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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 (*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 regulated kinase 1/2 (ERK1/2) 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.

Keywords

SARI; MDA-7/IL-24; apoptosis; IL-20/IL-22 receptors

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Introduction

Cancer is a progressive disease with advanced tumors frequently displaying resistance to multiple therapies, including chemotherapy, immunotherapy and radiation (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).

mda-7/IL-24, a novel member of the IL-10-related cytokine gene family (4), and *SARI* (Suppressor of AP-1, Regulated by IFN), a novel type I 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 radiation- 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 (ER) chaperone protein BiP/GRP78 and initiates a cascade of “*unfolded protein response (UPR)*” processes in tumor cells down-regulating anti apoptotic proteins, including Bcl-X_L and Mcl-1 (24, 25).

SARI expression is induced as early as 2 h 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 sparing their respective normal counterparts (5, 6). Infection with Ad.*SARI* (adenovirus expressing *SARI* regulated by a CMV 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* up-regulates *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 post-transcriptional 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* up regulation.

Materials and methods

Cell lines and stable clones

Normal SV-40-immortalized human prostate epithelial cells (P69) were obtained from Dr. Ware, VCU, 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 FM516-SV (referred to as FM516) were obtained from Dr. Diamond, Wistar Institute (26); WM35 radial growth phase primary melanoma cells were provided by Dr. Herlyn, Wistar Institute; WM238 and MeWo metastatic melanoma cells were from ATCC; and HO-1 and FO-1 metastatic melanoma cells were from Dr. Huberman, Argonne National Laboratories. The melanocyte and melanoma cells were cultured as described (26). H-TERT immortalized primary human fetal astrocytes (Im-PHFA) 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 pancreatic mesenchymal cell line LT2 was from Chemicon and cultured according to the manufacturer's instructions (28). Stable HeLa cells constitutively expressing antisense *SARI* (HeLa-SARIAs) were established as described (5). All cells were routinely monitored for mycoplasma contamination.

Nuclear Run-On Assays and Northern blotting

Nuclear run-on assays and northern blotting for *SARI* and *GAPDH* were performed as described (29).

Preparation of whole-cell lysates and Western blotting

Western blotting was performed as described (5, 6, 29). The primary antibodies included anti-SARI polyclonal antibody raised by immunizing rabbits with full-length bacterially expressed SARI protein, anti-MDA-7/IL-24 (GenHunter Corporation), anti-total and phospho-p38 (Thr-180/Tyr-182) (Cell Signaling Technology), IL-20R1, IL-20R2, IL-22R (R&D systems) and anti-EF1 α (Upstate Biotechnology).

RNA extraction, Northern blot analysis, RT-PCR

Total cellular RNA was isolated by the guanidinium/phenol extraction method and Northern blotting was performed as described (30). The cDNA probes were full-length human *SARI*, and *GAPDH*. *SARI* and *mda-7/IL-24* Taqman probes were from Applied Biosystems and quantitative real time-PCR was performed as described (25).

Cloning of SARI promoter and 3' UTR

The *SARI* promoter (~3-kb fragment) was cloned by amplifying genomic DNA isolated from PHFA-Im cells using primers 5'CGGGCTTCAGGGTTTGCTAGGTCT'3 (sense) and 5'CCAGAGGGCCAAGTTTCAGTTTCTCC'3 (antisense). Random deletion mutants were generated by PCR amplification of *SARI* promoter using gene-specific primers as described (29). The full-length *SARI* promoter construct and deletion mutants were cloned into the chloramphenicol acetyltransferase (CAT) reporter vector pGL3 basic (Promega) to produce the *SARI*/pGL3 or *SARI* deletion mutant/pGL3 constructs. The 3'UTR region was amplified from the mRNA of IFN-induced PHFA-Im cells using primers 5'TCAGGAAGCAGCCTTAGC'3 (sense) and 5'CCAGGAGTGTGTTAGATAC'3 (Antisense). The *SARI* 3'UTR construct was cloned into the chloramphenicol acetyltransferase (CAT) reporter vector pGL3 basic (Promega) producing the 3'-UTR-Luc construct.

Transient transfections and luciferase assays

Transient transfections and luciferase assays were performed as described (5, 6, 29). In brief, transfections were carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. For Luciferase assays, cells were transiently cotransfected with reporter gene constructs (*SARI* full length or deleted promoter/pGL3Luc or 3'-UTR-Luc) or the pGL3-basic empty vector (Promega, Madison, WI) and pRL-TK-luc plasmid coding for *Renilla* luciferase. Cells were incubated for 24 h and then treated with Ad.vec or Ad.*mda-7* for the indicated time, after which the activities of firefly and *Renilla* luciferases were measured using the Dual-Luciferase reporter assay system (Promega).

Cell viability and proliferation assay

Cell proliferation was determined by standard 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (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 μ l. Once tumors reached a measurable size (~ 100 mm³), 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 $\pm$ 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 three-tumor contexts (Fig. 1A–1C). 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) (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.*p53*, induced *SARI* mRNA (Fig. 1B). This experiment was performed to determine if expression of another tumor suppressor gene, namely wild type p53, which induces death in HeLa cells would induce *SARI* mRNA. This data confirms that *SARI* mRNA is induced in HeLa cells following ectopic expression of *mda-7/IL-24* and not wild type p53. Ad.*mda-7* induction of *SARI* protein expression was observed in a temporal manner in malignant glioma, prostate carcinoma and melanoma (Fig. 2A–2C). In contrast, normal immortal counterparts (PHFA-Im, P69 and FM516) failed to display any change in *SARI* protein following infection with Ad.*mda-7*. In parallel with the change in expression of *SARI* after infection with Ad.*mda-7*, we observed cancer-specific growth inhibition in all three-cancer types, with no appreciable change in the growth of their normal counterparts (Fig. 2D). Overall, this data suggests that the cancer-specific toxicity of *mda-7/IL-24* correlates with induction of *SARI* expression.

Inhibition of K-ras-downstream extracellular regulated kinase 1/2 (ERK1/2) signaling results in *SARI* induction in Ad.*mda-7*-infected pancreatic cancer cells

Although 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 *mda-7/IL-24* mRNA into functional protein (28, 30). One potential mechanism for this block could be constitutive activation of K-ras that reduces association of *mda-7/IL-24* mRNA with polysomes (30, 32–34). In K-ras mutant pancreatic carcinomas, which represent the vast majority of tumors (85–95%) (34–36), inhibiting K-ras expression using antisense phosphorothioate oligonucleotides specifically targeting the K-ras gene, sensitizes tumor cells to Ad.*mda-7*-induced apoptosis (30). In contrast, blocking K-ras gene expression in wild-type K-ras BxPC-3 cells does not sensitize these cells to Ad.*mda-7*, suggesting additional genetic/epigenetic changes may also contribute to resistance to *mda-7/IL-24* in this particular pancreatic tumor cell (28).

ERK1/2 inhibitors in K-ras mutant pancreatic carcinoma cells inhibit *in vitro* growth and suppress *in vivo* subcutaneous 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 μ M concentration or Ad.*mda-7* at 100 pfu/cell infection alone failed to statistically inhibit growth of pancreatic cancer cells, while 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 3B). 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, 3B and 3C).

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 h, 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). Additionally, since 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) (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 h 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* vs. 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 to *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 4D). 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 seven intratumoral injections over a 3-week period, with 1×10^8 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 non-significant 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* post-transcriptionally 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 PGL-3-basic-Luc vector and transfected it into HeLa cells. Promoter reporter activity was assessed 24 h 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 post-transcriptional level following infection with Ad.*mda-7*.

To explore post-transcriptional 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* the Luc-3'UTR clones showed significantly higher luciferase activity (Ad.*mda-7*/Ad.vec) as compared to 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 h 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 h, whereas Ad.*mda-7* infection extended half-life to 3.5 h 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 two 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 (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 μ M His-MDA-7 for 24 h resulted in *SARI* mRNA expression as assessed by qRT-PCR in both DU-145 (contains all three 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 (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 overexpressing A549 cells produced *SARI* protein (Fig. 6F, left panel) and subsequently displayed reduced cell viability (Fig. 6F, right panel), while pcDNA3.1 control A549 cells failed to respond to His-MDA-7. These results highlight the obligatory nature 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' anti-proliferative 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 h) 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, while 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 an unfolded protein response (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) (42, 43), or treatment with SB203580 ablated *mda-7/IL-24*-induction of *SARI* (Fig. 7B, 7C). 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 h (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 CCN1, 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 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. Non-toxic doses of perillyl alcohol (POH) 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). Additionally, 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 non-infected cancer cells, but not in normal cells, through an antitumor “*bystander*” effect (22, 23, 52). Additionally, 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) (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, since 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.

Understanding the regulation and mode of action(s) of tumor suppressor genes such as *mda-7/IL-24* and *SARI* are pivotal for creating non-toxic strategies to treat local and metastatic disease. Antitumor effects due to 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 therapeutics to effectively treat multiple cancers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Forced expression of *mda-7*/IL-24 induces *SARI* mRNA expression in multiple cancers
 The indicated cells were infected as indicated with Ad.vec or Ad.mda-7 (or Ad.p53) at 20 pfu/cell (HeLa, LNCaP) or 100 pfu/cell (other cells) for 24 h. For Quantitative mRNA detection, total RNA was purified, blotted onto nylon membranes and Northern blotting was done with a *SARI* cDNA. **A)** Im-PHFA, malignant glioma and neuroglioma cells. **B)** P69, prostate cancer and HeLa cells. **C)** FM516 and melanoma cells. *GAPDH* mRNA was used as loading control.

Fig. 2. Forced expression of *mda-7*/IL-24 induces SARI protein expression causing cancer cell death

The indicated cell lines were infected with Ad.vec or Ad.*mda-7* at 100 pfu/cell for the indicated time after which cell lysates were collected. For detecting protein expression, 50 μ g of total protein was run on SDS-PAGE, transferred onto a nitrocellulose membrane and stained with the indicated antibodies, and protein expression was determined by Western blotting in **A**) Im-PHFA, malignant glioma and neuroglioma, **B**) P69 and prostate cancer cells, and **C**) FM516 and melanomas. **D**) Cells were infected with Ad.vec or Ad.*mda-7* at 100 pfu/cell for the indicated time after which cell proliferation/viability was assayed by MTT assay. All experiments were performed at least three times, and data represent mean $\pm$ S.D. * $p < 0.05$ cell viability is significantly less than cells infected for 0 h (one way ANOVA with Newman Keuls).

Fig. 3. Inhibition of K-ras-downstream ERK1/2 signaling plus Ad.mda-7 infection induces SARI expression in K-ras mutant pancreatic cancer cells

A) Cells were treated with PD98059 (50 μM) for 2 h and then infected with Ad.vec or Ad.mda-7 (100 pfu/cell) for 24 h. For Quantitative mRNA detection, total RNA was purified, blotted onto a nylon membrane, and Northern blotting was done with a SARI cDNA. GAPDH mRNA was used as loading control. **B)** LT-2, PANC-1 and MIA-PaCa-2 cells were treated as described in (A) and total lysates were collected. For protein expression 50 μg of total protein was run on SDS-PAGE, transferred onto nitrocellulose membranes, stained with the indicated antibodies and protein expression was determined by Western blotting. **C)** LT-2, PANC-1, MIA-PaCa-2 cells were treated with PD98059 (50 μM) for 2 h, infected with Ad.vec or Ad.mda-7 for 48 h, and then cell proliferation/cell viability was measured by MTT assay. Experiments were performed at least three times, and data represent mean ± S.D. * p<0.05 cell viability is significantly less than vector infected controls (one way ANOVA with Newman Keuls). **D)** PANC-1 and MIA-PaCa-2 cells were treated with 30 nmol/L GST-MDA-7 for 48 h and lysates were collected and SARI expression was examined using Western blotting as in (B).

Fig. 4. Knockdown of *SARI* promotes resistance to *mda-7/IL-24*-mediated apoptosis

Ai). HeLa clones stably expressing antisense *SARI* (HeLa SARIAs) were infected with Ad.mda-7 (48 h). For quantitative mRNA detection, total RNA was purified, blotted onto a nylon membrane, and Northern blotting was done with an *mda-7/IL-24* or *SARI* cDNA. *GAPDH* mRNA was used as a loading control. **Aii).** HeLa clones 4 and 6 stably expressing antisense *SARI* (HeLa SARIAs) were treated with IFN- β as indicated and cell number was determined by cell counting. **B)** HeLa cells stably expressing antisense *SARI* (HeLa SARIAs clones 1, 2, 4 and 6) were infected with 100 pfu/cell of Ad.vec or Ad.mda-7 as indicated and cell viability was determined by cell counting. **C)** HeLa cells stably expressing pcDNA3.1 or antisense *SARI* (HeLa SARIAs clones 2, 4 and 6) were infected with 100 pfu/cell of Ad.vec or Ad.mda-7 for 24 h after which cells were harvested and colony formation assays were performed. Representative photomicrographs are shown. **D)** HeLa cells stably expressing pcDNA3.1 or antisense *SARI* (HeLa SARIAs cl 2) were infected with 100 pfu/cell of Ad.vec or Ad.mda-7 for 24 h after which cells were harvested for soft agar colony formation assays. All experiments were performed at least three times; data presented as mean $\pm$ S.D. * $p < 0.05$ number of clones is significantly more than cells infected with Ad.vec (one way ANOVA with Newman Keuls). **E)** Tumor xenografts were established by injecting HeLa SARIAs cl 1 and cl 2 stably overexpressing antisense *SARI* in athymic nude mice, and tumors were injected with the indicated Ads over a 3-week period (total of seven injections). At the end of the experiment tumor weight was determined. Columns represent means (with at least five mice in each group); bars, $\pm$ SD. *Inset*: Representative photograph of an animal of each representative group. Average tumor volume of Ad.mda-7-treated HeLa SARIAs cl 1 group was significantly less than the average tumor volume of the Ad.vec-treated HeLa SARIAs cl 1 group, $p < 0.01$.

Fig. 5. *mda-7/IL-24* regulates *SARI* expression post-transcriptionally

A) *SARI* full length promoter/pGL3-Luc or *SARI* deletion mutant promoter/pGL3-Luc constructs were transfected into HeLa cells. Twenty-four h later the cells were treated with IFN- β (1000 U/ml) or infected with Ad.vec (100 pfu/cell) or Ad.*mda-7* (100 pfu/cell) for 24 h after which cell lysates were collected for luciferase assays. **B) i):** HeLa cells were infected with Ad.vec or Ad.*SARI* at 10 pfu/cell and nuclei were prepared from the treated cells. The isolated nuclei were used to label preinitiated RNA transcription with [α - 32 P] 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 panel 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 h followed by infection with Ad.vec or Ad.*mda-7* (100 pfu/cell for 24 h) and cell lysates were collected for luciferase assays. The data is 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 $\pm$ S.D. * $p < 0.05$ 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 h) after which Actinomycin D (0.5 μ g/ml) was added for the indicated times. Total RNA was collected to perform Northern blotting using a *SARI* cDNA.

Fig. 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 h. Total RNA was extracted using Qiagen RNeasy kit, reverse transcription and quantitative PCR was carried out using Applied Biosystems 7500. **B)** DU-145, HO-1 and A549 cells were treated with 5 μM His-MDA-7 for 24 h and lysates were collected and Western blotting was performed as described in (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 non-targeted siRNAs (NT siRNA). These cells were treated with 5 μM His-MDA-7 (24 h) and lysates were collected and Western blotting was performed as described in (3B). **D)** DU-145 and HO-1 cells were treated with 5 μM His-MDA-7 (48 h) and cell proliferation/viability was measured by MTT assays. All experiments were performed at least three times, and data represent mean ± S.D. * $p < 0.05$ cell growth is significantly less than cells treated with non-targeted (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 (3B), with Actin as a control for protein loading. **F)** Genetically modified A549 cells were treated with 5 μM of His-MDA-7 (48 h) and lysates were collected and Western blotting was performed using MDA-7/IL-24, SARI and Actin antibodies as described in (3B) (left panel). In the right panel, 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 (6E). **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 and 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.

Fig. 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 h) and then infected with Ad.vec or Ad.mda-7 (100 pfu/cell) for 48 h 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 h 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 h and then infected with Ad.mda-7 (48 h) 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.