transduced lines, however, reach the maximum number of cells in G2-M at 9 hours after irradiation, after which the population of cells in this cell-cycle phase decreases to the initial levels in the next 15 hours (Fig. 6C, left). In contrast with this, the control cells seem to accumulate in G2-M phase at a slower rate, with the maximum reached 18 hours after irradiation. The faster decrease of the number of cells in G2-M phase of MASTL knockdown cells is accompanied by an increase in the G1-phase population (Fig. 6C, middle) and a marked decrease in the number of cells in S-phase (Fig. 6C, right). These results suggest that decreased levels of MASTL lead to altered dynamics in cell-cycle regulation and an inappropriate G2-M block in response to irradiation.

Next, we analyzed the consequences of the faster exit from the G2-M arrest in MASTL knockdown cells. The nuclei of irradiated MASTL cells showed a marked increase in the number of chromatin bridges between cells (Fig. 6D and E), suggesting an improper timing of cell division. The improper segregation of chromosomes is frequently observed in cells with defects in the spindle assembly checkpoint (SAC; ref. 23). Strikingly, one of the proteins involved in the SAC, namely budding uninhibited by benzimidazoles 1 (BUB1), was also found to be less phosphorylated in MASTL knockdown cells in the phosphoproteomics analysis. This suggests that BUB1 might be a critical target for MASTL-mediated phosphorylation leading to its activation. We therefore also tested the effect of decreased BUB1 protein levels on the response to radiation, Fig. 6F shows that also BUB1 knockdown leads to radiosensitization of SW1573 cells (Fig. 6F and Supplementary Fig. S5C). All in all these results suggest that absence of MASTL and thereby an improper activation of BUB1 might lead to an improper SAC and a premature restart of cell division after radiation.

Discussion

In lung cancer treatment, radiotherapy is often combined with concomitant application of platinum-based chemotherapeutics (24). Platinum-associated toxicities, however, can be severe and often the treatment protocol has to be adapted to deal with this. We hypothesized that there might be many druggable genes that influence radiation-induced damage, which could be exploited to
enhance the efficacy of radiotherapy, preferentially with much less toxicities.

Here, we report an unbiased genome-wide loss-of-function screen to identify novel targets for radiosensitization in NSCLC. Using a library of siRNAs targeting approximately 21,000 unique human genes, we identified that the knockdown of 433 genes putatively sensitize NSCLC cells to ionizing radiation. Indeed, we could confirm that two genes from our hit list, FOXM1 and MASTL, are also modulators of the response to irradiation. Importantly, knockdown of these genes had large effects on radiosensitivity in cancer cells, but only minor effects were observed in normal primary fibroblasts, which might increase the therapeutic window.

FOXM1 has only recently been implicated in the response to DNA damage in pancreatic and breast cancer cell lines (21). On top of that, FOXM1 has been shown to mediate resistance to different drugs, such as herceptin, paclitaxel, cisplatin, and epirubicin (25–28). We showed that FOXM1 knockdown also leads to radiosensitization in NSCLC cell lines. All these studies indicate that FOXM1 regulates cellular processes that are of importance for the response to a variety of therapeutics currently used in the clinic. It would therefore be of great interest to investigate the effect of FOXM1 inhibitors on the response to combinations of the specific treatments.

Next to FOXM1, we could also validate that knockdown of MASTL leads to radiosensitization. From the limited number of

Figure 6.
Knockdown of MASTL affects cell-cycle progression of SW1573 upon irradiation. A, growth curve of SW1573 control and shMASTL-expressing cells treated with doses of 2 Gy of irradiation. B, cell-cycle distribution of SW1573 control cells and SW1573 cells transduced with shMASTL. C, FACS analysis of SW1573 cells, which were fixed at the indicated time points after irradiation with 2 Gy. Bars representing control cells are indicated in black, whereas the grey bars represent the cells expressing shMASTL. The percentages of cells in G2-M (left), G1 (middle), and S-phase (right) are indicated separately. All values represent the mean of triplicate experiments ± SD. D, representation of the observed chromatin bridges between DAPI stained nuclei. The top image represents nuclei of control cells and the bottom image shows nuclei of MASTL knockdown cells. E, quantification of the chromatin bridges of untreated or irradiated cells. Bars represent averages of independent counts of at least eight different fields of cells. F, clonogenic survival after irradiation with 2 Gy of control and BUB1 knockdown cells.
studies on MASTL up to date, it has become clear that this protein plays an important role in the G2–M phase of the cell cycle (29, 30), and our data confirm that. Depletion of G2M kinase, the Xenopus homolog of MASTL, prevented mitotic entry in Xenopus egg extracts (29, 30). This finding was later confirmed in human cells, where nearly complete knockdown of MASTL led to a severe delay in mitotic entry (31, 32). Strikingly, cells with a partial knockdown of MASTL could enter mitosis normally, but exited prematurely, leading to cytokinesis defects (31, 32). In our study, in which a partial knockdown of MASTL was achieved, a faster exit of cells from the radiation-induced G2–M arrest was observed, suggesting that these cells are exiting mitosis prematurely after encountering DNA damage. This is further strengthened by our observation of more chromatin bridges in MASTL knockdown cells after irradiation. The observed cell-cycle arrest in cells exposed to a fractionated dose scheme of irradiation is therefore likely to be caused by a response to the cytokinesis defects that were also observed in earlier studies (31, 32).

The radiosensitizing effect of MASTL knockdown did not seem to be driven by ENSA, the currently known substrate for MASTL (22, 33), as we could not confirm an altered phosphorylation state of this protein after MASTL knockdown. Our phosphoproteomics analysis further suggests that the initial phosphorylation of DNA damage response proteins is not altered in irradiated cells with lowered MASTL expression. Hence, these proteins do not seem to be MASTL substrates as well. In contrast, the phosphorylation of a number of cell-cycle–related proteins was significantly reduced after MASTL knockdown. Strikingly, it has previously been suggested that G2M kinase has a role in DNA damage checkpoint activation and recovery in a cell-free system (34). Our study suggests that MASTL knockdown has an effect on cell-cycle progression of irradiated cells, likely through the regulation of cell-cycle–related proteins, leading to an inappropriate G2–M arrest in response to irradiation. A prime candidate for the observed cytokinesis defects in MASTL knockdown cells would be BUB1, an essential component of the SAC. It has been shown that loss of BUB1 in Drosophila leads to many defects in mitosis, of which chromatin bridge formation is the predominant phenotype (35). The increase in chromatin bridges in irradiated MASTL knockdown cells might therefore be due to a diminished activation of BUB1, thereby causing a defective SAC and leading to an improper timing of the start of anaphase. Our data show that diminished BUB1 levels mimic the radiosensitizing effect seen in MASTL knockdown cells. Altogether, this suggests that deregulated BUB1 activation might be involved in the radiosensitizing effect of MASTL knockdown.

Strikingly, the radiosensitizing effect of MASTL knockdown had a major effect on cells treated by a fractionated schedule in vitro, which implies that it might be a potent targeted radiosensitizer. Remarkably, there are no small-molecule inhibitors described for MASTL, even though it is a kinase. Our work might be therefore a starting point to develop a novel class of targeted inhibitors that may act as radiosensitizers to enhance the effect of radiation on tumor cells with MASTL as prime candidate.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: R. Nagel, V.W. van Beusechem, R.H. Brakenhoff
Development of methodology: R. Nagel, I.H. van der Meulen, J. Hodzic, V.W. van Beusechem, R.H. Brakenhoff
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R. Nagel, M. Siugter-van Walsum, J. van den Berg, S.R. Piersma, C.R. Jimenez, V.M. van Beusechem
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References


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