mda-7 (IL-24): Signaling and Functional Roles

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ABSTRACT

*One hallmark of neoplasia is abnormal differentiation. Induction of differentiation, by chemical or biological methods, provides a possible therapeutic intervention. "Differentiation therapy" is well documented in several model systems. These include melanoma, in which treatment with interferon- β and the protein kinase C activator mezerein induces irreversible growth arrest and terminal differentiation culminating in programmed cell death. Subtraction hybridization between terminally differentiated and untreated melanoma cells identified melanoma differentiation-associated gene-7 (*mda-7*), which is selectively induced during the process of melanoma terminal differentiation. Since its identification seven years ago, *mda-7* has been the object of intense focus because of its unique biological properties. Firstly, *mda-7* is a secreted protein having cytokine-like properties and belonging to the IL-10 cytokine family. Based on this consideration, *mda-7* was renamed IL-24. Secondly, if delivered by means of an adenoviral vector, *mda-7* induces selective apoptosis in cancer cells of diverse origin, while sparing their normal cellular counterparts. As such, *mda-7* has become a novel tool for cancer gene therapy and is currently undergoing phase II clinical trials to determine its clinical efficacy in patients. The present review examines the biological properties of *mda-7* and the signaling pathways that contribute to its unique cancer-specific apoptosis-inducing properties.*

INTRODUCTION

Two important hallmarks of neoplastic transformation are aberrant growth and abnormal differentiation (44,65,66). Thus, induction of growth arrest and differentiation is a potential therapeutic intervention for cancer (5,30,64,73,75). Several chemical agents that modulate intracellular signal transduction pathways have differentiation-inducing properties in cancers of diverse tissue types (44). It is hypothesized that cancer cells retain the appropriate genetic information necessary for terminal cell differentiation, but these genes are either not expressed or they are expressed at sub-threshold levels that preclude biological activity (5,30,65,75). When exposed to the appropriate agent(s) or environmental cues, the necessary genes are expressed at threshold levels and cancer cell proliferation is inhibited,

culminating in terminal differentiation (5,13,30,50,73). Unraveling the molecular basis of differentiation and identifying clinically efficacious agents (with minimal or no toxicity) would pave the way for improved cancer therapy.

A well-established model for studying differentiation therapy in cancer is human melanoma (14,30,75). The development of malignant melanoma, with the exception of nodular type melanoma, is a multistep process involving the conversion of a melanocyte into a nevus, a dysplastic nevus, a radial growth phase (RGP) primary melanoma, a vertical growth phase (VGP) primary melanoma and, finally, a metastatic melanoma (23). Several in vitro cultures of malignant human melanoma cells cease proliferating and display a differentiated phenotype, analogous to normal melanocytes, upon treatment with retinoic acid (RA), 12-O-tetradecanoyl-phorbol-13-acetate (TPA), cyclic adenosine monophosphate (cAMP), dimethyl sulfoxide (DMSO), or mycophenolic acid (see references in Reference 44). Combined treatment of metastatic human melanoma cells, such as HO-1, with recombinant human fibroblast interferon (IFN)- β and the protein kinase C activator mezerein (MEZ) results in a rapid irreversible cessation of growth, changes in cell morphology, modifications in gene expression, alterations in surface antigen expression, and terminal cell differentiation (13,14,18,20,21,29). This treatment protocol also extinguishes tumorigenic potential when IFN- β + MEZ treated HO-1 cells are injected into athymic nude mice (unpublished data).

To determine the mechanism of terminal cell differentiation in HO-1 cells, multiple molecular approaches are being used to define the complete spectrum of gene expression changes associated with—and potentially mediating—differentiation. Classical subtraction hybridization procedures and improved modifications of this scheme, such as differentiation induction subtraction hybridization (DISH), reciprocal subtraction differentiation RNA display (RSDD), and rapid subtraction hybridization (RaSH), as well as cDNA microarrays, are being used to identify genes that are selectively up-regulated by IFN- β + MEZ treatment in HO-1 cells as compared to untreated cells (25,26,35,36). These approaches have uncovered a diverse spectrum of melanoma differentiation associated (*mda*) genes, both

known and unknown, which are up-regulated during terminal differentiation. The known *mda* genes can be classified into several categories, including IFN-inducible genes, MEZ-inducible genes, immune system-associated genes, differentiation and growth regulating genes, transcription regulation and signal transduction genes, cytoskeleton and extracellular matrix genes, and mitochondrial genes (25,32,33,35,36). Additionally, several growth regulatory genes are down-regulated during terminal differentiation, such as *c-myc*, cyclin A, cyclin B, *cdc2*, and histone H1 and H4. Unknown up-regulated *mda* genes, i.e., genes not previously represented in current databases, were also identified, including p21 CIP1/WAF-1/*mda-6*, *mda-2*/germ-cell-specific transcription repressor Tctex-1, *mda-5*, *mda-7* and *mda-9*/syntenin. The function of these genes is gradually being elucidated. One originally novel gene, *mda-5*, is a putative RNA helicase with double-stranded RNA-dependent ATPase activity and a caspase-recruitment domain (37). Another is *mda-7*, a cytokine-like molecule (also known as IL-24) that has ubiquitous cancer growth-suppressor properties (31,34).

WHAT IS *mda-7* ?

The *mda-7* gene is selectively up-regulated during terminal differentiation of human melanoma cells (31). The *mda-7* cDNA is 1718-bp in length with an open reading frame encoding a polypeptide of 206 amino acids with a predicted size of 23.8 kDa (31). The predicted reading frame is flanked by 5'- and 3'-untranslated sequences of 274 bp and 823 bp, respectively. The 3'-UTR contains three consensus elements (AUUUA) involved in mRNA stability and three polyadenylation signals (AAUAAA). The *mda-7* gene is composed of seven exons and six introns and is located in chromosome 1q32 within an interleukin (IL)-10 related gene cluster containing four genes (IL-10, IL-19, IL-20, and *mda-7*) in linear order spanning 195 kb of genomic DNA (27). Sequence analysis reveals that *mda-7* has a 49-amino acid N-terminal hydrophobic signal peptide that allows the molecule to be cleaved and secreted. MDA-7 has three putative glycosylation sites at amino acids 95, 109, and 126 resulting in several molecular sizes of the secreted product. Analysis of the protein structure reveals that MDA-7 is a member of the four-helix bundle family of cytokine molecules with greatest homology to the IL-10 subfamily, which now includes IL-19, IL-20, IL-22 (IL-TIF), and AK-155 (4,16,27,41,93,96). IL-10 and *mda-7* share <20% sequence identity, however the presence of an IL-10 signature sequence in *mda-7* indicates that it belongs to the IL-10 subfamily. Analysis of the expression profile of *mda-7* in different human tissues shows that *mda-7* expression is restricted to those tissues associated with the immune system, such as spleen, thymus, and peripheral blood leukocytes, suggesting cytokine-like properties of the *mda-7* molecule (27). Based on these observations, *mda-7* has been renamed IL-24 by the Human Gene Organisation (HUGO) nomenclature system.

mda-7 displays at least two distinct biological functions. At low concentration, *mda-7* functions predominantly as a cytokine (3). However, when overexpressed at supra-physiological levels using a replication-incompetent adenoviral vector, *mda-7* shows cancer-cell specific growth inhibitory properties without negatively affecting normal cells (34,54,56,77). These two important func-

tional attributes of *mda-7* have stimulated growing interest in understanding the mechanism of action of this interesting molecule.

mda-7 LIKE MOLECULES IN OTHER SPECIES

Southern blot analysis of DNAs from different species using a *mda-7* cDNA probe identified homologous sequences in the genomic DNAs of yeast, monkey, cow, dog, and cat, suggesting that *mda-7* is an evolutionarily conserved gene (31). Three subsequent studies have identified *mda-7*-like molecules in rat and mouse (72,76,96). *C49a* was identified by differential display PCR performed between wound-edge RNA isolated from rat excisional skin wounds and non-wounded controls (76). The protein product, consisting of 183 amino acid residues, has 58.7% identity to the complete MDA-7 protein and 69% identity (82% positives) in a conserved 492-bp coding segment. Wounded rat dermal cells expressed markedly elevated levels of *c49a*/C49A before and during the proliferative phase of repair, suggesting a role in proliferation rather than growth inhibition. An additional *mda-7*-like molecule named *mob-5* was also isolated by differential display PCR between rat embryo fibroblast cells Rat1 and Rat1:iRas cells containing an inducible oncogenic Ha-*ras* (96). In Rat1 cells, *mob-5* expression was induced by oncogenic Ha-*ras* and Ki-*ras*. MOB-5 protein is essentially identical to the C49A protein except for two amino acid mismatches. MOB-5 is a secreted protein and since its expression is induced as a function of induction of the oncogenic *ras* pathway it was proposed that *mob-5* is involved in proliferation. From these observations, it is clear that although C49A and MOB-5 proteins have significant similarity to the MDA-7 protein the biological function of these molecules are different in rat and human.

The mouse *mda-7* like molecule, termed FISP (IL-4-induced secreted protein), was identified in type 2 T helper lymphocytes (Th2) by representational difference analysis between Th2 cells and Th1 cells (72). It encodes a secreted protein of 220 amino acids, which displays 93% identity with MOB-5/C49A and 69% identity with MDA-7. FISP expression is induced during Th2 differentiation and its expression is induced by TCR signaling involving protein kinase C activation and STAT-6-dependent IL-4R signaling. There are several common properties to *mda-7* and FISP, including expression of both molecules predominantly in the immune system. FISP mRNA expression could not be detected in any other tissues apart from Th2 lymphocytes. Both *mda-7* and FISP are induced during the process of differentiation and their expression is induced by a combinatorial treatment with a cytokine and a protein kinase C activator. Although the function of FISP is not known it appears as if this gene is more closely related to *mda-7* than *c49a*/*mob-5*.

REGULATION OF *mda-7* EXPRESSION

To understand the regulatory mechanism of *mda-7* gene expression during melanoma differentiation, the promoter region of the *mda-7* gene was cloned (52,53). Interestingly, it was observed that *mda-7* gene expression is regulated predominantly at a posttranscriptional level during differentiation of melanoma cells. Transfection of a *mda-7*-promoter-luciferase construct into

HO-1 or MeWo melanoma cells resulted in basal activity that was not induced further by IFN- β + MEZ treatment (53). However, *mda-7* mRNA could not be detected in untreated melanoma cells but it was markedly induced by IFN- β + MEZ treatment. This observation led to the hypothesis that IFN- β + MEZ functions by stabilizing the level of *mda-7* mRNA. The 3'-UTR region of *mda-7* contains AU-rich elements (ARE) that correlate with rapid mRNA turnover and posttranscriptional control (1,61,74). AREs are also detected in the 3'-UTR of other cytokine molecules, such as interferon- α , granulocyte macrophage-colony stimulating factor (GM-CSF), and tumor necrosis factor alpha (TNF- α) (74). The importance of the 3'-UTR in increasing the stability of *mda-7* mRNA was strengthened by the observation that when the SV40 polyadenylation signal sequence in the luciferase reporter plasmid was substituted with the 3'-UTR of *mda-7* the luciferase message became unstable in proliferating and reversibly differentiated cells. In contrast, the luciferase message was stabilized when the *mda-7*-3'-UTR construct was expressed in terminally differentiated HO-1 cells. Treatment with the protein synthesis inhibitor cycloheximide resulted in increased accumulation of *mda-7* mRNA in untreated, IFN- β - or MEZ-treated cells, indicating that specific labile factor(s) may target the ARE sequences in the *mda-7* 3'-UTR for rapid mRNA degradation.

Transcriptional control of *mda-7* expression is regulated predominantly by two transcription factor families, AP-1 and C/EBP (52). The promoter region of the *mda-7* gene contains several recognition sites for these two transcription factors. Overexpression of c-Jun (a member of the AP-1 family), C/EBP- α or C/EBP- β resulted in significant induction of the *mda-7* promoter activity in HO-1 cells. Overexpression of a dominant negative mutant of c-Jun (TAM67) abrogated the basal *mda-7* promoter activity, indicating an involvement of AP-1 in controlling constitutive promoter activity. Electrophoretic mobility shift assays (EMSA) revealed increased binding of AP-1 and C/EBP complexes to the *mda-7* promoter following IFN- β + MEZ treatment. IFN- β + MEZ treatment also resulted in an increase in the level of c-JUN and C/EBP- β proteins underscoring their importance not only in the control of *mda-7* promoter activity, but also in the process of terminal differentiation.

mda-7 AND MELANOMA

Since *mda-7* is induced during terminal differentiation of melanoma cells its expression pattern and biological behavior have been extensively examined in this model (9,11,27,31,43). Expression of *mda-7* mRNA is undetectable in untreated HO-1 melanoma cells (31). However, *mda-7* mRNA expression is markedly induced by IFN- β + MEZ treatment in these cells as early as 24 hours, and the expression continues to increase during terminal differentiation. Treatment of HO-1 cells with IFN- β + MEZ for 24 hours followed by growth for 72 hours in the absence of inducer resulted in a similar expression level of *mda-7* as with continuous treatment, indicating that once the cells were committed to terminal differentiation *mda-7* expression continued. Treatment with MEZ alone resulted in a modest but detectable increase in *mda-7* mRNA expression. Interestingly, although *mda-7* mRNA expression could not be detected in

melanoma cells, *mda-7* was constitutively expressed in SV40-transformed human melanocyte cells (FM516-SV). Immunohistochemical studies using anti-*mda-7* antibody detected cytoplasmic *mda-7* protein in normal human epidermal melanocytes, as well as in melanocytes in paraffin-embedded sections of normal skin and nevi (9). However, MDA-7 expression could not be detected in RGP, VGP, or metastatic melanomas. These results suggest that elevated *mda-7* expression in the context of a melanocyte is still compatible with growth, whereas induction of *mda-7* expression in human melanoma cells correlates with irreversible growth arrest and terminal cell differentiation. These observations also suggest that *mda-7* might have a selective, yet-to-be-identified physiological function in melanocytes, which may not be compatible with melanoma cell growth.

To evaluate the relationship between *mda-7* expression and melanoma progression, *mda-7* and GAPDH levels were determined by comparative reverse transcription polymerase chain reaction (RT-PCR) in actively growing melanocytes, primary melanomas (RGP and early VGP), and metastatic melanomas (31). Normal melanocytes express on average 3.7-fold more *mda-7* than actively growing metastatic melanomas ($P < 0.001$) and 2.2-fold more *mda-7* than actively growing non-metastatic primary melanomas ($P < 0.02$). The expression of *mda-7* during melanoma progression was analyzed by "MatrigelTM-assisted" (BD Biosciences, San Jose, CA, USA) tumorigenic growth. This approach comprises the co-injection of nontumorigenic or weakly tumorigenic RGP or early VGP primary human melanomas with Matrigel in nude mice (39). Tumors develop rapidly in Matrigel co-injected animals and sublines can be developed that readily grow as solid tumors in secondary nude mouse recipients, without the need for co-injection of Matrigel. Although no significant change in *mda-7* expression was evident in an RGP primary melanoma cell line (WM35) before or after Matrigel selection, two independent early VGP primary human melanoma cell lines (WM793 and WM1341B) expressed less *mda-7* after Matrigel selection. Immunoreactivity for MDA-7 could be detected in primary melanomas (9,11). More intense staining was seen in the thinner tumors, whereas very light or no staining was seen in thick primary tumors (9). In addition, both the percentage of MDA-7-positive cells and the intensity of immunolabeling decreased significantly in the invasive portion of the tumor (11). Immunoreactivity for MDA-7 could be detected in only 1 of 15 cases of lymph node metastasis of melanoma (9). In a separate study, intense MDA-7 immunoreactivity was seen in dendritic cells of germinal centers of four of the 20 lymph node metastasis examined (11). However, the relevance of this finding is not known. The conclusion reached from these studies is that *mda-7* expression is gradually lost during melanoma progression, thereby resulting in a growth advantage in evolving tumor cells. In these contexts, *mda-7* could be regarded as a tumor suppressor gene whose loss of expression is involved in melanoma progression.

What happens when *mda-7* is overexpressed in melanoma cells? Transient transfection of *mda-7* in C8161 human melanoma cells provided the first demonstration of growth inhibitory properties of *mda-7* in human melanoma cells (31). These findings were substantiated by experiments using a replication-incompetent adenovirus expressing *mda-7* (Ad.*mda-7*) (9,43). Infection with Ad.*mda-7* resulted in significant inhibition of growth in melanoma cells but not in FM516-SV or nor-

mal human melanocytes. In a comparative study, Ad.*mda-7* was as potent as Ad.*p53* or Ad.*p21*, a cyclin-dependent kinase inhibitor that is a downstream wild-type p53 target gene, in its growth inhibitory effect on melanoma cells (43). Analysis of programmed cell death (apoptosis) by cell cycle analysis, Annexin V staining and DNA fragmentation analysis revealed an increase in the apoptotic cell population as a function of Ad.*mda-7* infection in melanoma cells, but not in FM516-SV or early passage normal human melanocytes (NHuMEL) (43). In addition, Ad.*mda-7* infection also resulted in an increase in melanoma cells in the G₂/M phase of the cell cycle (9,43). The resistance of FM516-SV or NHuMEL to the killing effect of Ad.*mda-7* is an inherent property of these cells rather than an experimental artifact, since Ad.*mda-7* effectively infected both cell types and resulted in the production of abundant *mda-7* mRNA and protein (43). In addition, infection with Ad.*mda-7* also resulted in the secretion of MDA-7 protein in the media by both normal and tumor cells (43). Studies were performed to ascertain the potential involvement of apoptosis-associated genes in Ad.*mda-7* induction of apoptosis in melanoma cells (43). Decreases were apparent in the ratio of anti-apoptotic (BCL-2 and BCL-XL) proteins to pro-apoptotic (BAX and BAK) proteins, suggesting that this change in equilibrium between the two classes of molecules may be a major determinant of induction of apoptosis by Ad.*mda-7* in human melanoma cells.

One interesting observation is that there is an inverse relationship between the expression levels of *mda-7*, which is down-regulated, and inducible nitric oxide synthase (iNOS), which is up-regulated, in melanoma. Infection of melanoma cells with Ad.*mda-7* results in profound suppression in iNOS expression (10). A recent study has shown that inhibition of iNOS by aminoguanidine (AMG) induces apoptosis in melanoma cells but not in normal melanocytes, underscoring the importance of nitric oxide for melanoma survival (70). Inhibition of iNOS results in down-regulation of *bcl-2* and up-regulation of *bax*, caspases, and GADD45 among other apoptosis-related molecules. Understanding how Ad.*mda-7* down-regulates iNOS will provide additional insights into the apoptosis-inducing function of this molecule.

***mda-7* INDUCES GROWTH SUPPRESSION AND APOPTOSIS IN DIVERSE CANCER CELLS**

Transfection with an *mda-7* expression vector documented the wide-ranging antitumor inhibitory properties of *mda-7* (34,54). *mda-7*-induced growth suppression in a diverse array of human cancer cell lines, including breast, colon, nasopharyngeal, prostate, melanoma, glioblastoma multiforme, and osteosarcoma (34,54). In contrast, when *mda-7* was transfected into normal cells, including human mammary epithelial cells and skin fibroblasts, only a marginal reduction in growth was observed (34). These results firmly established that *mda-7* functions as a growth-suppressor specifically in the context of cancer cells. The activity of *mda-7* was independent of the status of other tumor suppressor genes, such as p53, Rb, or p16^{ink4}, indicating that *mda-7* is capable of inducing growth-suppression in diverse cancer cell lines irrespective of their tumor-suppressor status (34,54).

Experiments with Ad.*mda-7* provided further documentation of the growth-inhibitory properties of *mda-7*. Ad.*mda-7* in-

fection resulted in growth inhibition in several human breast cancer cell lines irrespective of their p53 status (wild type, mutant, or null), while sparing normal human mammary epithelial cells (HMEC), immortalized human breast epithelial cells HBL-100 and human endothelial cells (56,77). Analysis of the mechanism underlying the growth-inhibiting properties of Ad.*mda-7* revealed that it induces apoptosis in breast cancer cell lines, as evidenced by DNA fragmentation analysis, flow cytometry, and terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assays (77). Infection with Ad.*mda-7* resulted in an increase in the pro-apoptotic protein Bax in breast cancer cells, but not in HMEC cells, and the ratio between Bax and anti-apoptotic protein Bcl-2 was significantly higher in breast cancer cells (77). No changes were apparent in the levels of additional apoptosis-modifying proteins, such as Bak, Bad, Bag-1 or Bcl-xL, following Ad.*mda-7* infection of MCF-7 (wild-type p53) or T47D (mutant p53) breast carcinoma cells. Overexpression of Bcl-2 protected breast cancer cells from Ad.*mda-7* mediated apoptosis, indicating that Ad.*mda-7* might exert its apoptotic effect by modulating mitochondrial functions. In addition, the cancer growth suppressive effects of Ad.*mda-7* were demonstrated in vivo (56,77). Infection of MCF-7 cells with Ad.*mda-7* prior to injection into nude mice resulted in a statistically significant suppression of tumor development, as defined by tumor volume and tumor weight (77). Similarly, repeated injection of established human cervical carcinoma (HeLa) xenografts in nude mice with Ad.*mda-7* inhibited tumor growth and disease progression (54). These experimental findings document that Ad.*mda-7* can inhibit human tumor formation and progression directly in vivo in nude mice.

Additional insights into the mechanism underlying the apoptosis-inducing properties of Ad.*mda-7* were obtained by studying non-small cell lung cancer cells (67). Ad.*mda-7* infection induced apoptosis and G₂/M cell cycle arrest in both p53 wild type containing lung cancer cells (A549 and H460) and p53 null cells (H1299) while sparing normal human lung fibroblasts (NHFL). Interestingly, Ad.*mda-7* infection resulted in a significant increase in the levels of p53 (5.4 fold), Bax (2.7 fold) and Bak (2.5 fold) proteins in A549 and H460 cells as compared to infection with a luciferase expressing adenovirus control. However, this induction was not observed in H1299 or NHFL cells and the expression level of Bcl-2 did not change in any of these cells. In cancer cell lines, the induction of apoptosis was associated with activation of the caspase cascade, including cleavage of caspase-9 and caspase-3 and cleavage of PARP, a caspase substrate. This activation of the caspase cascade occurred earlier (48 h post-infection) in p53 wild-type cells than in p53 null cells (72 h post-infection). In NHFL cells, activation of the caspase cascade was not observed. Treatment with a pancaspase inhibitor, Z-VAD.FMK, significantly inhibited apoptosis induced by Ad.*mda-7* in all the cancer cell lines. Since Ad.*mda-7* could kill both p53 wild-type and p53 null cells, it was concluded that *mda-7*-mediated tumor cell killing occurred independently of p53, Bax and Bak expression. However, the p53-dependent pathway seen in p53 wild-type tumor cells might additionally participate in apoptotic mechanisms, thereby amplifying the apoptotic effects of *mda-7*. This hypothesis is supported by the observation that the caspase cascade is activated earlier in p53 wild-type cells than in p53 null cells. Additional

experiments in DU-145 prostate cancer cells, which are Bax deficient and p53 mutated (62), showed that Ad.*mda-7* also induces apoptosis in these cells (54) and activates the caspase cascade (67). Moreover, no induction of p53 or Bak could be detected in these cells. These observations indicate that in specific cases *mda-7* can induce apoptosis in a Bax-independent manner. Cleavage of caspase 3 and caspase 9 following Ad.*mda-7* infection provides further support that apoptosis is mediated by the mitochondrial (intrinsic) pathway rather than the death receptor (extrinsic) pathway.

In the mitochondrial pathway, death signals result in mitochondrial activation, which initiates changes in mitochondrial membrane permeability and subsequent release of pro-apoptotic factors like cytochrome c (cyto c), apoptosis-inducing factor (AIF), second mitochondrial-derived activator of caspase (smac), HtAr2, and endonuclease G (7,19,46,48,79,81,87,89). Cytosolic cyto c forms an essential part of the apoptosis complex "apoptosome," which is composed of cyto c, Apaf-1, and procaspase 9. Formation of the apoptosome leads to activation of caspase 9, which then processes and activates other caspases, including caspase 3, to orchestrate the biochemical execution of cells (47). The key regulatory proteins in the mitochondria-mediated apoptotic events are Bcl-2, Bcl-xL, Bax, and Bak. Bcl-2 and Bcl-xL appear to directly or indirectly preserve the integrity of the outer mitochondrial membrane, thus preventing cyto c release, whereas Bax and Bak promote cyto c release from mitochondria (85,89). Several studies indicate that Ad.*mda-7* can induce apoptosis in specific cancer cells in a Bax-dependent and Bax-independent manner as well as independent of Bak, Bcl-2, and caspase 3 (54,56,67). These observations raise the relevant question as to how the mitochondrial pathway is activated by Ad.*mda-7* in diverse cancer cells.

Infection of melanoma cells with Ad.*mda-7* induced a maximum change in Bcl-xL expression (43). Thus, Ad.*mda-7*-mediated changes in Bcl-xL levels might be important in altering the balance between pro-apoptotic and anti-apoptotic molecules resulting in induction of apoptosis. Ad.*mda-7* infection also leads to cleavage of caspase 8 and Bid in lung cancer cells (58). Activation of caspase 8 occurs through the extrinsic pathway (38). Activated caspase 8 cleaves Bid and the truncated Bid translocates to mitochondria and induces homo-oligomerization of Bax or Bak resulting in the release of cyto c, thus establishing a cross-talk between the intrinsic and extrinsic pathways (45,51,90,91). In these contexts, it is possible that the secreted MDA-7 protein may activate the extrinsic pathway, which in turn activates the intrinsic pathway thereby promoting apoptosis. Further studies need to be performed to answer these questions and shed light on the mechanism of cancer specific induction of apoptosis by Ad.*mda-7*.

SIGNALING PATHWAYS MODIFIED BY Ad.*mda-7*

The signaling pathways responsible for the apoptosis-inducing effect of Ad.*mda-7* are gradually being elucidated (Figure 1). Pretreatment with inhibitors of diverse signaling pathways followed by Ad.*mda-7* infection revealed that inhibition of MAPK pathways, especially the p38 MAPK pathway by its specific inhibitor SB203580, abrogates the apoptosis-inducing effect of Ad.*mda-7* (71). Activation of p38 MAPK is directly related to the induction of apoptosis in several model systems (ref. in 71). In FO-1 melanoma cells, but not in FM516-SV immortalized normal melanocytes, Ad.*mda-7* infection resulted in phosphorylation of p38 MAPK and its downstream target HSP27 (71).

Figure 1. Overview of the signaling pathways associated with Ad.*mda-7* and MDA-7 activity in cancer cells and the immune system. P- indicates phosphorylation. VEGF: vascular endothelial growth factor; TGF- β : transforming growth factor- β ; iNOS: inducible nitric oxide synthase; PKR: double-stranded RNA dependent protein kinase R; MAPK: mitogen activated protein kinase; eIF2 α : eukaryotic translation initiation factor 2 α ; STAT: signal transducer and activator of transcription; GADD: growth arrest and DNA damage inducible; PHA: phytohemagglutinin; LPS: lipopolysaccharide; IL: interleukin; TNF- α : tumor necrosis factor- α ; IFN- γ : interferon- γ ; GM-CSF: granulocyte macrophage-colony stimulating factor.

This observation indicates that the phosphorylation of p38 MAPK is a critical event in the cancer-selective apoptosis-inducing effect of Ad.*mda-7*. Several studies have shown that apoptosis triggered by p38 MAPK activation is mediated by the induction of growth arrest and DNA damage inducible (GADD) family of genes (see references in Reference 71). This gene family consists of GADD34, GADD45 α , GADD45 β , GADD45 γ , and GADD153, which encode highly acidic nuclear proteins with similar and unusual charge characteristics (82,94). GADD34 is a 73-kDa protein that interacts with a diverse array of proteins within the cell (ref. in 71). Some of these interactions facilitate growth suppression/apoptosis while others indicate its involvement in translation initiation, DNA recombination or repair, mRNA transport, and transcriptional regulation. GADD45, a p53-regulated gene, codes for a 21-kDa protein that interacts with the products of two other p53-regulated genes, p21^{WAF1/CIP1}/MDA-6 and PCNA (proliferating cell nuclear antigen), and has been implicated in specific aspects of nucleotide excision repair (refs. in 71). GADD45 has also been shown to interact with MTK1 MAPKKK and thus mediates activation of p38 and JNK MAP kinases in response to environmental stress (82). GADD153, also known as CHOP10 (C/EBP-homologous protein), is a transcription factor containing the basic region-leucine zipper domain that heterodimerizes with members of the C/EBP family of transcription factors and interferes with C/EBP-mediated transcription (63). In addition, GADD153 itself can enhance gene transcription by binding to a DNA element or by interactions with other transcription factors like AP-1 (83,84). Overexpression of each GADD gene causes growth inhibition and/or apoptosis, and combined overexpression of the GADD genes leads to synergistic or cooperative antiproliferative effects (94). Based on the observations that GADD34 is induced during terminal differentiation of melanoma cells (35) and that GADD family genes are modulated by p38 MAPK pathway, the expression pattern of GADD family of genes following Ad.*mda-7* infection was analyzed (71). Ad.*mda-7* infection resulted in marked induction of GADD153, GADD45 α , and GADD34, and a modest induction of GADD45 γ in melanoma cell lines in a time- and dose-dependent manner, but not in normal melanocytes. This induction correlated with the induction of apoptosis in these cell lines. Inhibition of p38 MAPK pathway, both with SB203580 or by an adenovirus expressing a dominant negative p38 MAPK, abrogated the induction of the GADD family of genes by Ad.*mda-7* and also protected melanoma cells from apoptosis. In addition, inhibition of the GADD family of genes by an antisense approach also rescued cells from Ad.*mda-7*-mediated cell death (71). These findings suggest that activation of the p38 MAPK pathway followed by induction of the GADD family of genes plays a crucial role in the cancer-selective apoptosis-inducing effect of Ad.*mda-7* (Figure 1).

A prominent effect of Ad.*mda-7* infection of melanoma cells is down-regulation of the anti-apoptotic protein BCL-2 (43). Previous studies demonstrate that GADD153 functions by down-regulating the *bcl-2* promoter (55). Based on these considerations, it was hypothesized that inhibition of the p38 MAPK pathway, resulting in inhibition of GADD153 induction, should prevent Ad.*mda-7*-mediated Bcl-2 down-regulation. Indeed, treatment with SB203580 prevented the down-regulation of Bcl-

2 by Ad.*mda-7*, strengthening the hypothesis that p38 MAPK and GADD family of genes are essential for the killing effects of Ad.*mda-7* in melanoma cells (71). The induction of the GADD family of genes following Ad.*mda-7* infection is not restricted to melanoma cells, since this induction is also observed in Ad.*mda-7*-infected glioblastoma multiforme, prostate cancer, and breast cancer cells, and in pancreatic cancer cells infected with Ad.*mda-7* in combination with antisense *K-ras* (unpublished data).

Ad.*mda-7* induces apoptosis of lung cancer cells via up-regulation of double-stranded RNA-dependent protein kinase (PKR) (58). PKR is recognized as a mediator of antiviral and antitumor responses in target cells. Two dsRNA-binding domains reside in the NH₂ terminus, and interaction with dsRNA or other activators modifies the conformation of PKR allowing it to undergo autophosphorylation and activation (28,86,95). Once activated, PKR is able to phosphorylate a variety of substrate targets, the most well characterized being eIF-2 α , which can lead to the inhibition of protein synthesis, growth suppression, and induction of apoptosis (6,69,80). Infection with Ad.*mda-7* resulted in phosphorylation of PKR as well as its downstream targets eIF-2 α , Tyk2, Stat1, Stat3, and p38MAPK (58). Ad.*mda-7* infection also produced apoptosis that was associated with activation of caspase 3, 8, and 9 and cleavage of Bid and PARP. The activation of PKR appeared to be upstream of caspase activation because pretreatment with caspase inhibitors failed to block PKR phosphorylation. However, treatment with 2-amino purine (2-AP, a specific serine/threonine kinase inhibitor) blocked Ad.*mda-7*-mediated apoptosis induction as well as activation of PKR and eIF-2 α (58). The involvement of PKR in Ad.*mda-7*-mediated apoptosis was confirmed in PKR null (-/-) mouse embryonic fibroblasts (MEF). Infection with Ad.*mda-7* induced apoptosis in PKR wild-type MEFs but not in PKR null (-/-) MEFs emphasizing the importance of PKR in Ad.*mda-7*-mediated cell death. The common findings between these studies (58,71) is activation of p38 MAPK following Ad.*mda-7* infection. It is possible that in melanoma cells p38 MAPK activation is downstream of PKR activation, although in melanoma cells the post-p38 signal transduction changes appear to be more important in Ad.*mda-7*-induced apoptosis. It has been documented that eIF-2 α phosphorylation activates the transcription factor ATF4, which activates GADD153 (12,22). Thus, there is a significant level of cross talk between the PKR and p38 MAPK signal transduction pathways. Further studies are required to identify the upstream molecules activating PKR and p38 MAPK pathways in order to delineate the relevant signal transduction pathways involved in Ad.*mda-7*-induced apoptosis (Figure 1).

MDA-7 AND PANCREATIC CANCER

An interesting model in which to study the signaling mechanism of Ad.*mda-7*-mediated apoptosis is pancreatic cancer. Pancreatic cancer is a complex disease in which multiple subsets of genes undergo genetic change, either activation or inactivation, during tumor development and progression (15,24,59). A frequent genetic alteration in pancreatic cancer involves activation of the *K-ras* oncogene (85%–95% of all cases). Infection with Ad.*mda-7* alone does not kill pancreatic cancer cells either with *mutK-ras* or *wtK-ras* (78). However, a

combination of Ad.*mda-7* and antisense (AS) K-*ras* phosphorothioate oligodeoxynucleotides (PS ODN) results in significant growth inhibition and apoptosis only in *mutK-ras* pancreatic cancer cells. This novel observation provides a basis for developing rational targeted therapies for selectively killing pancreatic cancer cells and holds promise for increasing the efficacy of therapy for additional cancers (17). Combinatorial treatment of these cells results in a significant reduction in Bcl-2 protein levels and a concomitant increase in Bax protein levels. Of note, MDA-7 protein could not be detected by Western blot analysis in the cell lysates of pancreatic cancer cells 24 hours after infection with Ad.*mda-7* despite detection of *mda-7* mRNA in both *mutK-ras* and *wK-ras* pancreatic cancer cells (78). However, MDA-7 protein could be detected in the cell lysates of *mutK-ras* pancreatic cancer cells following treatment with the combination of Ad.*mda-7* and AS K-*ras* PS ODN. These results suggest that *mutK-ras* may negatively affect MDA-7 protein processing in *mutK-ras* pancreatic cancer cells. It is possible that the downstream signaling pathways of K-*ras*, such as MAPK, p13-kinase and/or c-Jun N-terminal kinase, may interfere with the ability of Ad.*mda-7* to induce apoptosis. If this hypothesis is correct, pharmacological inhibition of any or all of these pathways in concert with Ad.*mda-7* infection might prove to be an effective therapeutic approach for pancreatic cancer. The experiments performed in different cancer cell lines indicate that although Ad.*mda-7* induces apoptosis in diverse cancer cell lines, there may be tissue-specific differences in signaling events following Ad.*mda-7* infection. In these contexts, a complete understanding of the biological behavior of *mda-7* in different cancer and normal cell types will require additional experimentation.

mda-7 AS AN ANTI-ANGIOGENIC FACTOR

In addition to its direct effect on cancer cells resulting in apoptosis, Ad.*mda-7* also inhibits tumor growth by inhibiting angiogenesis. Although Ad.*mda-7* does not have any deleterious effect on the growth of normal human vascular endothelial cells (HUVEC), infection with Ad.*mda-7* inhibits differentiation and tube formation by HUVEC in vitro (68). Ad.*mda-7* infection also down-regulates the expression of vascular endothelial growth factor (VEGF) and transforming growth factor (TGF)- β mRNA, known modulators of angiogenesis, in human lung cancer cells. Subcutaneous tumor xenografts in nude mice injected with Ad.*mda-7* showed low levels of CD31 staining, which is a marker highly expressed by neoangiogenic endothelial cells. These results confirm that Ad.*mda-7* can induce cancer cell growth inhibition by multiple pathways, including induction of apoptosis and suppression of angiogenesis, further documenting the potential of *mda-7* for cancer gene therapy.

BIOLOGICAL FUNCTION OF *mda-7* AS A CYTOKINE

Based on its sequence and structural analysis, *mda-7* is now classified as a cytokine, IL-24 (27,42,60). If this assumption is correct, *mda-7* should have receptors to which it would bind, signal, and exert its diverse biological effects. The IL-10 family

of cytokines signal through receptors that are dimers of an R1 type of receptor (with a long cytoplasmic domain) and an R2 type of receptor (with a short cytoplasmic domain) (40,49). IL-10 signals through IL-10R1 and IL-10R2; IL-20, which is involved in the proliferation of keratinocytes, signals through IL-20R1 and IL-20R2; and IL-22, which mediates acute phase response in hepatocytes, signals through IL-22R1 and IL-10R2 (2,41,93). Two recent studies have identified receptors that can bind to the MDA-7/IL-24 protein (8,88). In one study, stable transfection of 293T cells with an alkaline phosphatase-conjugated *mda-7*/IL-24 (MDA-7/IL-24-AP) expression construct produced conditioned media that contained secreted MDA-7/IL-24-AP (88). When added to Cos-E5 cells transiently transfected with multiple combinations of IL-10/IL-20 and IL-22 receptors, it was possible to determine which receptors bind MDA-7/IL-24-AP (88). Maximum binding activity was induced by MDA-7/IL-24-AP in those cells transfected with IL-20R1/IL-20R2 and IL-22R1/IL-20R2, suggesting that these receptor complexes mediate MDA-7/IL-24 signal transduction. Electrophoretic mobility shift assays (EMSA) in human keratinocyte cell lines revealed that, similar to other IL-10 family members, MDA-7/IL-24 could induce the activation of signal transducers and activators of transcription (STAT), notably STAT-1 and STAT-3 (88).

In a second study, supernatants of HEK293 cells transfected with an *mda-7*-flag construct served as the source of MDA-7 (8). HT-29 intestinal epithelial cells, which endogenously express IL-22R and IL-10R2, were transfected with a luciferase expression construct downstream of five copies of STAT-binding sites. The cells were also transfected with either IL-20R1 or IL-20R2 or both, pretreated or not with anti-IL-22R antiserum for 1 hour and then treated with MDA-7. Luciferase activity was measured 2 hours after MDA-7 treatment. When HT-29 cells were transfected with IL-20R2 cDNA, MDA-7 induced luciferase activity that was completely blocked by an anti-IL-22R antiserum, suggesting that MDA-7 can activate STATs through IL-22R/IL-20R2 receptor complexes. When the cells were transfected with both IL-20R1 and IL-20R2, MDA-7 induced luciferase production that was not blocked by anti-IL-22R antiserum, indicating that this activity was independent from the IL-22R. Transfection with IL-20R1 alone failed to induce any luciferase activity by MDA-7, suggesting that IL-20R1/IL-20R2 and IL-22R1/IL-20R2 receptor complexes are necessary for MDA-7 mediated signal transduction. Phosphorylation of STAT3 was demonstrated by Western blot analysis following MDA-7 treatment.

A critical question is the relevance of STAT activation in mediating *mda-7*'s biological activities. The two studies described above did not evaluate the effect of MDA-7/IL-24 on biological properties of treated cells, i.e., growth and/or survival. Since the concentration of MDA-7 administered in the receptor studies is low compared to that observed following transfection with an *mda-7* expression vector or following infection with Ad.*mda-7*, it is possible that the physiological consequences of MDA-7/IL-24 will be different under these conditions. It is possible that in order to induce its growth-inhibiting and apoptosis-inducing effects a critical threshold concentration of MDA-7/IL24 must be achieved. This argument is strengthened by the observation that the apoptosis inducing effect of *mda-7* is most profound when it is delivered by means of a replication-incompetent adenovirus

vector that produces supra-physiological concentrations of this molecule (27,43). It should also be noted that infection with Ad.*mda-7* results in phosphorylation of STAT1 and STAT3 (58). However, the significance of this activation in Ad.*mda-7*-mediated apoptosis remains to be elucidated.

A potential role for *mda-7*/IL-24 as a cytokine and its involvement in the immune system has been highlighted in two recent studies (3,92). *mda-7*/IL-24 expression could be induced in peripheral blood mononuclear cells (PBMC) upon treatment with PHA or LPS (3). In addition, *mda-7*/IL-24 mRNA could be detected by real-time RT-PCR in monocytes and its expression was upregulated in monocytes by treatment with LPS or in T cells, especially in CD4⁺ naive and memory cells, following activation by anti-CD3 mAb (92). The mouse counterpart of *mda-7*, FISP, was shown to have exclusive expression in Th2 lymphocytes (72). However, in humans the expression pattern in T cells is different from that in mouse. At earlier time points (6 h), *mda-7* expression was down-regulated in cells undergoing T1 differentiation (mediating cellular immunity) and slightly up-regulated in cells undergoing T2 differentiation (regulating humoral immunity). At 66 hours, the expression increased in cells committed to T1 differentiation (92). Treatment of PBMC with MDA-7/IL-24, purified from the conditioned media of a 293 cell line stably expressing *mda-7*, resulted in the induction of IL-6, IFN- γ , TNF- α , IL-1 β , IL-12, and GM-CSF (3). These inductions could be blocked, either completely or partially, by simultaneous administration of IL-10, which might be due to the sharing of receptor subunits by IL-10 family and due to a 10-fold higher affinity of IL-10 for its receptor (92). It is intriguing to note that while IL-10 and *mda-7*/IL-24 belong to the same family, functionally they elicit opposite effects, IL-10 being a major suppressor of the immune response and inflammation (57), while *mda-7*/IL-24 is immunomodulatory. However, treatment with MDA-7/IL-24 does not modulate the proliferative functions of PBMC (3). It was hypothesized that the secondary cytokines induced by MDA-7/IL-24 might activate antigen presenting cells to present tumor antigen, thereby triggering an antitumor immune response (3). MDA-7/IL-24 expression could be detected in melanocytes but not in melanomas, indicating that loss of *mda-7*/IL-24 expression in progressing melanoma might contribute to the ineffective immune response to melanoma.

CONCLUSION

mda-7/IL-24 is an intriguing molecule with diverse biological activities. When expressed at low, presumably physiological levels, this gene product may function as a cytokine, whereas when expressed at supra-physiological levels by adenovirus administration, *mda-7*/IL-24 specifically targets cancer cells for programmed cell death. In contrast, *mda-7*/IL-24 has no discernible pathological effect in normal cells. These unique properties have resulted in Ad.*mda-7* entering the clinical arena as a potential gene therapy for multiple cancer indications. Initial studies look very promising and firmly establish that *mda-7*/IL-24 is safe in patients and very effective in inducing cancer cell apoptosis when administered intratumorally. To more effectively use Ad.*mda-7* in the clinical setting, it is imperative to define the mechanism of action and the molecular basis for the cancer-spe-

cific selectivity of *mda-7*/IL-24. As newer studies are published, different aspects of the regulation and function of *mda-7* are gradually being delineated. However, many questions remain to be answered. Is the growth-suppressing effect of *mda-7* independent of its cytokine function? Is it necessary for *mda-7* to be secreted following Ad.*mda-7* infection to exert its apoptotic activity? Is the threshold level of MDA-7 a major determinant of biological activity? Considering how far we have come from the initial cloning of *mda-7* as a melanoma differentiation-associated gene (30,31) to clinical trials in patients, we are very optimistic that insights into the mechanism of action will be forthcoming in the not too distant future. This knowledge will increase our ability to more effectively use *mda-7*/IL-24 for cancer therapy.

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REFERENCES

- Aharon T. and R. J. Schneider. 1993. Selective destabilization of short-lived mRNAs with the granulocyte-macrophage colony-stimulating factor AU-rich 3' noncoding region is mediated by a cotranslational mechanism. *Mol. Cell Biol.* 13:1971-1980.
- Blumberg, H., D. Conklin, W. F. Xu, A. Grossmann, T. Brender, S. Carollo, M. Eagan, D. Foster, et al. 2001. Interleukin 20: discovery, receptor identification, and role in epidermal function. *Cell* 104:9-19.
- Caudell, E.G., J.B. Mumm, N. Poindexter, S. Ekmekcioglu, A.M. Mhashikar, X.H. Yang, M.W. Retter, P. Hill, et al. 2002. The protein product of the tumor suppressor gene, melanoma differentiation-associated gene 7, exhibits immunostimulatory activity and is designated IL-24. *J. Immunol.* 168:6041-6046.
- Chaiken, I.M. and W.V. Williams. 1996. Identifying structure-function relationships in four-helix bundle cytokines: towards de novo mimetics design. *Trends Biotechnol.* 14:369-375.
- Degos, L. 1997. Differentiation therapy in acute promyelocytic leukemia: European experience. *J. Cell Physiol.* 173:285-287.
- Der, S.D., Y.L. Yang, C. Weissmann, and B.R. Williams. 1997. A double-stranded RNA-activated protein kinase-dependent pathway mediating stress-induced apoptosis. *Proc. Natl. Acad. Sci. USA* 94:3279-3283.
- Du, C., M. Fang, Y. Li, L. Li, and X. Wang. 2000. Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. *Cell* 102:33-42.
- Dumoutier, L., C. Leemans, D. Lejeune, S.V. Kutenko, and J.C. Renauld. 2001. Cutting edge: STAT activation by IL-19, IL-20 and *mda-7* through IL-20 receptor complexes of two types. *J. Immunol.* 167:3545-3549.
- Ekmekcioglu, S., J. Ellerhorst, A.M. Mhashikar, A.A. Sahin, C.M. Read, V.G. Prieto, S. Chada, and E.A. Grimm. 2001. Down-regulated melanoma differentiation associated gene (*mda-7*) expression in human melanomas. *Int. J. Cancer* 94:54-59.
- Ekmekcioglu, S., J.A. Ellerhorst, L. Broemling, A.M. Mhashikar, A.A. Sahin, V.G. Prieto, S. Chada, and E.A. Grimm. 2002. MDA-7 regulates iNOS expression in human melanomas. Proceedings of the 93rd Annual Meeting of the American Association for Cancer Research [Abstract #3347].
- Ellerhorst, J.A., V.G. Prieto, S. Ekmekcioglu, L. Broemeling, S. Yekell, S. Chada, and E.A. Grimm. 2002. Loss of MDA-7 expression with progression of melanoma. *J. Clin. Oncol.* 20:1069-1074.

12. Fawcett, T.W., J.L. Martindale, K.Z. Guyton, T. Hai, and N.J. Holbrook. 1999. Complexes containing activating transcription factor (ATF)/cAMP-responsive-element-binding protein (CREB) interact with the CCAAT/enhancer-binding protein (C/EBP)-ATF composite site to regulate Gadd153 expression during the stress response. *Biochem. J.* 339:135-141.
13. Fisher, P.B. and S. Grant. 1985. Effects of interferon on differentiation of normal and tumor cells. *Pharmacol. Ther.* 27:143-166.
14. Fisher, P.B., D.R. Prignoli, H. Herms, Jr., I.B. Weinstein, and S. Pestka. 1985. Effects of combined treatment with interferon and mezerein on melanogenesis and growth in human melanoma cells. *J. Interferon Res.* 5:11-22.
15. Friess, H., J. Kleeff, M. Korc, and M.W. Buchler. 1999. Molecular aspects of pancreatic cancer and future perspectives. *Dig. Surg.* 16:281-290.
16. Gallagher, G., H. Dickensheets, J. Eskdale, L.S. Izotova, O.V. Mirochnitchenko, J.D. Peat, N. Vazquez, S. Pestka, et al. 2000. Cloning, expression and initial characterization of interleukin-19 (IL-19), a novel homologue of human interleukin-10 (IL-10). *Genes Immun.* 1:442-450.
17. Gazdar, A.F. and J.D. Minna. 2001. Targeted therapies for killing tumor cells. *Proc. Natl. Acad. Sci. USA* 98:10028-10030.
18. Graham, G.M., L. Guarini, T.A. Moulton, S. Datta, S. Ferrone, P. Giacomini, R.S. Kerbel, and P.B. Fisher. 1991. Potentiation of growth suppression and modulation of the antigenic phenotype in human melanoma cells by the combination of recombinant human fibroblast and immune interferons. *Cancer Immunol. Immunother.* 32:382-390.
19. Green, D.R. and J.C. Reed. 1998. Mitochondria and apoptosis. *Science* 281:1309-1312.
20. Guarini, L., M. Temponi, G.M. Edwards, J.R. Vita, P.B. Fisher, and S. Ferrone. 1989. In vitro differentiation and antigenic changes in human melanoma cell lines. *Cancer Immunol. Immunother.* 30:262-268.
21. Guarini, L., G.M. Graham, H. Jiang, S. Ferrone, S. Zucker, and P.B. Fisher. 1992. Modulation of the antigenic phenotype of human melanoma cells by differentiation-inducing and growth-suppressing agents. *Pigment Cell Res. (Suppl.)* 123-131.
22. Harding, H.P., I. Novoa, Y. Zhang, H. Zeng, R. Wek, M. Schapira, and D. Ron. 2000. Regulated translation initiation controls stress-induced gene expression in mammalian cells. *Mol. Cell* 6:1099-1108.
23. Herlyn, M. 1990. Human melanoma: development and progression. *Cancer Metastasis Rev.* 9:101-112.
24. Hilgers, W., and S.E. Kern. 1999. Molecular genetic basis of pancreatic adenocarcinoma. *Genes Chromosomes Cancer* 26:1-12.
25. Huang, F., J. Adelman, H. Jiang, N.I. Goldstein, and P.B. Fisher. 1999. Differentiation induction subtraction hybridization (DISH): a strategy for cloning genes displaying differential expression during growth arrest and terminal differentiation. *Gene* 236:125-131.
26. Huang, F., J. Adelman, H. Jiang, N.I. Goldstein, and P.B. Fisher. 1999. Identification and temporal expression pattern of genes modulated during irreversible growth arrest and terminal differentiation in human melanoma cells. *Oncogene* 18:3546-3552.
27. Huang, E. Y., M. T. Madireddi, R. V. Gopalkrishnan, M. Leszczyniecka, Z. Su, I. V. Lebedeva, D. Kang, H. Jiang, et al. 2001. Genomic structure, chromosomal localization and expression profile of a novel melanoma differentiation associated (*mda-7*) gene with cancer specific growth suppressing and apoptosis inducing properties. *Oncogene* 20:7051-7063.
28. Jagus, R., B. Joshi, and G.N. Barber. 1999. PKR, apoptosis and cancer. *Int. J. Biochem. Cell Biol.* 31:123-138.
29. Jiang, H., Z.-Z. Su, J. Boyd, and P.B. Fisher. 1993. Gene expression changes associated with reversible growth suppression and the induction of terminal differentiation in human melanoma cells. *Mol. Cell Different.* 1:41-66.
30. Jiang, H., J. Lin, and P.B. Fisher. 1994. A molecular definition of terminal cell differentiation in human melanoma cells. *Mol. Cell Different.* 2:221-239.
31. Jiang, H., J.J. Lin, Z.Z. Su, N.I. Goldstein, and P.B. Fisher. 1995. Subtraction hybridization identifies a novel melanoma differentiation associated gene, *mda-7*, modulated during human melanoma differentiation, growth and progression. *Oncogene* 11:2477-2486.
32. Jiang, H., J. Lin, Z.Z. Su, M. Herlyn, R.S. Kerbel, B.E. Weissman, D.R. Welch, and P.B. Fisher. 1995. The melanoma differentiation-associated gene *mda-6*, which encodes the cyclin-dependent kinase inhibitor p21, is differentially expressed during growth, differentiation and progression in human melanoma cells. *Oncogene* 10:1855-1864.
33. Jiang, H., J. Lin, S.M. Young, N.I. Goldstein, S. Waxman, V. Davila, S.P. Chellappan, and P.B. Fisher. 1995. Cell cycle gene expression and E2F transcription factor complexes in human melanoma cells induced to terminally differentiate. *Oncogene* 11:1179-1189.
34. Jiang, H., Z.Z. Su, J.J. Lin, N.I. Goldstein, C.S. Young, and P.B. Fisher. 1996. The melanoma differentiation associated gene *mda-7* suppresses cancer cell growth. *Proc. Natl. Acad. Sci. USA* 93:9160-9165.
35. Jiang, H., D.C. Kang, D. Alexandre, and P.B. Fisher. 2000. RaSH, a rapid subtraction hybridization approach for identifying and cloning differentially expressed genes. *Proc. Natl. Acad. Sci. USA* 97:12684-12689.
36. Kang, D.C., R. LaFrance, Z.Z. Su, and P.B. Fisher. 1998. Reciprocal subtraction differential RNA display: an efficient and rapid procedure for isolating differentially expressed gene sequences. *Proc. Natl. Acad. Sci. USA* 95:13788-13793.
37. Kang, D.C., R.V. Gopalkrishnan, Q. Wu, E. Jankowsky, A.M. Pyle, and P.B. Fisher. 2002. *mda-5*: An interferon-inducible putative RNA helicase with double-stranded RNA-dependent ATPase activity and melanoma growth-suppressive properties. *Proc. Natl. Acad. Sci. USA* 99:637-642.
38. Kischkel, F.C., D.A. Lawrence, A. Chuntharapai, P. Schow, K.J. Kim, and A. Ashkenazi. 2000. Apo2L/TRAIL-dependent recruitment of endogenous FADD and caspase-8 to death receptors 4 and 5. *Immunity* 12:611-620.
39. Kobayashi, H., S. Man, J.R. MacDougall, C.H. Graham, C. Lu, and R.S. Kerbel. 1994. Variant sublines of early-stage human melanomas selected for tumorigenicity in nude mice express a multicytokine-resistant phenotype. *Am. J. Pathol.* 144:776-786.
40. Kotenko, S.V., C.D. Krause, L.S. Izotova, B.P. Pollack, W. Wu, and S. Pestka. 1997. Identification and functional characterization of a second chain of the interleukin-10 receptor complex. *EMBO J.* 16:5894-5903.
41. Kotenko, S.V., L.S. Izotova, O.V. Mirochnitchenko, E. Esterova, H. Dickensheets, R.P. Donnelly, and S. Pestka. 2001. Identification of the functional interleukin-22 (IL-22) receptor complex: the IL-10R2 chain (IL-10Rbeta) is a common chain of both the IL-10 and IL-22 (IL-10-related T cell-derived inducible factor, IL-TIF) receptor complexes. *J. Biol. Chem.* 276:2725-2732.
42. Kotenko, S.V. 2002. The family of IL-10-related cytokines and their receptors: related, but to what extent? *Cytokine Growth Factor Rev.* 21:71-18.
43. Lebedeva, I.V., Z.Z. Su, Y. Chang, S. Kitada, J.C. Reed, and P.B. Fisher. 2002. The cancer growth suppressing gene *mda-7* induces apoptosis selectively in human melanoma cells. *Oncogene* 21:708-718.
44. Leszczyniecka, M., T. Roberts, P. Dent, S. Grant, and P.B. Fisher. 2001. Differentiation therapy of human cancer: basic science and clinical applications. *Pharmacol. Ther.* 90:105-156.
45. Li, H., H. Zhu, C.J. Xu, and J. Yuan. 1998. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* 94:491-501.
46. Li, L.Y., X. Luo, and X. Wang. 2001. Endonuclease G is an apoptotic DNase when released from mitochondria. *Nature* 412:95-99.
47. Li, P., D. Nijhawan, I. Budihardjo, S. M. Srinivasula, M. Ahmad, E.S. Alnemri, and X. Wang. 1997. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* 91:479-489.
48. Liu, X., C.N. Kim, J. Yang, R. Jemmerson, and X. Wang. 1996. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell* 86:147-157.
49. Liu, Y., S.H. Wei, A.S. Ho, R. de Waal Malefyt, and K.W. Moore. 1994. Expression cloning and characterization of a human IL-10 receptor. *J. Immunol.* 152:1821-1829.
50. Lovat, P.E., H. Irving, M. Annicchiarico-Petruzzelli, F. Bernassola, A.J. Malcolm, A.D. Pearson, G. Melino, and C.P. Redfern. 1997. Retinoids in neuroblastoma therapy: distinct biological properties of 9-cis- and all-trans-retinoic acid. *Eur. J. Cancer* 33:2075-2080.
51. Luo, X., I. Budihardjo, H. Zou, C. Slaughter, and X. Wang. 1998. Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. *Cell* 94:481-490.
52. Madireddi, M.T., P. Dent, and P.B. Fisher. 2000. AP-1 and C/EBP transcription factors contribute to *mda-7* gene promoter activity during human melanoma differentiation. *J. Cell Physiol.* 185: 36-46.
53. Madireddi, M.T., P. Dent, and P.B. Fisher. 2000. Regulation of *mda-7* gene expression during human melanoma differentiation. *Oncogene* 19:1362-1368.
54. Madireddi, M.T., Z.Z. Su, C.S. Young, N.I. Goldstein, and P.B. Fisher. 2000. *Mda-7*, a novel melanoma differentiation associated gene with promise for cancer gene therapy. *Adv. Exp. Med. Biol.* 465:239-261.
55. McCullough, K.D., J.L. Martindale, L.O. Klotz, T.Y. Aw, and N.J. Holbrook. 2001. Gadd153 sensitizes cells to endoplasmic reticulum stress by down-regulating Bcl2 and perturbing the cellular redox state. *Mol. Cell Biol.* 21:1249-1259.
56. Mhashikar, A.M., R.D. Schrock, M. Hindi, J. Liao, K. Sieger, F. Kourouma,

- X.H. Zou-Yang, E. Onishi, et al. 2001. Melanoma differentiation associated gene-7 (*mda-7*): a novel anti-tumor gene for cancer gene therapy. *Mol. Med.* 7:271-282.
57. Moore, K.W., R. de Waal Malefyt, R.L. Coffman, and A. O'Garra. 2001. Interleukin-10 and the interleukin-10 receptor. *Annu. Rev. Immunol.* 19:683-765.
58. Pataer, A., S.A. Vorburger, G.N. Barber, S. Chada, A.M. Mhashilkar, H. Zou-Yang, A.L. Stewart, S. Balachandran, et al. 2002. Adenoviral transfer of the melanoma differentiation-associated gene 7 (*mda-7*) induces apoptosis of lung cancer cells via up-regulation of the double-stranded RNA-dependent protein kinase (PKR). *Cancer Res.* 62:2239-2243.
59. Perugini, R.A., T.P. McDade, F.J. Vitimberga, Jr., and M.P. Callery. 1998. The molecular and cellular biology of pancreatic cancer. *Crit. Rev. Eukaryot. Gene Expr.* 8:377-393.
60. Pestka, S., S.V. Kutenko, and P.B. Fisher. 2002. IL-22 and *mda-7* (IL-24), members of the family of IL-10-related cytokines. In H.L. Henry and A.W. Norman (Eds.), *Encyclopedia of Hormones*. Academic Press, San Diego. (In press.)
61. Rajagopalan, L.E. and J.S. Malter. 1997. Regulation of eukaryotic messenger RNA turnover. *Prog. Nucleic Acid Res. Mol. Biol.* 56:257-286.
62. Rampino, N., H. Yamamoto, Y. Ionov, Y. Li, H. Sawai, J.C. Reed, and M. Perucho. 1997. Somatic frameshift mutations in the BAX gene in colon cancers of the microsatellite mutator phenotype. *Science* 275:967-969.
63. Ron, D. and J.F. Habener. 1992. CHOP, a novel developmentally regulated nuclear protein that dimerizes with transcription factors C/EBP and LAP and functions as a dominant-negative inhibitor of gene transcription. *Genes Dev.* 6:439-453.
64. Sachs, L. 1987. Cell differentiation and bypassing of genetic defects in the suppression of malignancy. *Cancer Res.* 47:1981-1986.
65. Sachs, L. 1989. Cell differentiation and tumour suppression. *Ciba Found. Symp.* 142:217-231.
66. Sachs, L. 1990. The control of growth and differentiation in normal and leukemic blood cells. *Cancer* 65:2196-2206.
67. Saeki, T., A. Mhashilkar, S. Chada, C. Branch, J.A. Roth, and R. Ramesh. 2000. Tumor-suppressive effects by adenovirus-mediated *mda-7* gene transfer in non-small cell lung cancer cell in vitro. *Gene Ther.* 7:2051-2057.
68. Saeki, T., A. Mhashilkar, S. Swanson, X.H. Zou-Yang, K. Sieger, S. Kawabe, C.D. Branch, L. Zumstein, et al. 2002. Inhibition of human lung cancer growth following adenovirus-mediated *mda-7* gene expression in vivo. *Oncogene* 21:4558-4566.
69. Saelens, X., M. Kalai, and P. Vandenabeele. 2001. Translation inhibition in apoptosis: caspase-dependent PKR activation and eIF2- α phosphorylation. *J. Biol. Chem.* 276:41620-41628.
70. Salvucci, O., M. Carsana, I. Bersani, G. Tragni, and A. Anichini. 2001. Anti-apoptotic role of endogenous nitric oxide in human melanoma cells. *Cancer Res.* 61:318-326.
71. Sarkar, D., Z.-Z. Su, I.V. Lebedeva, M. Sauane, R.V. Gopalkrishnan, K. Valerie, P. Dent, and P.B. Fisher. 2002. *mda-7* (IL-24) mediates selective apoptosis in human melanoma cells by inducing the coordinated overexpression of the GADD family of genes by means of p38 MAPK. *Proc. Natl. Acad. Sci. USA* 99:10054-10059.
72. Schaefer, G., C. Venkataraman, and U. Schindler. 2001. Cutting edge: FISP (IL-4-induced secreted protein), a novel cytokine-like molecule secreted by Th2 cells. *J. Immunol.* 166:5859-5863.
73. Scott, R.E. 1997. Differentiation, differentiation/gene therapy and cancer. *Pharmacol. Ther.* 73:51-65.
74. Shaw, G. and R. Kamen. 1986. A conserved AU sequence from the 3' untranslated region of GM-CSF mRNA mediates selective mRNA degradation. *Cell* 46:659-667.
75. Soballe, P.W. and M. Herlyn. 1994. Cellular pathways leading to melanoma differentiation: therapeutic implications. *Melanoma Res.* 4:213-223.
76. Soo, C., W.W. Shaw, E. Freymiller, M.T. Longaker, C.N. Bertolami, R. Chiu, A. Tieu, and K. Ting. 1999. Cutaneous rat wounds express c49a, a novel gene with homology to the human melanoma differentiation associated gene, *mda-7*. *J. Cell Biochem.* 74:1-10.
77. Su, Z.Z., M.T. Madireddi, J.J. Lin, C.S. Young, S. Kitada, J.C. Reed, N.I. Goldstein, and P.B. Fisher. 1998. The cancer growth suppressor gene *mda-7* selectively induces apoptosis in human breast cancer cells and inhibits tumor growth in nude mice. *Proc. Natl. Acad. Sci. USA* 95:14400-14405.
78. Su, Z., I.V. Lebedeva, R.V. Gopalkrishnan, N.I. Goldstein, C.A. Stein, J.C. Reed, P. Dent, and P.B. Fisher. 2001. A combinatorial approach for selectively inducing programmed cell death in human pancreatic cancer cells. *Proc. Natl. Acad. Sci. USA* 98:10332-10337.
79. Susin, S.A., H.K. Lorenzo, N. Zamzami, I. Marzo, B.E. Snow, G.M. Brothers, J. Mangion, E. Jacotot, et al. 1999. Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature* 397:441-446.
80. Sudhakar, A., A. Ramachandran, S. Ghosh, S.E. Hasnain, R.J. Kaufman, and K.V. Ramaiah. 2000. Phosphorylation of serine 51 in initiation factor 2 α (eIF2 α) promotes complex formation between eIF2 α (P) and eIF2B and causes inhibition in the guanine nucleotide exchange activity of eIF2B. *Biochemistry* 39:12929-12938.
81. Suzuki, Y., Y. Imai, H. Nakayama, K. Takahashi, K. Takio, and R. Takahashi. 2001. A serine protease, HtrA2, is released from the mitochondria and interacts with XIAP, inducing cell death. *Mol. Cell* 8:613-21.
82. Takekawa, M. and H. Saito. 1998. A family of stress-inducible GADD45-like proteins mediate activation of the stress-responsive MTK1/MEKK4 MAP-KKK. *Cell* 95:521-530.
83. Ubeda, M., X.Z. Wang, H. Zinsner, I. Wu, J.F. Habener, and D. Ron. 1996. Stress-induced binding of the transcriptional factor CHOP to a novel DNA control element. *Mol. Cell Biol.* 16:1479-1489.
84. Ubeda, M., M. Vallejo, and J.F. Habener. 1999. CHOP enhancement of gene transcription by interactions with Jun/Fos AP-1 complex proteins. *Mol. Cell Biol.* 19:7589-7599.
85. Vander Heiden, M.G. and C.B. Thompson. 1999. Bcl-2 proteins: regulators of apoptosis or of mitochondrial homeostasis? *Nat. Cell Biol.* 1:E209-216.
86. Vattam, K.M., K.A. Staschke, and R.C. Wek. 2001. Mechanism of activation of the double-stranded-RNA-dependent protein kinase, PKR: role of dimerization and cellular localization in the stimulation of PKR phosphorylation of eukaryotic initiation factor-2 (eIF2). *Eur. J. Biochem.* 268:3674-3684.
87. Verhagen, A.M., P.G. Ekert, M. Pakusch, J. Silke, L.M. Connolly, G.E. Reid, R.L. Moritz, R.J. Simpson, and D.L. Vaux. 2000. Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. *Cell* 102:43-53.
88. Wang, M., Z. Tan, R. Zhang, S.V. Kutenko, and P. Liang. 2002. Interleukin 24 (MDA-7/MOB-5) signals through two heterodimeric receptors, IL-22R1/IL-20R2 and IL-20R1/IL-20R2. *J. Biol. Chem.* 277:7341-7347.
89. Wang, X. 2001. The expanding role of mitochondria in apoptosis. *Genes Dev.* 15:2922-2933.
90. Wei, M.C., T. Lindsten, V.K. Mootha, S. Weiler, A. Gross, M. Ashiya, C.B. Thompson, and S.J. Korsmeyer. 2000. tBID, a membrane-targeted death ligand, oligomerizes BAK to release cytochrome c. *Genes Dev.* 14:2060-2071.
91. Wei, M.C., W.X. Zong, E.H. Cheng, T. Lindsten, V. Panoutsakopoulou, A.J. Ross, K.A. Roth, G.R. MacGregor, et al. 2001. Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. *Science* 292:727-730.
92. Wolk, K., S. Kunz, K. Asadullah, and R. Sabat. 2002. Cutting edge: immune cells as sources and targets of the IL-10 family members? *J. Immunol.* 168:5397-5402.
93. Xie, M.H., S. Aggarwal, W.H. Ho, J. Foster, Z. Zhang, J. Stinson, W.I. Wood, A.D. Goddard, and A.L. Gurney. 2000. Interleukin (IL)-22, a novel human cytokine that signals through the interferon receptor-related proteins CRF2-4 and IL-22R. *J. Biol. Chem.* 275:31335-31339.
94. Zhan, Q., K.A. Lord, I. Alamo, Jr., M.C. Hollander, F. Carrier, D. Ron, K.W. Kohn, B. Hoffman, et al. 1994. The gadd and MyD genes define a novel set of mammalian genes encoding acidic proteins that synergistically suppress cell growth. *Mol. Cell Biol.* 14:2361-2371.
95. Zhang, F., P.R. Romano, T. Nagamura-Inoue, B. Tian, T.E. Dever, M.B. Mathews, K. Ozato, and A.G. Hinnebusch. 2001. Binding of double-stranded RNA to protein kinase PKR is required for dimerization and promotes critical autophosphorylation events in the activation loop. *J. Biol. Chem.* 276:24946-24958.
96. Zhang, R., Z. Tan, and P. Liang. 2000. Identification of a novel ligand-receptor pair constitutively activated by ras oncogenes. *J. Biol. Chem.* 275:24436-24443.

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