Novel Mechanism of MDA-7/IL-24 Cancer-Specific Apoptosis through SARI Induction

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Abstract

Subtraction hybridization combined with induction of cancer cell terminal differentiation in human melanoma cells identified melanoma differentiation–associated gene-7/interleukin-24 (mda-7/IL-24) and SARI (suppressor of AP-1, induced by IFN) that display potent antitumor activity. These genes are not constitutively expressed in cancer cells and forced expression of mda-7/IL-24 (Ad.mda-7) or SARI (Ad.SARI) promotes cancer-specific cell death. Ectopic expression of mda-7/IL-24 induces SARI mRNA and protein in a panel of different cancer cells, leading to cell death, without harming corresponding normal cells. Simultaneous inhibition of K-ras downstream extracellular signal-regulated kinase 1/2 signaling in pancreatic cancer cells reverses the translational block of MDA-7/IL-24 and induces SARI expression and cell death. Using SARI-antisense-based approaches, we demonstrate that SARI expression is necessary for mda-7/IL-24 antitumor effects. Secreted MDA-7/IL-24 protein induces antitumor " bystander" effects by promoting its own expression. Recombinant MDA-7/IL-24 (His-MDA-7) induces SARI expression, supporting the involvement of SARI in the MDA-7/IL-24-driven autocrine loop, culminating in antitumor effects. Moreover, His-MDA-7, after binding to its cognate receptors (IL-20R1/IL-20R2 or IL-22R/IL-20R2), induces intracellular signaling by phosphorylation of p38 MAPK, leading to transcription of a family of growth arrest and DNA damage inducible (GADD) genes, culminating in apoptosis. Inhibition of p38 MAPK fails to induce SARI following Ad.mda-7 infection. These findings reveal the significance of the mda-7/IL-24-SARI axis in cancer-specific killing and provide a potential strategy for treating both local and metastatic disease. Cancer Res; 74(2); 563–74. ©2013 AACR.

Introduction

Cancer is a progressive disease with advanced tumors frequently displaying resistance to multiple therapies, including chemotherapy, immunotherapy, and radiotherapy (1). Despite intensive investigation, solid and hematopoietic tumors remain formidable clinical challenges, resulting in high incidences of morbidity and mortality. Reprogramming tumor cells to undergo programmed cell death (apoptosis) represents a promising and powerful strategy for treating both local and metastatic disease (1). Approaches for achieving this objective involve replacement of defective tumor suppressor genes and/or expression/activation of apoptosis-inducing and/or toxic autophagy-inducing genes or their products in tumor cells (2, 3).

Melanoma differentiation associated gene-7/interleukin-24 (mda-7/IL-24), a novel member of the IL-10–related cytokine gene family (4), and SARI (suppressor of AP-1, induced by IFN), a novel type 1 IFN-inducible early response gene (5, 6), were cloned using subtraction hybridization combined with induction of cancer cell terminal differentiation. mda-7/IL-24 was found to have nearly ubiquitous antitumor properties in vitro and in vivo (7, 8), which led to successful entry into the clinic where safety and clinical efficacy when administered by adenovirus (Ad.mda-7) was shown in a phase I clinical trial in humans with advanced carcinomas and melanomas (9). mda-7/IL-24 uniquely induces apoptosis or toxic autophagy in cancer cells without harming normal cells. Forced mda-7/IL-24 expression in cancer cells also inhibits angiogenesis (10, 11), promotes antitumor immune responses (12–14), sensitizes cancer cells to radiotherapy- and chemotherapy-induced killing (15–21), and elicits potent “antitumor bystander activity” through autocrine/paracrine secretion (22, 23). The MDA-7/IL-24 protein interacts with the endoplasmic reticulum chaperone protein Bip/GRP78 and initiates a cascade of “unfolded protein response (UPR)” processes in tumor cells downregulating antiapoptotic proteins, including Bcl-XL and Mcl-1 (24, 25).

SARI expression is induced as early as 2 hours after IFN-β treatment, suggesting a potential role as an early mediator of IFN-β action. Stable HeLa cervical cancer cells expressing...
antisense SARI were resistant to IFN-β-mediated growth inhibition, which suggests that SARI may have tumor-suppressing effects (5). Moreover, expression of SARI mRNA was detected in normal cells of diverse lineage, including melanocytes, astrocytes, breast and prostate epithelial cells, and pancreatic mesothelial cells, whereas expression was absent in multiple cancer cell lines of the same tissue of origin, further supporting a putative function as a tumor suppressor gene (5). Forced expression of SARI resulted in tumor-selective growth inhibition and induction of apoptosis in prostate cancer, malignant glioma, and metastatic melanoma cells, while sparring their respective normal counterparts (5, 6). Infection with Ad.SARI (adenovirus expressing SARI regulated by a cytomegalovirus promoter) inhibited DU-145 xenograft growth in nude mice (5). Although the precise molecular mechanism by which SARI provokes tumor-suppressing activity requires further clarification, preliminary studies reveal that forced expression of SARI inhibits DNA binding of activator protein (AP-1) complexes and consequently inhibits AP-1–dependent gene expression (6).

We now document cross talk between these two potent tumor suppressor genes; mda-7/IL-24 and SARI. Infection with Ad.mda-7 upregulates SARI mRNA as well as SARI protein, suggesting that SARI might be a downstream facilitator of mda-7/IL-24–mediated cancer-specific cell death. Moreover, both in vitro and in vivo data document that stable transfectants of HeLa that express SARI antisense sequences display resistance to Ad.mda-7 infection. Moreover, we demonstrate that mda-7/IL-24 regulates SARI by posttranscriptional modification rather than through a direct transcriptional mechanism. Finally, our data shows that inhibition of p38 MAP kinase activity, a known target of mda-7/IL-24, ablates mda-7/IL-24–induced SARI upregulation.

Materials and Methods

Cell lines and stable clones

Normal SV-40–immortalized human prostate epithelial cells (P69) were obtained from Dr. Joy Ware (Virginia Commonwealth University, Richmond, VA) and DU-145, PC3, and LNCaP human prostate carcinoma cells were obtained from American Type Culture Collection (ATCC) and cultured as described (25). Normal human SV-40–T-antigen immortalized melanocytes FMS16-SV (referred to as FMS16) were obtained from Dr. Leila Diamond (Wistar Institute, Philadelphia, PA; ref. 26); WM35 radial growth phase primary melanoma cells were provided by Dr. Meenhard Herlyn (Wistar Institute); WM238a and MeWo metastatic melanoma cells were from ATCC; and HO-1 and FO-1 metastatic melanoma cells were from Dr. Eliezer Huberman (Argonne National Laboratories, Lemont, IL). The melanocyte and melanoma cells were cultured as described (26). hTERT immortalized primary human fetal astrocytes (PHFA-Im) were produced in our laboratory (19) and U87MG, U251, G18, U373, and H4 human malignant glioma/neuroglioma cells were obtained from ATCC and cultured as described (27). Cervical carcinoma cells, HeLa, were from ATCC and cultured as described (5). MIA-PaCa-2 and Panc-1 pancreatic carcinoma cells were from ATCC and cultured as described (28). The human immortalized pancre-
Cell viability and proliferation assay

Cell proliferation was determined by standard MTT assays (5, 6, 18). Colony formation assays were done as described (25). Cell growth/viability was monitored by trypan blue dye exclusion assay (5).

Anchorage-independent growth assay in soft agar

Invasion assays and anchorage-independent growth in soft agar assays were done as described (23, 27).

Tumor xenograft studies

Tumor xenografts were established subcutaneously in the left flanks of female athymic nude mice by injecting 2 × 10^6 HeLa SARIAs cl 1 or cl 2 cells (stably overexpressing antisense SARI) mixed at a 1:1 ratio of Matrigel in a total volume of 200 μL. Once tumors reached a measurable size (~100 mm^3), the mice were randomly divided into 2 groups and were treated with either Ad. mda-7 or Ad.vec control for 3 weeks (total 7 injections). When the control tumors reached the maximum allowable limit, mice were sacrificed and tumor weight was determined.

Statistical analysis

The data are presented as the mean ± SD of the values from 3 to 5 independent determinations and statistical analysis was conducted using the Student t test in comparison with corresponding controls. Probability value <0.05 was considered statistically significant. One-way ANOVA was used to test the difference between the means of several subgroups of a variable (Prism Statistical Software).

Results

Adenovirus-transduced mda-7/IL-24 induces SARI in multiple cancers

We initially determined mRNA and protein expression of SARI after ectopic expression of mda-7/IL-24 in immortal normal and cancer cells of different lineages including normal immortal melanocyte/melanoma, normal immortal primary human fetal astrocyte/malignant glioma, and normal immortal prostate epithelial/prostate carcinoma. Northern blotting indicated that infection with Ad.mda-7 (replication-deficient adenovirus-expressing mda-7/IL-24) at 100 pfu/cell resulted in profound induction of SARI mRNA selectively in cancer cells in all 3 tumor contexts (Fig. 1A–C). Infection with 100 pfu/cell of Ad.mda-7 was chosen to avoid issues with differences in infectivity and consequently gene transduction (31) based on expression of Coxsackie adenovirus receptors (CAR). The rationale for using this input of virus is also supported by determining mda-7/IL-24 copy number, using RNAseP as a reference gene, following Ad.mda-7 infection (100 pfu/cell) of DU-145 cells. This protocol resulted in 3.56 × 10^4 copies of mda-7/IL-24 mRNA, which is comparable to the low therapeutic dose used in the clinical trials (2 × 10^10 viral particles per treatment resulted in 2 × 10^4 copies of mda-7/IL-24 mRNA; ref. 9; data not shown). Due to increased sensitivity to Ad. mda-7–induced toxicity (because of elevated transgene transduction), we infected HeLa and LNCaP cells with 20 pfu/cell of Ad. mda-7 and observed a similar effect on SARI mRNA induction (Fig. 1B). Interestingly, in HeLa cells infection with Ad.mda-7,
but not Ad.\textit{p}53, induced \textit{SARI} mRNA (Fig. 1B). This experiment was performed to determine if expression of another tumor suppressor gene, namely wild-type \textit{p}53, which induces death in \textit{HeLa} cells would induce \textit{SARI} mRNA. This data confirms that \textit{SARI} mRNA is induced in \textit{HeLa} cells following ectopic expression of \textit{mda-7}/IL-24 and not wild-type \textit{p}53. \textit{Ad.mda-7} induction of \textit{SARI} protein expression was observed in a temporal manner in malignant glioma, prostate carcinoma, and melanoma (Fig. 2A–C). In contrast, normal immortal counterparts (PHFA-Im, P69, and FM516) failed to display any change in \textit{SARI} protein expression using antisense phosphorothioate oligonucleotides that reduces \textit{ras} gene expression in wild-type \textit{K-ras} mutant pancreatic carcinomas, which represent the vast majority of tumors (85–95%; refs. 34–36), inhibiting \textit{K-ras} expression using antisense phosphorothioate oligonucleotides specifically targeting the \textit{K-ras} gene, sensitizes tumor cells to \textit{Ad.mda-7}-induced apoptosis (30). In contrast, blocking \textit{K-ras} gene expression in wild-type \textit{K-ras} BxPC-3 cells does not sensitize these cells to \textit{Ad.mda-7}, suggesting additional genetic/epigenetic changes may also contribute to resistance to \textit{mda-7}/IL-24 in this particular pancreatic tumor cell (28).

Inhibition of \textit{K-ras}-downstream extracellular signal-regulated kinase 1/2 (ERK1/2) signaling results in \textit{SARI} induction in \textit{Ad.mda-7}-infected pancreatic cancer cells. Although \textit{Ad.mda-7} shows biological activity in most primary and established cancer cells, it exerts no discernible effect on pancreatic cancer cells because of a pancreatic cancer-specific inherent "translational block" that prevents conversion of \textit{mda-7}/IL-24 mRNA into functional protein (28, 30). One potential mechanism for this block could be constitutive activation of \textit{K-ras} because of a pancreatic cancer–specific inherent "translational block" that prevents conversion of \textit{mda-7}/IL-24 mRNA into functional protein (28, 30).

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tumor growth in xenograft murine models (28, 30). Therefore, we tested the effect of simultaneous inhibition of ERK1/2 using PD98059 (MEK inhibitor) at a concentration not affecting growth in combination with Ad.mda-7 infection. Interestingly, inhibiting K-ras-downstream ERK1/2 signaling by PD98059 at 50 μmol/L concentration or Ad.mda-7 at 100 pfu/cell infection alone failed to statistically inhibit growth of pancreatic cancer cells, whereas simultaneous treatment with PD98059 and Ad.mda-7 led to profound growth suppression. Only the combination treatment induced SARI mRNA and protein, and MDA-7/IL-24 protein expression in PANC-1 and MIA-PaCa-2 cells, suggesting that the "translational block" was reversed by ERK1/2 inhibition (Fig. 3A and B). Immortal pancreatic epithelial cells LT2 expressed basal levels of SARI (both mRNA and protein) and infection with Ad.mda-7 or treatment with PD98059 alone or in combination did not increase SARI levels or suppress growth, which supports the cancer-selective toxicity of mda-7/IL-24 as well as cancer-selective induction of SARI by mda-7/IL-24 in pancreatic carcinoma cells (Fig. 3A–C).

To test if MDA-7/IL-24 protein was capable of directly inducing SARI and subsequently cell death in pancreatic cancer cells, we treated PANC-1 and MIA-PaCa-2 cells with GST-MDA-7 protein. Following treatment with GST-MDA-7 at a concentration of 30 nmol/L for 48 hours, a dose chosen based on prior studies (37), cell lysates were analyzed for SARI expression (Fig. 3D). Interestingly, treatment of pancreatic cancer cells with exogenous MDA-7/IL-24 protein (GST-MDA-7) that enters cells through a yet to be defined mechanism (37) significantly induced cell death in both PANC-1 and MIA-PaCa-2 cells, suggesting that MDA-7/IL-24 protein delivered inside the cell can induce pancreatic cancer cell death (Fig. 3C). In addition, because GST-MDA-7 induces SARI protein expression in pancreatic cancer cells, induction of SARI mRNA and protein expression by MDA-7/IL-24 occurs through a process that is independent of the translational block in pancreatic cancer cells.
SARI expression is essential for mda-7/IL-24–induced cancer cell death

To investigate a potential cause-and-effect relationship between mda-7/IL-24 and SARI expression in mediating cancer-specific cytotoxicity, HeLa cells that ectopically express SARI antisense sequences were infected with Ad.mda-7. We selected 4 different HeLa clones expressing antisense SARI (HeLa SARIAs; ref. 5) displaying variable, inducible levels of SARI expression after Ad.mda-7 expression (Fig. 4Ai). Initially, we compared SARI message induction in HeLa SARIAs clones 48 hours after infection with Ad.mda-7 at 100 pfu/cell. HeLa SARIAs cl 2 and HeLa SARIAs cl 4 did not show SARI expression, whereas HeLa SARIAs cl 1 showed reduced induction of SARI versus vector (pcDNA3.1) control. HeLa cells and HeLa SARIAs cl 6 showed equivalent induction of SARI as the control HeLa pcDNA3.1 clone (Fig. 4Ai); compared with GAPDH levels. When we analyzed IFN-β–mediated growth inhibition in these clones, HeLa SARIAs cl 6 was sensitive to IFN-β, but HeLa SARIAs cl 4 was resistant (Fig. 4Aii). SARI was reported to be an IFN-β–inducible gene that plays a significant role in IFN-β–mediated growth inhibition (5) and this data supports that hypothesis.

It was evident using cell proliferation assays that HeLa SARIAs cl 1 and cl 6 were sensitive to mda-7/IL-24, whereas HeLa SARIAs cl 2 and cl 4 were resistant to Ad.mda-7 (Fig. 4B). A similar trend was observed using colony formation and anchorage independent agar growth assays (Fig. 4C and D). These data suggest that SARI plays a prominent role in both mda-7/IL-24–induced cell killing and IFN-β–mediated toxicity in HeLa cells. To investigate further the dependence of mda-7/IL-24 on SARI expression in vivo, HeLa SARIAs cl 1 and cl 2 cells were injected subcutaneously to establish tumor xenografts in athymic nude mice (38). After palpable tumors of 75 mm³ developed, in 7 to 10 days, the animals received 7 intratumoral injections over a 3-week period, with 1 × 10⁷ pfu of Ad.vec or Ad.mda-7. In HeLa SARIAs cl 1, a significant growth-inhibitory effect was observed after injection of Ad.mda-7, whereas a nonsignificant antitumor growth-inhibitory effect (based on...
tumor weight) was observed with injection of Ad.mda-7 in HeLa SARIAs cl 2 xenografts (Fig. 4E). Taken together, these results emphasize the significance of SARI expression in eliciting MDA-7/IL-24–mediated cell killing in cancer cells.

**mda-7/IL-24 posttranscriptionally regulates SARI expression**

To explore the molecular mechanism by which mda-7/IL-24 regulates the expression of SARI, we cloned an ~3-kb promoter region of SARI into the pGL3-basic-Luc vector and transfected it into HeLa cells. Promoter reporter activity was assessed 24 hours after treating with IFN-β or infecting with Ad.mda-7 or Ad.vec. Infection with Ad.mda-7 failed to induce the full-length or truncated SARI promoter (Fig. 5A), whereas an increase in SARI mRNA expression (Fig. 5Bii) and SARI protein expression (data not shown) was evident in HeLa cells infected with Ad.mda-7. In contrast, the level of SARI induction measured by nuclear run-on assays was only modestly changed after infection with Ad.mda-7 (Fig. 5Bi). In the case of IFN-β treatment, only the full-length and specific regions of the SARI promoter (Nt-924 and to a lesser extent Nt-694, Nt-594, Nt-494, and Nt-344) enhanced luciferase activity (Fig. 5A). These data indicate possible regulation of SARI mRNA at a posttranscriptional level following infection with Ad.mda-7.

To explore posttranscriptional control of SARI mRNA induction after Ad.mda-7 infection, we cloned the 3′-UTR region of SARI mRNA into a PGL3-basic-Luc vector (Luc-3′UTR) and transfected it into HeLa cells. Upon infection with Ad.mda-7, ACT D (h) 0 0.5 1 1.5 2 2.5 3 3.5 4

**Figure 5.** mda-7/IL-24 regulates SARI expression posttranscriptionally. A, SARI full length promoter/pGL3-Luc or SARI deletion mutant promoter/PGL3-Luc constructs were transfected into HeLa cells. Twenty-four hours later, the cells were treated with IFN-β (1,000 U/mL) or infected with Ad.vec (100 pfu/cell) or Ad.mda-7 (100 pfu/cell) for 24 hours, after which cell lysates were collected for luciferase assays. B, i, HeLa cells were infected with Ad.vec or Ad.mda-7, 10 pfu/cell and nuclei were prepared from the treated cells. The isolated nuclei were used to label preinitiated RNA transcription with [α-32P]UTP in vitro, and the purified RNA was hybridized to a dot blot carrying an equivalent amount of a SARI cDNA. The transcription rate of GAPDH served as control. ii, HeLa cells were treated as described in A and total RNA was purified, blotted onto a nylon membrane, and Northern blotting was done with a SARI, mda-7/IL-24, or GAPDH cDNA. C, 3′-UTR region of SARI mRNA was cloned into PGL3-basic-Luc vector (Luc-3′UTR) and three independent clones (along with control vector) were transfected into HeLa cells for 24 hours followed by infection with Ad.vec or Ad.mda-7 (100 pfu/cell for 24 hours) and cell lysates were collected for luciferase assays. The data are presented as the ratio of Ad.mda-7 to Ad.vec treated cells. All experiments were performed at least three times, and data represent mean ± SD. *, P < 0.05 RLU is significantly higher than PGL-3 Luc control cells. D, P69 cells were infected with Ad.vec or Ad.mda-7 (20 pfu/cell for 24 hours), after which Actinomycin D (0.5 μg/mL) was added for the indicated times. Total RNA was collected to perform Northern blotting using a SARI cDNA.
the Luc-3’UTR clones showed significantly higher luciferase activity (Ad.mda-7/Ad.vec) as compared with the control vector (Fig. 5C). To determine whether mda-7/IL-24 stabilizes SARI mRNA, P69 cells (which exhibit basal SARI expression) were infected with Ad.vec or Ad.mda-7 for 24 hours followed by actinomycin D (Act D) treatment. Cells were then incubated further and harvested at different time points. Total RNA was isolated, and SARI mRNA expression was examined by Northern blotting to measure mRNA decay rates. With infection of Ad.vec the half-life of SARI mRNA was 1.5 hours, whereas Ad. mda-7 infection extended half-life to 3.5 hours, suggesting that mda-7/IL-24 results in increased stability of SARI mRNA.

**Recombinant His-MDA-7/IL-24 induces SARI expression**

Recombinant MDA-7/IL-24 protein (His-MDA-7) robustly induces expression of endogenous mda-7/IL-24, generating signaling events necessary for "bystander killing" (22). At physiological levels, MDA-7/IL-24 binds to MDA-7/IL-24 cognate receptor complexes consisting of 2 sets of heterodimeric chains, IL-20R1/IL-20R2 or IL-22R/IL-20R2 (38–40). The binding and signaling through these receptor complexes results in phosphorylation of STAT-3 following treatment with purified MDA-7/IL-24 protein (39–41). We selected a panel of cells that express a functional pair of IL-20/IL-22 receptors, IL-20R1/IL-20R2, IL-22R/IL-20R2, or IL-20R1/IL-22R (Supplementary Fig. S1), or lack a functional set of cognate receptors (A549 lung carcinoma: contain only IL-20R1) and do not respond to exogenously applied His-MDA-7. Treatment of DU-145, HO-1, and A549 cells with 5 μmol/L His-MDA-7 for 24 hours resulted in SARI mRNA expression as assessed by qRT-PCR in both DU-145 (contains all 3 receptor subtypes) and HO-1 (contains IL-20R1 and IL-22R) cells, with no appreciable increase in A549 cells (Fig. 6A). Moreover, immunoblotting of total cell lysates from DU-145 and HO-1 cells showed SARI protein with a concomitant induction in endogenous MDA-7/IL-24 protein, without induction of SARI protein in A549 cells (Fig. 6B). Experiments were then performed to confirm that IL-20R2 or IL-22R receptors are required for SARI induction in cells containing IL-20R1 receptors. Three siRNAs duplexes were tested for IL-20R2 and IL-22R (Supplementary Fig. S2), and the most effective one was used for further testing. After IL-20R2 silencing in DU-145 and IL-22R silencing in HO-1 cells, His-MDA-7 failed to induce MDA-7/IL-24 protein expression in total lysates and there was no subsequent induction of SARI protein expression (Fig. 6C). These receptor-modified cells also failed to show a reduction in cell viability following IL-20R2 or IL-22R silencing, respectively, when treated with His-MDA-7 (Fig. 6D).

To authenticate the direct role of the heterodimeric cognate IL-20/IL-22 receptor pairs in mediating response to mda-7/IL-24 and induction of SARI expression A549 cells, which express IL-20R1, were genetically modified to stably express either IL-20R2 or IL-22R (Fig. 6E). When treated with recombinant His-MDA-7, both genetically modified IL-20R2 and IL-22R over-expressing A549 cells produced SARI protein (Fig. 6F, left) and subsequently displayed reduced cell viability (Fig. 6F, right), whereas pcDNA3.1 control A549 cells failed to respond to His-MDA-7. These results highlight the obligatory nature of IL-20R1/IL-20R2 receptor pair and further support for the first time signaling by the heterodimeric combination of IL-22R/IL-20R1 in mediating responses of cancer cells to secreted MDA-7/IL-24 protein through a "bystander" antiproliferative and killing effect in human tumor cells. These observations also further strengthen the link between expression of mda-7/IL-24 and SARI.

We also determined if glutathione S-transferase (GST)–fused MDA-7/IL-24 (19, 21, 37) could induce SARI expression. Interestingly, SARI expression was induced by GST-MDA-7 (30 nmol/L for 48 hours) in heterodimeric cognate receptor positive DU-145 and HO-1 cells as well as in A549 receptor complex negative tumor cells (Fig. 6G). These results support our hypothesis that GST-MDA-7 upon entering cells via an IL-20/IL-22 receptor–independent mechanism can induce SARI protein, subsequently leading to cell death (Fig. 6G). To determine if induction of endogenous mda-7/IL-24 is mandatory for promoting SARI expression, we treated DU-145 cells stably expressing mda-7/IL-24 shRNA with His-MDA-7 or GST-MDA-7. Interestingly, GST-MDA-7 induced SARI protein expression, whereas His-MDA-7 failed to induce SARI expression in mda-7-shRNA expressing DU-145 cells, suggesting that SARI induction can also be induced through pathways that do not require endogenous mda-7/IL-24 generation (Fig. 6H).

**Inhibition of mda-7/IL-24 signaling ablates SARI-mediated cell killing**

In human melanoma cells, mda-7/IL-24 mediates a UPR, leading to activation of p38 MAPK activity and the induction of GADD genes culminating in apoptosis (42). In this context, inhibition of p38 MAPK activity results in development of resistance to mda-7/IL-24–mediated cytotoxicity (42, 43). Accordingly, we examined whether MDA-7/IL-24–induced signaling mediated by p38 MAPK and GADD activation was necessary for SARI induction and cancer cell death. Treatment of DU-145 and HeLa cells with a pharmacological inhibitor of p38 MAPK (SB203580) increased resistance to Ad.mda-7–induced growth inhibition (Fig. 7A). Northern blotting indicated that inhibition of p38 MAPK by a dominant negative construct (Ad.dnp38; refs. 42 and 43), or treatment with SB203580 ablated mda-7/IL-24 induction of SARI (Fig. 7B and C). These results confirm that active p38 MAPK is required for mda-7/IL-24 induction of SARI expression.

**Discussion**

Reprogramming tumor cells to undergo apoptosis or toxic autophagy holds significant promise as a general strategy for treating local and metastatic disease (2, 3). Our discovery of mda-7/IL-24 and SARI as upregulated genes using subtraction hybridization applied to induction of melanoma cell terminal differentiation and apoptosis (44–46) has taken us closer to achieving this goal. A distinctive feature of mda-7/IL-24 as a cancer therapeutic is its ability to selectively kill a wide range of cancer cells, through apoptosis and toxic autophagy, without harming normal cells (2, 8, 21, 43, 47, 48). We demonstrated that SARI, an IFN-β–induced gene (5, 6), also preferentially induced apoptosis in cancer cells, without displaying toxicity toward normal cells (5); highlighting mutually overlapping roles of...
mda-7/IL-24 and SARI. We presently delineate a dependent relationship between these two tumor suppressor proteins, MDA-7/IL-24 and SARI. mda-7/IL-24 induces SARI mRNA and SARI protein expression, and through genetic and pharmacological manipulations we demonstrate that mda-7/IL-24 implements its cell killing through SARI induction. Using a panel of melanoma, prostate, and glioblastoma/neuroglioma cells and their normal counterparts, we demonstrate a profound increase in SARI expression following adenovirus-mediated delivery of mda-7/IL-24. Interestingly, induction of SARI expression occurred and inhibited the growth of cancer cells, without affecting their normal counterparts. Using both gain-of-function and loss-of-function genetic approaches we demonstrate that SARI expression is necessary for mda-7/IL-24–induced cancer-specific killing. In these contexts, SARI represents a downstream target of MDA-7/IL-24 in cancer cells, which functions as a key determinant of cancer-specific killing by this IL-10-family cytokine gene member.

IFN-β induces SARI expression as early as 2 hours (4), indicating that it is a type I IFN-inducible early response gene. SARI is a bZIP domain-containing nuclear protein that interacts with c-Jun via its leucine zipper domain (5). SARI binds with c-Jun and suppresses DNA-binding activity of AP-1 complexes inhibiting AP-1–mediated gene transcription in breast cancer cells causing cell-cycle arrest by suppressing G1 cyclin expression and reducing cyclin-dependent kinase activity (49). SARI induction also inhibits AP-1–dependent gene expression, including CCNI, which is a secretory integrin-binding protein critical for tumor cell proliferation, migration, and invasion (6).

Changes in protein expression through differential translation of mRNAs represent potential mediators of the complex

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**Figure 6.** Recombinant His-MDA-7/IL-24 induces SARI expression. A, the indicated cells were treated with various concentrations of His-MDA-7 protein for 24 hours. Total RNA was extracted using Qiagen RNeasy kit, reverse transcription, and qPCR was carried out using Applied Biosystems 7500. B, DU-145, HO-1, and A549 cells were treated with 5 μmol/L His-MDA-7 for 24 hours and lysates were collected and Western blotting was performed as described in Fig. 3B, C, IL-20R2 expression in DU-145, and IL-22R expression in HO-1 cells were suppressed using respective targeted siRNAs or treated with nontargeted siRNAs (NT siRNA). These cells were treated with 5 μmol/L His-MDA-7 (24 hours) and lysates were collected and Western blotting was performed as described in Fig. 3B. D, DU-145 and HO-1 cells were treated with 5 μmol/L His-MDA-7 (48 hours) and cell proliferation/viability was measured by MTT assays. All experiments were performed at least three times, and data represent mean ± SD. *, P < 0.05 cell growth is significantly less than cells treated with nontargeted (NT) control siRNA (one-way ANOVA with Newman Keuls). E, A549 cells were stably transfected with IL-20R2 or IL-22R and expression of IL-20R1, IL-20R2, IL-22R, and actin in cell lysates was determined by Western blotting as described in Fig. 3B, with actin as a control for protein loading. F, genetically modified A549 cells were treated with 5 μmol/L of His-MDA-7 (48 hours) and lysates were collected and Western blotting was performed using MDA-7/IL-24, SARI, and Actin antibodies as described in Fig. 3B (left). Right, the effect of His-MDA-7 on proliferation/viability by MTT assay of stable pcDNA3.1 A549 cells, stable IL-20R2 A549 cells, and stable IL-22R A549 cells. A549 constitutively express IL-20R1 receptors (E). G, expression of SARI protein in various cells treated with GST-MDA-7 or His-MDA-7. DU-145, HO-1, and A549 cells were treated with GST-MDA-7 or His-MDA-7 and expression of SARI was assessed using Western blotting. H, DU-145 cells stably expressing mda-7 shRNA were treated with His-MDA-7 or GST-MDA-7 and expression of MDA-7/IL-24 and SARI was assessed using Western blotting. EF-1α was used as a loading control for this experiment. All experiments shown in this figure were performed at least three times.
phenotypes observed in cancer cells during tumor development and progression (50, 51). Translation controlling elements frequently interact with oncogenes and components of defined signal transduction pathways in cancer, exerting positive or negative effects on translation of specific mRNAs (50, 51). Pancreatic cancer cells are inherently resistant to mda-7/IL-24, resulting from an mRNA “translational block” limiting production of MDA-7/IL-24 protein (28, 30, 32, 33). This restricted translation occurs because of decreased association of mda-7/IL-24 mRNA with polysomes, a phenomenon that can be mediated through aberrant signaling such as constitutive activation of the Ras pathway, which is a common event in pancreatic cancer cells (28, 30, 35). Reversal of the “translational block” in mutant K-ras pancreatic cancer cells can be induced by treatment with ROS-generating agents (28, 30, 32–35). Reversing the “translational block” by facilitating mRNA association with polysomes culminates in apoptosis induction in pancreatic cancer cells. Nontoxic doses of perillyl alcohol sensitize resistant pancreatic carcinoma cells, but not normal immortal pancreatic mesenchymal cells, to mda-7/IL-24-mediated gene therapy by enhancing ROS production, thereby reversing the “translational block” (33, 35). Suppression of the ras pathway enhances the ability of specific mRNAs to associate with polysomes thereby enhancing protein translation (32). Abrogating K-ras expression, and consequently, its downstream MEK1/2 signaling pathway results in the conversion of mda-7/IL-24 mRNA into protein by inducing translational enhancement of this mRNA (32, 33, 35). In the absence of MDA-7/IL-24 protein in pancreatic cancer cells, SARI induction is inhibited. Blocking ERK1/2 using PD98059 (MEK inhibitor) by itself failed to induce SARI expression, but when mutant K-ras pancreatic cancer cells were treated with PD98059 and infected with Ad.mda-7 SARI expression was induced. Alternatively, recombinant GST-MDA-7/IL-24 protein induced SARI expression in a Ras pathway-independent and cognate MDA-7/IL-24 receptor-independent manner. These results support the development of rational combinatorial approaches with potential to enhance therapeutic activity in pancreatic cancer using concurrent inhibition of the Ras pathway combined with Ad.mda-7 or targeted delivery of GST-MDA-7 (38). In addition, approaches can also be developed that do not rely on Ras inhibition, using a SARI-based therapeutic approach.

Secreted MDA-7/IL-24 protein following infection with Ad. mda-7 induces growth inhibition and apoptosis in surrounding noninfected cancer cells, but not in normal cells, through an antitumor “bystander” effect (22, 23, 52). In addition, secreted MDA-7/IL-24 protein induces endogenous MDA-7/IL-24 protein expression in both normal and cancer cells through an autocrine/paracrine loop (Fig. 7D; ref. 22). This autocrine/paracrine loop underlies the observed profound distant tumor growth inhibitory/apoptotic effects observed with MDA-7/IL-24 therapy (47, 48, 52, 53). Using recombinant MDA-7/IL-24 (His-MDA-7) protein, we provide proof-of-concept that secreted MDA-7/IL-24 via binding to its cognate receptors induces intracellular SARI expression. Activation of cognate IL-20/IL-22 receptors leads to phosphorylation of p38 MAPK, which activates transcription of GADD genes (42), subsequently leading to apoptosis. Induction of SARI by MDA-7/IL-24 protein is p38 MAPK dependent, because inhibiting p38 MAPK using either genetic (Ad.dnp38) or pharmacological (SB203580) approaches prevents induction of SARI expression and cell death (Fig. 7D). These results further emphasize the significance of the MDA-7/IL-24-SARI axis in eliciting tumor-specific cell killing.

Figure 7. Inhibition of p38 MAPK ablates mda-7/IL-24 induction of SARI expression and cell death. A, DU-145 and HeLa cells were treated with the indicated concentration of SB203580 (2 hours) and then infected with Ad. vec or Ad.mda-7 (100 pfu/cell) for 48 hours, after which proliferation/cell viability was measured by MTT assay. B, DU-145 cells were infected with Ad.vec (−/− lane), Ad.dnp38, and/or Ad.mda-7 (100 pfu/cell) for 48 hours, after which total RNA was purified, blotted onto a nylon membrane, and Northern blotting was done with a SARI cDNA or GAPDH probe. C, the indicated cells were treated with DMSO (−) or SB203580 (SB+) for 2 hours and then infected with Ad.mda-7 (48 hours), after which total RNA was purified, blotted onto a nylon membrane, and Northern blotting was done with a SARI cDNA or GAPDH probe. D, schematic diagram describing MDA-7/IL-24–induced SARI-mediated cancer cell killing.
Understanding the regulation and mode of action(s) of tumor suppressor genes such as mda-7/IL-24 and SARI are pivotal for creating nontoxic strategies to treat local and metastatic disease. Antitumor effects because of expression of mda-7/IL-24, confirmed in cell culture and in multiple animal models led to its successful entry into the clinic in an unprecedented time (5 years), where safety and clinical efficacy when administered by adenovirus (Ad.mda-7) has been shown in a phase I clinical trial in humans with advanced carcinomas and melanomas (9, 14, 47–48, 52). This study brings us closer to understanding the significance of the mda-7/IL-24-SARI axis in tumor-specific killing and provides a rational path to potentially exploit this relationship to develop novel therapeutic strategies to effectively treat multiple cancers.

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

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Conception and design: R. Dash, P. Bhoopathi, B. Sarkar, P.B. Fisher
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R. Dash, P. Bhoopathi, P.B. Fisher

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