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***mda-7/IL-24*: Multifunctional cancer-specific apoptosis-inducing cytokine**

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Abstract

“Differentiation therapy” provides a unique and potentially effective, less toxic treatment paradigm for cancer. Moreover, combining “differentiation therapy” with molecular approaches presents an unparalleled opportunity to identify and clone genes mediating cancer growth control, differentiation, senescence, and programmed cell death (apoptosis). Subtraction hybridization applied to human melanoma cells induced to terminally differentiate by treatment with fibroblast interferon (IFN- β) plus mezerein (MEZ) permitted cloning of melanoma differentiation associated (*mda*) genes. Founded on its novel properties, one particular *mda* gene, *mda-7*, now classified as a member of the interleukin (IL)-10 gene family (IL-24) because of conserved structure, chromosomal location, and cytokine-like properties has become the focus of attention of multiple laboratories. When administered by transfection or adenovirus-transduction into a spectrum of tumor cell types, melanoma differentiation associated gene-7/interleukin-24 (*mda-7/IL-24*) induces apoptosis, whereas no toxicity is apparent in normal cells. *mda-7/IL-24* displays potent “bystander antitumor” activity and also has the capacity to enhance radiation lethality, to induce immune-regulatory activities, and to inhibit tumor angiogenesis. Based on these remarkable attributes and effective antitumor therapy in animal models, this cytokine has taken the important step of entering the clinic. In a Phase I clinical trial, intratumoral injections of adenovirus-administered *mda-7/IL-24* (Ad.*mda-7*) was safe, elicited tumor-regulatory and immune-activating processes, and provided clinically significant activity. This review highlights our current understanding of the diverse

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activities and properties of this novel cytokine, with potential to become a prominent gene therapy for cancer.

Keywords

mda-7/IL-24; Differentiation therapy of cancer; Programmed cell death; Antitumor bystander activity; Radiosensitization; Angiogenesis; Cell signaling; Phase I clinical trial

1. Introduction

Of the multitude of diseases afflicting mankind, cancer poses a major threat delimiting longevity and the quality of human life. Despite significant improvements in diagnosis and innovations in the therapy of specific cancers, effectively treating neoplastic diseases still present major challenges. The etiological factors mediating cancer development and progression are complex, involving genetic and epigenetic changes, and these processes are intimately associated with environmental factors, including diet, exposure to toxic, and carcinogenic chemicals and radiation (Fisher, 1984; Bishop, 1991; Knudson, 1993; Hartwell & Kastan, 1994; Leszczyniecka et al., 2001; Vogelstein & Kinzler, 2004). It is now accepted as axiomatic that the vast majority of cancers do not result from a single genetic change but rather reflect the compilation of multiple genomic modifications, such as alterations in the expression of dominantly acting oncogenes, recessive tumor suppressor genes, and unique genetic elements directly affecting progression of the cancer phenotype (Fisher, 1984; Bishop, 1991; Knudson, 1993; Kang et al., 1998; Vogelstein & Kinzler, 2004; Emdad et al., 2005; Michor et al., 2005; Su et al., 2005a). In these contexts, it is the totality of the multitude of changes that ultimately result in a loss of proliferative control and changes in properties of the evolving tumor cells (Fisher, 1984; Bishop, 1991; Hartwell & Kastan, 1994; Jiang et al., 1994a, 1994b; Vogelstein & Kinzler, 2004; Emdad et al., 2005; Michor et al., 2005; Su et al., 2005a).

The most common modalities used to treat cancer are surgery, chemotherapy, and radiotherapy (Gregoire et al., 1999; Raman & Small, 1999; Akduman et al., 2005; Bucci et al., 2005; DeAngelis, 2005). Limitations of chemotherapy include development of drug resistance, non-specific toxicity, and additional side effects preventing optimization of this approach (Harris, 1985a, 1985b; Harris & Hochhauser, 1992; Sawicka et al., 2004; Liscovitch & Lavie, 2005). Radiotherapy is also associated with negative side effects, and when used at doses frequently necessary to achieve a clinically beneficial effect, it may itself promote cancer development (Gregoire et al., 1999; Ross, 1999; Gregoire et al., 2002). The limitations of current cancer therapies underscore the need to develop less toxic and potentially more specific and effective forms of treatment (Kobayashi et al., 2005; Lin et al., 2005; Tripathy, 2005; Vassal, 2005).

Hallmarks of the cancer cell include aberrant growth and abnormal differentiation (Sachs, 1987, 1989; Borden et al., 1993; Jiang et al., 1994a; Leszczyniecka et al., 2001; Zelent et al., 2005). In many contexts, these defects are reversible and tumor cells actually contain the appropriate genetic information for inducing restrained growth and terminal differentiation. However, appropriate genes are either not expressed or subthreshold levels of proteins are present that are necessary to maintain normal growth and differentiation. A potentially less toxic approach for treating cancer involves reprogramming tumor cells to undergo irreversible growth arrest and terminal differentiation, referred to as “differentiation therapy” (Sachs, 1978, 1987, 1989, 1990; Fisher et al., 1985; Borden et al., 1993; Jiang et al., 1993, 1994a; Leszczyniecka et al., 2001; Miller & Waxman, 2002; Zelent et al., 2005). In this scheme, neoplastic cells exhibiting aberrant patterns of differentiation upon treatment with an appropriate agent(s) lose proliferative capacity and terminally differentiate. The

“differentiation therapy” stratagem was evaluated in HO-1 human melanoma cells, where treatment with recombinant human fibroblast interferon (IFN- β) and the protein kinase C activator mezerein (MEZ) resulted in irreversible cessation of growth, changes in cell morphology, modifications in gene expression, alterations in surface antigen expression, and terminal cell differentiation (Fisher et al., 1985; Guarini et al., 1989, 1992; Jiang & Fisher, 1993; Jiang et al., 1993, 1994a).

To characterize genes involved in terminal differentiation of HO-1 human melanoma cells, temporally spaced poly(A) RNAs from untreated and IFN- β + MEZ-treated HO-1 cells (Jiang & Fisher, 1993; Jiang et al., 1993) were collected and cDNA libraries were created (Fig. 1). These 2 cDNA libraries were subtracted resulting in construction of a temporally spaced subtracted cDNA library, enriched for genes modified during HO-1 cell terminal differentiation (Jiang & Fisher, 1993). Improved versions of this scheme, such as reciprocal subtraction differential RNA display (RSDD) (Kang et al., 1998; Sarkar et al., in press) and rapid subtraction hybridization (RaSH) (Jiang et al., 2000; Kang et al., 2001, Boukerche et al., 2004, in press; Kang et al., in press), as well as cDNA microarrays (Huang et al., 1999a, 1999b), have revealed a broad spectrum of melanoma differentiation associated (*mda*) genes and differentiation induction subtraction hybridization (*DISH*) genes, which are either up-regulated or temporally down-regulated upon treatment of HO-1 cells with either IFN- β , MEZ, or IFN- β + MEZ. Originally, novel up-regulated genes identified by these techniques included p21^{CIP1/WAF-1}/*mda-6* (Jiang & Fisher, 1993; Jiang et al., 1994b, 1995a), *mda-2*/the male germ cell-specific transcription repressor Tctex-1 (Jiang & Fisher, 1993), *mda-5* (Jiang et al., 1994a; Kang et al., 2002, 2004), melanoma differentiation associated gene-7/interleukin-24 (*mda-7/IL-24*; Jiang & Fisher, 1993; Jiang et al., 1995c; Fisher et al., 2003; Sauane et al., 2003a, 2003b; Fisher, 2005; Lebedeva et al., 2005a), and *mda-9*/syntenin (Lin et al., 1996, 1998; Fernandez-Larrea et al., 1999; Koroll et al., 2001; Sarkar et al., 2004; Boukerche et al., 2005). Continuing studies indicate the functional importance and relevance of these genes to many significant physiological processes. *mda-6*, which is p21, is a universal cyclin-dependent kinase inhibitor that is intimately associated with cell cycle regulation and growth control (Jiang et al., 1994b, 1995a, 1995b, 1995c). *mda-5* is a putative RNA-helicase with double-stranded RNA-dependent ATPase activity and a caspase recruiting domain involved in interferon response and viral infection (Kang et al., 2002; Andrejeva et al., 2004; Kang et al., 2004). *mda-7/IL-24* is a novel cytokine that has a broad range of antitumor properties (Sarkar et al., 2002a; Fisher et al., 2003; Sauane et al., 2003b; Chada et al., 2004b; Gopalkrishnan et al., 2004; Cunningham et al., 2005; Fisher, 2005; Lebedeva et al., 2005a; Tong et al., 2005). *mda-9*/syntenin associates with syndecans involved in cell adhesion and early endosome formation and has recently been found to contribute to metastasis (Lin et al., 1996, 1998; Fernandez-Larrea et al., 1999; Koroll et al., 2001, 2002; Helmke et al., 2004; Sarkar et al., 2004; Boukerche et al., in press). Apart from these genes, several other growth-regulatory genes that are down-regulated during differentiation, such as c-myc, cyclin A, cyclin B, human ribosomal protein L23a, cdc2, and histone H1 and H4, have been identified that also contribute to the spectrum of molecular changes occurring during terminal differentiation in human melanoma cells (Jiang et al., 1995b, 1997). The present review focuses on *mda-7/IL-24*, its discovery and functional role in tumor suppression, as a cytokine, and the proposed signaling pathways responsible for inducing apoptosis in a cancer-specific manner, including a discussion of potential cell surface and intracellular targets of activity. Additionally, an overview of its properties as an immune modulating agent, radiation-enhancing molecule, and anti-angiogenic agent is also provided. This review concludes with a discussion of the present status of clinical trials with *mda-7/IL-24* administered intratumorally by means of a replication incompetent adenovirus (Ad.*mda-7*; INGN 241) (Fisher et al., 2003; Gopalkrishnan et al., 2004; Cunningham et al., 2005; Fisher, 2005; Lebedeva et al., 2005a; Tong et al., 2005).

2. Identification and structural analysis of melanoma differentiation associated gene-7/interleukin-24

mda-7/IL-24 was first identified by subtraction hybridization from HO-1 human melanoma cells induced to irreversibly growth arrest and terminally differentiate by combined treatment with IFN- β + MEZ (Fig. 1) (Jiang et al., 1993,1995c). RNA and immunohistochemical analyses confirmed expression of *mda-7/IL-24* mRNA and protein in melanocytes with a progressive decline in expression during the process of melanoma progression from radial to vertical growth phase (VGP) primary melanoma to metastatic disease (Jiang et al., 1995c; Ekmekcioglu et al., 2001; Ellerhorst et al., 2002). These observations support a putative role of *mda-7/IL-24* as a tumor suppressor gene, where loss of expression is a critical step in the process of melanoma progression from a non-invasive primary tumor to an invasive malignancy with metastatic potential (Ellerhorst et al., 2002). *mda-7/IL-24* is localized on human chromosome 1q32–33, a genomic area spanning 195-kb and containing a family of genes associated with the interleukin (IL)-10 family of cytokines, including IL-10, IL-19, IL-20, and IL-24 (*mda-7*) (Huang et al., 2001; Pestka et al., 2004). The mRNA encoding *mda-7/IL-24* is ~2-kb encoding a polypeptide of ~23.8-kDa (Jiang et al., 1995c). The open reading frame is flanked by 5'- and 3'-untranslated sequences of 274 and 823 bp, respectively. The 3'-UTR contains 3 consensus AU-rich elements and 3 polyadenylation signals (AAUAAA) playing a crucial role in the post-transcriptional stability of mRNA (Madireddi et al., 2000b; Huang et al., 2001). The *mda-7/IL-24* gene is composed of 7 exons and 6 introns (Huang et al., 2001). Sequence analysis also reveals the presence of a 49-amino acid signal peptide that allows the molecule to be cleaved and secreted. Sequence analysis of *mda-7/IL-24* reveals 3 putative glycosylation sites at amino acids 95, 109, and 126 resulting in different forms and molecular sizes of secreted *mda-7/IL-24* (Fig. 2).

mda-7/IL-24 belongs to the 4-helix bundle family of cytokine molecules most closely related to the IL-10 subfamily (Kotenko, 2002; Pestka et al., 2004). Tertiary structure predictions, based on computer simulations, generate a compact globular structure consisting of 4 helical regions interspersed by loops of unpredicted structure (Kotenko, 2002; Pestka et al., 2004). The predicted organization of *mda-7/IL-24* reveals greatest homology to the IL-10 subfamily, which includes IL-19, IL-20, IL-22, and IL-26/AK-155 (Chaiken & Williams, 1996; Gallagher et al., 2000; Xie et al., 2000; Huang et al., 2001; Kotenko, 2002; Pestka et al., 2004). Comparison of the amino acid sequence of IL-10 and *mda-7/IL-24* indicates only 23% homology; however, the presence of an IL-10 signature sequence in *mda-7/IL-24* supports its being a member of the IL-10 subfamily (Fig. 2) (Kotenko, 2002; Pestka et al., 2003, 2004). Expression analysis of *mda-7/IL-24* demonstrates restricted expression to tissues associated with the immune system, such as thymus, spleen, and peripheral blood leukocytes (PBMC) (Fig. 3), further suggesting cytokine-like properties of this molecule (Huang et al., 2001). Based on its chromosomal location, structure, and expression profile, *mda-7* has been renamed IL-24 (*mda-7/IL-24*) by the Human Gene Organization (HUGO) (Caudell et al., 2002; Sarkar et al., 2002a, 2000b; Sauane et al., 2003b; Lebedeva et al., 2005a).

Southern blot analysis of DNA from different species using a cDNA probe identified homologous sequences in genomic DNA of yeast, monkey, cow, dog, and cat, suggesting that *mda-7/IL-24* is an evolutionary conserved gene (Jiang et al., 1995c). Further studies by several groups have established the presence of *mda-7/IL-24* orthologues in other species, *c49a/mob-5* in rat fibroblasts (Soo et al., 1999; Zhang et al., 2000; Wang et al., 2002; Wang & Liang, 2005) and FISP in Th2 cells in mouse (Schaefer et al., 2001) (Fig. 4). Of interest, the functions of the *mda-7/IL-24* rat orthologue appear to be different than that of the human and potentially mouse version of this gene.

Using the differential display polymerase chain reaction (DD-PCR), new sets of genes up- or down-regulated during wound repair in rat fibroblasts were identified (Soo et al., 1999). A 260-nucleotide fragment of a gene designated *c49a* showed significant up-regulation 12 hr post-wounding in a rat cutaneous wound model. This fragment led to the cloning of the rat *c49a* cDNA, which is 1107-nucleotides in length and shares 82% homology with *mda-7/IL-24* (Soo et al., 1999) (Fig. 4). Like *mda-7/IL-24*, the 3'-untranslated region of rat *c49a* also contains copies of the AUUUA sequence motif involved in mRNA destabilization. Alignment of the amino acid sequences of *c49a* and *mda-7/IL-24* protein reveals ~58.7% homology, suggesting that rat *c49a* and *mda-7/IL-24* may be related molecules, rather than true homologues (Soo et al., 1999; Sauane et al., 2003b) (Fig. 4). Because *c49a* expression is seen in wounded rat dermal cells, it is believed to play a role in proliferation. An additional *mda-7/IL-24*-like molecule named *mob-5* was isolated by DD-PCR between rat embryo fibroblast cells Rat1 and Rat1:ras cells containing an inducible oncogenic *Ha-ras* gene (Zhang et al., 2000). MOB-5 protein is identical to the *c49a* protein, except for 2 amino acid mismatches. MOB-5 is a secreted protein and its expression is induced by oncogenic *H-ras* and *K-ras* and a role for this gene has been proposed in proliferation (Zhang et al., 2000; Wang et al., 2002; Wang & Liang, 2005). Thus, both C49A and MOB-5 resemble MDA-7/IL-24 protein but they may play a role in stimulating rather than inhibiting growth (Soo et al., 1999; Wang et al., 2002; Shinohara & Rothstein, 2004; Wang & Liang, 2005). These observations suggest that the function of *mda-7/IL-24* in humans is distinct from that of its rat orthologue.

A mouse MDA-7/IL-24-like protein called FISP has also been identified using representational difference analysis between type 2 and type 1 helper T-lymphocyte (Th2 and Th1, respectively) cells as an IL-4-induced secreted protein in Th2 helper lymphocytes (Th2) (Schaefer et al., 2001). FISP is selectively expressed in lymphocytes under Th2 differentiation conditions and its expression is induced in CD4+ enriched Th2 cells, whereas no expression is observed in Th1 cells. FISP expression is regulated by the T-cell receptor and IL-4 and involves protein kinase C and STAT6 signaling pathways (Schaefer et al., 2001). FISP is a secreted protein of 220 amino acids with a predicted molecular mass of 25-kDa (Schaefer et al., 2001). It shares 93% homology with rat C49A/MOB-5 and 69% identity with MDA-7/IL-24 at the protein level (Fig. 4). Although MDA-7/IL-24 and FISP share several common properties, expression in the immune system, induced expression in response to differentiation and treatment with cytokine and protein kinase activators, the precise role of FISP in these processes remains to be established (Schaefer et al., 2001). Further work is clearly needed to address this question and to determine the precise function of FISP in Th1 and Th2 differentiation.

Direct support for similar functional attributes between human *mda-7/IL-24* and mouse *mda-7/IL-24* (mIL-24; FISP) in the context of antitumor activity has recently been provided in mice using mouse ML-1 hepatoma cells (Chen et al., 2005). Intramuscular electroporation of mIL-24 was shown to suppress mouse ML-1 hepatoma cell growth in vivo in female BALB/cJ mice. This effect was observed when ML-1 cells were administered by subcutaneous dorsal injection or when ML-1 cells were directly injected into the spleen, which resulted in tumor metastasis in the liver. These studies confirm the tumor growth-suppressive properties of mouse *mda-7/IL-24* (mIL-24; FISP) in syngeneic mice and highlight potentially similar antitumor properties of both human MDA-7/IL-24 and murine mIL-24. These findings are intriguing and provide additional evidence that human MDA-7/IL-24 is more closely related functionally to murine mIL-24 than to the rat orthologue of this gene, *c49a/mob-5*, which appears to have growth stimulatory properties (Soo et al., 1999; Wang et al., 2002). Further experimentation is required to explain how the rat version of *mda-7/IL-24* acquired its divergent function from that of the human and murine genes.

3. Expression analysis of melanoma differentiation associated gene-7/interleukin-24 and its regulation

Analysis of *mda-7/IL-24* expression in normal and cancer cells indicated a lack of constitutive expression in most cellular contexts (Huang et al., 2001; Caudell et al., 2002; Garn et al., 2002; Wolk et al., 2002). However, expression was evident, using poly(A)⁺ RNA and Northern blotting in spleen, thymus, and peripheral blood leukocytes (PBMC), that is, cells of the immune system (Fig. 3) (Huang et al., 2001; Caudell et al., 2002; Wolk et al., 2002). Further experimentation with PBMC following treatment with various activators of the immune response revealed that lipopolysaccharide (LPS) or concanavalin A enhances transcription, translation, and secretion of *mda-7/IL-24* in vitro (Wang et al., 2002). LPS treatment also induced expression of *mda-7/IL-24* in monocytes from a healthy patient. Concanavalin A activation of T-cells promoted *mda-7/IL-24* expression, suggesting a role of *mda-7/IL-24* as a putative cytokine. Further studies are necessary to clarify a presumed role of *mda-7/IL-24* as an immune-modulating cytokine (Garn et al., 2002).

Apart from tissues of the immune system, expression of *mda-7/IL-24* mRNA can be transiently induced in certain cell types under appropriate conditions that are not of melanocytic or hematopoietic origin (Huang et al., 2001). A 24-hr treatment of DU-145 (prostate carcinoma), HBL-100 (normal breast epithelium), MDA-MB-157 and MDA-MB-231 (breast carcinoma), HeLa (cervical carcinoma), NC (normal cerebellum astrocytes), GBM18 (glioblastoma multiforme), Saos-2 (osteosarcoma), and HONE-1 (nasopharyngeal carcinoma) cells with IFN- β + MEZ transiently induced *mda-7/IL-24* mRNA expression (Huang et al., 2001). In contrast, constitutive expression or induction was not apparent in other normal/tumor-derived cell lines including HuPEC (normal prostate epithelial), PC-3 and LNCaP (prostate carcinoma), MCF-7, T47D, MDA-MB-453 (breast carcinoma), T98G (glioblastoma multiforme), and SW613 (colon carcinoma) (Huang et al., 2001). These results confirm that *mda-7/IL-24* is not constitutively expressed in most normal or cancer cell types; however, by appropriate treatment (e.g., IFN- β + MEZ) this gene can be induced on an mRNA level confirming functional integrity of the *mda-7/IL-24* locus in both normal and cancer cells of non-melanocytic and hematopoietic origins (Huang et al., 2001).

Gene expression programs are dramatically altered during growth suppression and terminal differentiation, including the modified expression of genes regulating cell cycle progression, transcriptional control, cytoskeletal architecture, and novel genetic elements with undefined functions (Huang et al., 1999a, 1999b; Leszczyniecka et al., 2001). Expression analysis of *mda-7/IL-24* requires complete understanding of the regulatory mechanisms controlling transcription, translation, and other modifications in mRNA, including stability. The promoter region of *mda-7/IL-24* was cloned and it was found that expression was regulated post-transcriptionally during melanoma differentiation (Madireddi et al., 2000b). In HO-1 and MeWo human melanoma cells, uninduced basal full-length promoter activity did not change upon treatment with IFN- β + MEZ (Madireddi et al., 2000b). This suggested that modulation of *mda-7/IL-24* gene expression during differentiation in human melanoma cells might not be controlled on a transcriptional level. Terminal differentiation in human melanoma cells resulting from treatment with IFN- β + MEZ resulted in an elevation in the levels of *mda-7/IL-24* mRNA and protein, but no or limited mRNA was detected in cells treated with IFN- β or MEZ alone. This led to the hypothesis that IFN- β + MEZ might function to stabilize *mda-7/IL-24* mRNA and this stabilization may occur by post-transcriptional modifications (Madireddi et al., 2000b).

The cDNA of *mda-7/IL-24* contains 3 AU-rich sequences in its 3'-UTR (Madireddi et al., 2000b; Huang et al., 2001). Many transiently expressed genes, including lymphokines and other cytokine genes and proto-oncogene, such as *c-myc* and *c-fos*, contain AU-rich sequences

in their 3'-UTR. Another class 2 cytokine, IL-10, was found to be regulated in melanocytes and melanoma cells by AU-rich sequences in their 3'-UTR (Brewer et al., 2003). The presence of AU-rich sequences in eukaryotic mRNA correlates with rapid mRNA turnover and post-translational control (Aharon & Schneider, 1993; Rajagopalan & Malter, 1997; McCormick & Ganem, 2005). To investigate the importance of the 3'-UTR of *mda-7/IL-24* in regulating mRNA stability, a luciferase gene construct was generated containing the 3'-UTR of *mda-7/IL-24* (Madireddi et al., 2000b). Expression of this construct was enhanced when transfected into terminally differentiated HO-1 cells (IFN- β + MEZ treated). This finding supports the conclusion that the steady-state level of *mda-7/IL-24* mRNA is determined by post-translational degradation of this message, which decays in HO-1 cells that are uninduced or treated singly with IFN- β or MEZ, whereas in IFN- β + MEZ-treated HO-1 cells, *mda-7/IL-24* mRNA does not undergo degradation at a comparable rate (Madireddi et al., 2000b).

Transcription regulation of *mda-7/IL-24* occurs by binding 2 primary transcription factors at various sites in its promoter (Madireddi et al., 2000a). These transcription factors, identified by gel shift and super shift analyses, are AP-1 and C/EBP (Madireddi et al., 2000a). Increased binding of AP-1 and C/EBP was observed following IFN- β + MEZ treatment, whereas a dominant-negative form of *c-jun* (TAM67) (a member of the AP-1 family) abrogated *mda-7/IL-24* basal activity. Over-expression of *c-jun* or C/EBP increased the activity of the *mda-7/IL-24* promoter, suggesting that both of these factors play a central role in *mda-7/IL-24* gene expression via transcriptional activation (Madireddi et al., 2000a).

4. Melanoma differentiation associated gene-7/interleukin-24, a novel cytokine belonging to the interleukin-10 gene family

Due to the existence of a large number of cytokines and their utilization of overlapping signal transduction pathways, addressing issues of functional specificity at a physiological level as well as key differences in signaling mechanisms presents a complex and difficult problem. The recent recognition of the expanded IL-10 subfamily of cytokines and the finding that individual members have the capacity to bind common receptor subunits has made the process even more daunting to decipher within this particular subset of genes (Burdin et al., 1993; Josephson et al., 2000, 2001; Dumoutier & Renauld, 2002; Langer et al., 2004; Pestka et al., 2004). As noted in previous sections, based on sequence homologies, structural analysis, and chromosomal location, the gene originally named *mda-7* (Jiang et al., 1995c) has been redesignated IL-24 and recognized as a member of the increasing IL-10 subfamily (Caudell et al., 2002; Kotenko, 2002; Pestka et al., 2003, 2004; Sauane et al., 2003b). Further experimental evidence for this reclassification was provided by demonstrating secretion from PBMC and melanocytes (Caudell et al., 2002; Lebedeva et al., 2002), binding to cognate receptors (IL-20R1/IL-20R2 or IL-22R1/IL-20R2 heterodimers) (Dumoutier et al., 2001; Wang et al., 2002) and activation of the JAK/STAT (STAT 1 and 3) signaling pathway (Kotenko et al., 1997; Dumoutier et al., 2001; Wang et al., 2002). An examination of the various tissues and cell types expressing this cytokine has demonstrated restricted expression. Huang et al. (2001) reported expression in melanocyte, PBMC, and spleen-derived mRNAs from normal human tissues (Fig. 3). This study supported the initial report by Jiang et al. (1995c) relating to the isolation of the gene from a growth arrested and in vitro differentiated human melanoma cell line as well as its loss of expression in human melanoma progression models (Jiang et al., 1995c; Ellerhorst et al., 2002).

The cytokine-related functions of *mda-7/IL-24* through its secretion by melanocytes and loss of expression in a melanoma context are not fully understood. While no formal experimental demonstration has been made, it is possible that *mda-7/IL-24* acts as a paracrine factor and contributes to short-range signaling and performs immune-related functions in skin. The related cytokines, IL-19 and IL-20, are expressed in skin and particularly in keratinocytes, either in

normal or disease states, such as psoriatic lesions, and likely play a role in skin inflammation by inducing keratinocyte proliferation (Blumberg et al., 2001). The other members of this subfamily are not expressed in this tissue type. Presently, the responses mediated by *mda-7/IL-24* appear to be primarily pro-inflammatory, when secreted by primary blood mononuclear cells (PBMC), as will be described later in this review. The melanocyte-derived protein is likely to function similarly (pro-inflammatory activity), barring the possibility that localized target cells in the skin microenvironment behave and respond differently to this cytokine. The putative role of *mda-7/IL-24* as a tumor suppressor-like molecule, particularly in a human melanoma context, rests upon loss of expression associated with disease progression (Jiang et al., 1995c; Ellerhorst et al., 2002). The finding that iNOS and *mda-7/IL-24* expressions are inversely correlated and that increased expression of iNOS is involved in melanoma progression provides a mechanistic link to tumor-suppressive properties, although at the present time the basis of this activity requires further investigation (Ekmekcioglu et al., 2003). In general, the intriguing suppressive property of *mda-7/IL-24* expression in melanoma has not been definitively connected experimentally with its role as a secreted cytokine. Whether the recently discovered intracellularly localized activity of the molecule (Sauane et al., 2004a, 2004b; Sieger et al., 2004) plays some (or even a major) role in preventing melanocytes from undergoing malignant transformation compared to activity of the secreted cytokine form also remains to be determined.

As discussed previously, expression analysis of *mda-7/IL-24* by Northern blotting using primary human tissues indicated restricted tissue-specific expression of *mda-7/IL-24* in thymus, spleen, and PBMC (Fig. 3) (Huang et al., 2001; Caudell et al., 2002). A role for *mda-7/IL-24* as a cytokine and its involvement in the immune system has been highlighted by independent studies from 3 different groups. By real-time PCR, Wolk et al. (2002) analyzed the expression of *mda-7/IL-24* at an RNA level. Basal expression was confirmed in unstimulated monocytes, but not in other cell types such as T, NK, and B cells. Activation of monocytes by LPS treatment for 6 and 18 hr produced an ~ 10-fold and ~ 100-fold stimulation of expression of *mda-7/IL-24* over unstimulated monocytes, which were also grown for the same time points, respectively. Induction of *mda-7/IL-24* RNA in T-cell populations upon treatment with LPS was ~ 10-fold, although no increase occurred at 6 and 18 hr, but only at 66 hr. In contrast, no expression was apparent in NK or B cells either before or after stimulation (Wolk et al., 2002). Further analysis of expression in different subsets of T-helper cells (Type 1 or 2) revealed dual functionality, that is, activation in both lineages. In the initial phases of induction toward Th1, IL-22 is induced followed later by IL-26 at 42–66 hr post-stimulation. *mda-7/IL-24* is initially down-regulated in a Th1 milieu (at 6 hr) and up-regulated in Th2 cells (Wolk et al., 2002); however, at later times (66 hr) up-regulation of *mda-7/IL-24* is observed in a Th1 milieu. Expression of FISP, the mouse homologue of *mda-7/IL-24*, displayed highly specific Th2 expression, suggesting that the mouse and human genes might have distinct expression patterns (Schaefer et al., 2001).

Garn et al. (2002) confirmed expression of *mda-7/IL-24* in response to stimulation by various members of the IL-10 family of cytokines in mouse and human macrophages. *mda-7/IL-24* RNA and protein were present upon treatment of rat alveolar macrophages with LPS or IL-4, but not tumor necrosis factor alpha (TNF- α). Treating these cells with PMA, an activator of protein kinase C, promoted weak expression of *mda-7/IL-24* over basal levels. The NR8383 mouse macrophage cell line produces steady-state levels of *mda-7/IL-24*, and following treatment with IL-4, gene transcription and mRNA levels were increased (Garn et al., 2002) but protein levels remained constant. These researchers further demonstrated that intracellular pools of MDA-7/IL-24 protein exist and the level of this pool did not change significantly post-induction. In human macrophages, the levels of *mda-7/IL-24* mRNA increased significantly, peaking at 8 hr followed by a decline (Garn et al., 2002). The authors also found that addition of IL-10 inhibited *mda-7/IL-24* gene transcription. Induction of *mda-7/IL-24* mRNA correlated

with expression of IL-1, IL-6, and TNF- α in cultured human monocytes infected with influenza virus indicating a proinflammatory role for this molecule (Garn et al., 2002).

Caudell et al. (2002) have investigated the effect of purified MDA-7/IL-24 protein on PBMC. Treatment of monocytes with pure MDA-7/IL-24 protein led to secretion of IL-6, TNF- α , and IFN- γ at robust levels while trace amounts of GM-CSF, IL-2, IL-4, and IL-10 were also observed. Production of IFN- γ and TNF- α was completely blocked by simultaneous treatment with IL-10, but IL-6 expression was reduced by approximately one third (Caudell et al., 2002). These observations highlight the different roles of *mda-7/IL-24* and IL-10 on immune function, although both molecules belong to the same family of cytokines (Moore et al., 2001; Pestka et al., 2004). While IL-10 has anti-inflammatory and immune response suppressive roles, *mda-7/IL-24* plays an immunomodulatory and pro-inflammatory role. It is hypothesized that cytokines induced by *mda-7/IL-24* might activate antigen-presenting cells to present tumor antigens, thus triggering an antitumor immune response (Caudell et al., 2002). This possibility is supported by a recent Phase I clinical trial in patients with metastatic melanoma, in which injection of a tumor lesion resulted in a pronounced inflammatory response in the injected tumor and in distant metastases (Cunningham et al., 2005; Lebedeva et al., 2005a; Tong et al., 2005). However, despite these observations, the immunomodulatory role of *mda-7/IL-24* is not well established and further studies are required to clarify the role of *mda-7/IL-24* in regulating immune responses.

5. Receptors for melanoma differentiation associated gene-7/interleukin-24

Ligand receptor crosstalk plays a crucial role in transmitting signals from ligands and other environmental cues to the cell nucleus. Cytokines are known to transmit signals from the site of release to the effector cells through specific receptors present at the cell surface. *mda-7/IL-24* being a member of the IL-10 subfamily raises the obvious possibility of it having similar kinds of receptors as other members of the IL-10 subfamily of cytokines (Fickenscher et al., 2002; Pestka et al., 2004). Receptors of IL-10 belong to the class 2 cytokine receptor family, which comprises the various IFN receptor chains (Liu et al., 1994; Pestka et al., 2004). The IL-10 receptor was initially identified as a single R1 type of receptor with a long cytoplasmic domain, IL-10R1, which is the major signaling component (Liu et al., 1994; Pestka et al., 2004). Later it was found that the functional IL-10 receptor required a second chain of an R2 type of receptor, with a short membrane spanning cytoplasmic domain, IL-10R2 (Kotenko et al., 1997; Kotenko, 2002; Pestka et al., 2004). Subsequently, 3 R1 and 2 R2 types of receptor subunits of the IL-10 family were identified. The 3 R1 subunits are IL-10R1, IL-20R1, and IL-22R1 and the 2 R2 subunits are IL-10R2 and IL-20R2 (Josephson et al., 2000, 2001; Pestka et al., 2004). IL-20 receptors contain the long subunit IL-20R1 and IL-20R2, which join together on the surface of keratinocytes to form the functional IL-20 receptor (Josephson et al., 2000, 2001; Pestka et al., 2004). Recently, it was shown that IL-20 binds to 2 kinds of receptors, where the long chain can also be replaced by IL-22R1 (Blumberg et al., 2001; Pestka et al., 2004). In the case of the receptor for IL-22, the long chain IL-22R1 is complemented by IL-10R2 to form the functional receptor and thus play a role as a common chain in different cytokine receptors, in a manner similar to the common γ chain in the receptors for IL-2, IL-4 and others (Kotenko et al., 1997; Dumoutier & Renauld, 2002; Kotenko, 2002; Pestka et al., 2004). Receptors for IL-19 and *mda-7/IL-24* resemble the receptors for IL-20 as the functional receptor for IL-19 contains IL-20R1 and IL-20R2, whereas the receptors for *mda-7/IL-24* like the receptors of IL-20 signal through heterodimeric receptors IL-20R1/IL-20R2 and IL-22R1/IL-20R2 (Dumoutier et al., 2001; Wang et al., 2002; Pestka et al., 2004). Although many cytokines share receptors, receptor activation is ligand specific, and when activated by their ligands the receptors activate the JAK/STAT signaling pathway (Dumoutier et al., 2001; Pestka et al., 2004). Although alternative signaling pathways have not been investigated, Stat3 seems to be a major transcription factor mediating stimulatory effects. The tissue-specific structure

and organization of the specific combination of receptor subunits are likely to play a crucial role in determining the function of different members of the IL-10 family (Kotenko, 2002; Langer et al., 2004; Pestka et al., 2004).

6. Melanoma differentiation associated gene-7/interleukin-24 and melanoma

Melanoma represents an aggressive cancer that most frequently metastasizes to regional lymph nodes and to distant sites as the disease progresses (Herlyn et al., 2000; Bevona & Sober, 2002; Bogenrieder & Herlyn, 2002). This propensity for metastasis combined with resistance of melanoma metastases to therapy represents limitations to current therapeutic regimens (Eigentler et al., 2003; Lens & Elsen, 2003; Chung et al., 2004). In the United States, the incidence of melanoma is increasing at a faster rate than any other cancer and it is believed that as many as 1 in 75 currently born children may eventually develop superficial spreading type melanoma (McGary et al., 2002; Bevona et al., 2003; Carlson et al., 2003; Eigentler et al., 2003). Presently, surgery is an option for treating metastases, as chemotherapy and radiotherapy do not achieve cures in the majority of patients and less than 5% of melanoma patients with systemic metastases survive 5 years or more (Baron et al., 2003; Lens & Elsen, 2003; Meric et al., 2003; Nguyen, 2004). However, a high level of IFN- α has shown significant increase in lifetime but is not curative (Kirkwood et al., 2004). Many other forms of therapy have been evaluated with unimpressive results and there is a need to define new molecules and methods for treating metastatic melanoma (Lens & Elsen, 2003; Nguyen, 2004). Gene therapy involving tumor suppressor gene replacement or supplementation represents a new approach for combating this disease (Volk et al., 2003; Liu et al., 2004; Wolkersdorfer et al., 2004). In these contexts, it is important to understand the potential regulatory molecules that are involved in melanoma development, progression, and invasion (Bogenrieder & Herlyn, 2002; Boukerche et al., 2004). Our research groups and others have established that *mda-7/IL-24* mRNA and protein are expressed in melanocytes and they are the only skin cells expressing MDA-7/IL-24 protein constitutively (Jiang et al., 1995c; Ekmekcioglu et al., 2001; Huang et al., 2001; Ellerhorst et al., 2002).

Development of malignant melanoma in humans, with the exception of nodular-type melanoma, consists of a series of sequential alterations in the evolving tumor cells (Jiang et al., 1994a; Herlyn et al., 2000; Leszczyniecka et al., 2001; Baruch et al., 2005). These include conversion of a normal melanocyte to a nevus, followed by development of a dysplastic nevus, a radial growth phase (RGP) primary melanoma, a vertical growth phase (VGP) primary melanoma, and ultimately a metastatic melanoma. To evaluate the relationship between *mda-7/IL-24* expression and melanoma progression, *mda-7/IL-24* and GAPDH levels were determined by RT-PCR in actively growing melanocytes, RGP and VGP primary melanomas and metastatic melanoma cell lines, and in tissue samples (Jiang et al., 1995c). Normal melanocytes/nevi expressed more *mda-7/IL-24* than the majority of RGP primary melanomas (Fig. 5). Lower expression of *mda-7/IL-24* was evident in VGP primary and metastatic melanomas, with lowest levels expressed on average in metastatic melanomas (Fig. 5). The expression of *mda-7/IL-24* during melanoma progression was analyzed using a melanoma MatrigelTM assisted tumorigenic growth model (Jiang et al., 1995c). This approach involved the coinjection of non-tumorigenic or weakly tumorigenic RGP or early VGP primary human melanomas with MatrigelTM into nude mice (Kobayashi et al., 1994). This process results in tumor progression that correlates with acquisition of tumorigenic potential in nude mice by previously non-tumorigenic RGP and VGP primary human melanoma cells (Kobayashi et al., 1994). No change was noticed in *mda-7/IL-24* expression in a MatrigelTM-progressed RGP primary melanoma cell line, whereas the amount of *mda-7/IL-24* mRNA expressed in VGP primary human melanoma cell lines (WM793 and WM1341B) was less after Matrigel selection (Jiang et al., 1995c). These data correspond with the observation that ectopic transfer of *mda-7/IL-24* by plasmid or by means of a replication-incompetent adenovirus leads to growth

arrest and apoptosis in melanoma and other tumors suggesting that *mda-7/IL-24* may function as a tumor suppressor gene and a decline in the levels of *mda-7/IL-24* could play a crucial role in the progression of primary melanoma to invasive melanoma (Jiang et al., 1995c; Ekmekcioglu et al., 2001; Lebedeva et al., 2002). Confirmation of this possibility has come from studies by Ellerhorst et al. (2002), who studied the levels of MDA-7/IL-24 protein by immunohistochemistry during melanoma development and progression. Immunohistochemical analysis of MDA-7/IL-24 expression using tissue sections of melanomas indicated abundant expression of MDA-7/IL-24 protein in human nevi and in primary melanoma tumors. Additional data from this study indicated a decline in the levels of MDA-7/IL-24 protein as the melanoma progressed and invaded surrounding tissue (Ellerhorst et al., 2002). These results confirm that down-regulation of MDA-7/IL-24 protein occurs during progression of melanoma from primary non-invasive to advanced invasive stages of melanoma progression.

Based on the observation that expression of *mda-7/IL-24* decreases as a function of melanoma development and progression (Fig. 5), it was hypothesized that this gene might exhibit growth-suppressive properties when reactivated (Jiang et al., 1995c). To test this possibility, the effect of *mda-7/IL-24* gene replacement on tumor and normal cell growth was evaluated (Jiang et al., 1996). Transient transfection of *mda-7/IL-24* into human melanoma cell lines, such as HO-1 and C8161, as well as in transformed rat cells and a spectrum of additional human cancer cells, including carcinomas from the breast, cervix, colon, and prostate, resulted in a reduction in colony formation (Jiang et al., 1996). In contrast, *mda-7/IL-24* did not significantly alter the growth or colony formation of normal human and rat cells, respectively (Jiang et al., 1995c, 1996). These findings were confirmed and expanded using a replication incompetent adenovirus expressing *mda-7/IL-24* (Ad.*mda-7*), which resulted in significant inhibition of growth in melanoma and other tumor cells, but not in normal fibroblasts, epithelial cells, astrocytes, or melanocytes (Su et al., 1998,2001,2003a, 2005c; Madireddi et al., 2000c; Saeki et al., 2000,2002; Mhashilkar et al., 2001,2003; Lebedeva et al., 2002,2003a,2003b,2005a, 2005b; Sarkar et al., 2002a, 2002b; Pataer et al., 2002,2005; Sauane et al., 2003a,2003b, 2004a,2004b; Yacoub et al., 2003a,2003b, 2003c, 2004; Fisher et al., 2003; Chada et al., 2004b; Gopalakrishnan et al., 2004; Leath et al., 2004; Nishikawa et al., 2004; Dent et al., 2005; Fisher, 2005; Gopalan et al., 2005; Saito et al., 2005; Oida et al., 2005; Lebedeva et al., in press). Further analysis in melanoma cells and melanocytes indicated that growth suppression in melanoma cells was associated with selective induction of apoptosis (programmed cell death), without detrimental effects on normal early passage or immortal melanocytes (Lebedeva et al., 2002). Infection of human melanoma cells, but not normal melanocytes, with Ad.*mda-7* resulted in a temporal change in cell cycle and induction of Annexin V staining and DNA fragmentation, markers of apoptosis (Lebedeva et al., 2002). In a comparative study, Ad.*mda-7* was found to be as potent as Ad.*wtp53* or Ad.*p21*, a cyclin-dependent kinase inhibitor that is a downstream target of wild-type p53, in its growth inhibitory effects on melanoma cells (Lebedeva et al., 2002).

Recently, expression of inducible nitric oxide synthase (iNOS) was found to be increased in advanced stages of melanoma (Ekmekcioglu et al., 2000) and expression of *mda-7/IL-24* negatively regulated iNOS expression in malignant melanoma cell lines (Ekmekcioglu et al., 2003). Infection of melanoma cells with Ad.*mda-7* or recombinant MDA-7/IL-24 protein resulted in profound suppression of iNOS and this inverse expression of MDA-7/IL-24 and iNOS suggests a possible cause/effect relationship in melanoma (Ekmekcioglu et al., 2003). Further studies are necessary to determine if one of these molecules might function to control the expression of the other. Understanding how *mda-7/IL-24* regulates iNOS may provide insight into the apoptotic pathways regulated by this gene in melanoma.

7. Melanoma differentiation associated gene-7/interleukin-24 displays antitumor activity and cancer cell-specific apoptosis

As discussed above, *mda-7/IL-24* may contribute to the physiology of human melanocytes and melanomas and this gene has potent growth inhibitory properties when over-expressed in human melanoma cells (Jiang et al., 1995c, 1996; Lebedeva et al., 2002). Our groups and others have shown that *mda-7/IL-24* also has growth-suppressive properties in a wide variety of additional human cancer cell lines, without inducing harmful effects in normal cells (Table 1) (Jiang et al., 1996; Su et al., 1998, 2001, 2003a, 2005c; Madireddi et al., 2000c; Saeki et al., 2000; Mhashilkar et al., 2001, 2003; Lebedeva et al., 2002, 2003a, 2003b, 2005a, 2005b; Pataer et al., 2002; Sarkar et al., 2002a, 2002b; Chen et al., 2003; Fisher et al., 2003; Sauane et al., 2003a, 2003b, 2004a, 2004b; Yacoub et al., 2003a, 2003b, 2003c, 2004; Chada et al., 2004b; Gopalkrishnan et al., 2004; Leath et al., 2004; Nishikawa et al., 2004; Dent et al., 2005; Gopalan et al., 2005; Oida et al., 2005; Saito et al., 2005; Lebedeva et al., in press; Su et al., in press). The broad-spectrum antitumor activity of *mda-7/IL-24* can be distinguished from other extensively scrutinized tumor suppressor genes and its growth inhibitory properties are independent of the status of p53, pRB, p21, and additional tumor suppressor genes in cancer cells (Jiang et al., 1996; Su et al., 1998; Madireddi et al., 2000c; Lebedeva et al., 2002; Su et al., 2003a). For example, Ad.*mda-7* produced similar growth suppression in T47D and MCF7 cells (T47D is a mutant p53 containing human breast carcinoma cell line and MCF7 has wild-type p53 status) as well as in MDA-MB-157 cells (which are null for p53) (Su et al., 1998). Moreover, growth suppression by *mda-7/IL-24* can be dissociated from that observed with the p53, RB, and p16 suppressor genes and the mechanism is distinct from the mode of action of these tumor suppressor genes (Lebedeva et al., 2002; Fisher et al., 2003; Su et al., 2003a; Lebedeva et al., 2005a). In contrast, *mda-7/IL-24* does not affect growth in normal cells, including HBL-100 and non-established early passage skin fibroblasts, breast epithelial cells, ovarian epithelial cells, prostate epithelial cells, endothelial cells, melanocytes, and astrocytes, thereby providing support for the hypothesis that *mda-7/IL-24* has cancer-specific growth suppressing properties (Table 1) (Su et al., 1998, 2001, 2003a, 2005c; Madireddi et al., 2000c; Saeki et al., 2000, 2002; Mhashilkar et al., 2001, 2003; Lebedeva et al., 2002, 2003a, 2003b, 2005a; 2005b; Pataer et al., 2002; Sarkar et al., 2002a, 2002b; Fisher et al., 2003; Sauane et al., 2003a, 2003b, 2004a, 2004b; Yacoub et al., 2003a, 2003b, 2003c, 2004; Chada et al., 2004b; Gopalkrishnan et al., 2004; Leath et al., 2004; Nishikawa et al., 2004; Dent et al., 2005; Fisher, 2005; Gopalan et al., 2005; Oida et al., 2005; Saito et al., 2005; Lebedeva et al., in press; Su et al., in press).

To more efficiently administer *mda-7/IL-24* and to study the mechanism by which this gene specifically suppresses growth of tumor cells, a replication incompetent adenovirus was constructed (Su et al., 1998). Supra-physiological levels of expression inhibited growth by inducing apoptosis in many cancer cell lines, including melanoma, malignant glioma, osteosarcoma and carcinomas of the breast, cervix, colon, lung, ovary, and prostate, whereas no suppression of growth was observed in various normal early passage and established human cell lines suggesting that this effect on growth was specific to cancer cells (Su et al., 1998, 2001, 2003a, 2005c; Madireddi et al., 2000c; Saeki et al., 2000, 2002; Mhashilkar et al., 2001, 2003; Lebedeva et al., 2002, 2003a, 2003b, 2005a, 2005b; Pataer et al., 2002; Sarkar et al., 2002a, 2002b; Fisher et al., 2003; Sauane et al., 2003a, 2003b, 2004a, 2004b; Yacoub et al., 2003a, 2003b, 2003c, 2004; Chada et al., 2004b; Gopalkrishnan et al., 2004; Leath et al., 2004; Nishikawa et al., 2004; Dent et al., 2005; Fisher, 2005; Oida et al., 2005; Saito et al., 2005; Lebedeva et al., in press; Su et al., in press). In-depth analyses into the mechanism of action of *mda-7/IL-24* in eliciting cancer-specific killing by our laboratories and by other research groups have revealed the complexity of pathways that can be exploited by this gene in inducing programmed cell death (reviewed in Sarkar et al., 2002a; Fisher et al., 2003; Sauane

et al., 2003b; Dent et al., 2005; Fisher, 2005; Lebedeva et al., 2005a). As recently highlighted, Ad.*mda-7* induces apoptosis in a wide spectrum of cancer cells by exploiting diverse signaling abnormalities ultimately culminating in cell death (Fisher et al., 2003; Lebedeva et al., 2005a, in press). Studies are currently focusing on enhancing these cancer-specific killing properties by employing this novel cytokine with additional agents or treatment protocols, including chemotherapeutic agents, monoclonal antibodies, reactive oxygen species (ROS) inducers, and radiation (Kawabe et al., 2002; Lebedeva et al., 2003c, 2005b; Su et al., 2003a; Yacoub et al., 2003a, 2003b, 2003c, 2004; McKenzie et al., 2004; Nishikawa et al., 2004; Dent et al., 2005; Oida et al., 2005; Su et al., in press).

Initial therapy studies, in the context of MCF-7 human breast cancer and HeLa human cervical cancer cells, indicated that Ad.*mda-7* had antitumor activity in vivo in the context of nude mouse human tumor xenograft models (Su et al., 1998; Madireddi et al., 2000c). Breast cancer studies involved infection of MCF-7 human breast carcinoma cells in vitro with Ad.*mda-7* prior to injection into athymic nude mice, which resulted in an inhibition in tumor growth (Su et al., 1998). In the case of cervical cancer cells, HeLa cells were injected into nude mice and when tumors developed (100–150 mm²) they received repeated injections with Ad.*mda-7* or Ad.null and tumor growth was monitored (Madireddi et al., 2000c). Using this experimental protocol, tumor growth and cancer progression were inhibited and this effect persisted after discontinuing administration of Ad.*mda-7*. These studies provided definitive evidence that *mda-7*/IL-24 had antitumor activity in vivo in animal models. Additional studies indicated that Ad.*mda-7* had antitumor activity in vivo in human non-small cell lung carcinoma (NSCLC) when applied as a single injected agent (Saeki et al., 2002) or in combination with radiation (Nishikawa et al., 2004) or sulindac (Oida et al., 2005), and in human pancreatic cancer cells containing a mutated *K-ras* gene when applied with a combination of antisense phosphorothioate oligonucleotides targeting *K-ras* (Su et al., 2001) or when administered in combination with arsenic trioxide (Lebedeva et al., 2005b).

Two recent studies suggest that employing a strategy embodying adenovirus replication to deliver *mda-7*/IL-24 can enhance the antitumor activity of this cytokine (Sarkar et al., 2005; Zhao et al., 2005). Sarkar et al. (2005) used the cancer-specific progression elevated gene-3 (PEG-3) promoter (Su et al., 2000, 2005b) to develop conditionally replication competent adenoviruses (CRCAs) that upon replication simultaneously express *mda-7*/IL-24 uniquely in the context of breast cancer cells. Infection of this CRCA (designated Ad.PEG-E1A-*mda-7*) in normal mammary epithelial cells and breast cancer cells confirmed cancer-cell-selective adenoviral replication, *mda-7*/IL-24 expression, growth inhibition, and apoptosis induction. Injecting Ad.PEG-E1A-*mda-7* into human breast cancer xenografts established on both sides of athymic nude mice completely eradicated not only the primary injected tumor on one flank but also distant tumors (established on the opposite flank of the animal) thereby implementing a cure. In contrast, non-replicating viruses expressing *mda-7*/IL-24 or CRCAs not expressing *mda-7*/IL-24 displayed some antitumor activity, but this effect was greatly diminished in comparison with Ad.PEG-E1A-*mda-7* (Sarkar et al., 2005). This dual cancer-specific targeting strategy provides an effective approach for treating breast and other human neoplasms with potential for eradicating both primary tumors and metastatic disease. Zhao et al. (2005) constructed a CRCA using the ZD55 vector, which contains a deletion of the adenoviral E1B 55-kDa gene, to regulate replication in cancer cells with p53 dysfunction, to deliver *mda-7*/IL-24 (ZD55-IL-24). Infection of normal cells did not induce a cytolytic effect, although MDA-7/IL-24 protein was detected, indicating potential leakiness of this vector relative to targeting genes only in cancer cells. However, even in the presence of MDA-7/IL-24, no toxicity was evident in normal lung fibroblast cells, further supporting the cancer-specific activity of this novel cytokine. Infection of human colorectal cancer cells with ZD55-IL-24 resulted in activation of caspases 3 and 9, induction of bax, and apoptosis. Moreover, infection of established SW620 colorectal tumors with ZD55-IL-24 showed a much stronger antitumor

activity than observed with ONYX-015 (a virus preferentially replicating in cells with defective p53) or Ad-IL-24 (a non-replicating virus expressing *mda-7/IL-24*, similar to Ad.*mda-7*). These studies by Sarkar et al. (2005) and Zhao et al. (2005) demonstrate enhanced antitumor activity in vivo in nude mice when incorporating *mda-7/IL-24* into a replicating oncolytic adenovirus as opposed to simply administering this cytokine in the context of a non-replicating adenovirus. These types of vectors hold significant promise for augmenting the therapeutic potential of *mda-7/IL-24*.

8. Role of PKR in Ad.*mda-7*-induced cancer-specific growth inhibition and apoptosis induction

Pataer et al. (2002) provided evidence linking the tumor suppressor activity of overexpressed Ad.*mda-7* to up-regulation of the interferon-induced serine/threonine protein kinase (PKR) in a p53-independent manner in the context of NSCLC cells. Double-stranded RNA-dependent protein kinase PKR appears to mediate anti-tumorigenic activity through activation of specific biochemical pathways resulting in growth inhibition and apoptosis. Activation occurs due to signals leading to auto-phosphorylation. Once activated, PKR phosphorylates various targets, which play a crucial role in growth control and apoptosis induction, such as eIF-2 α , Stat1, Stat3, and p38 mitogen-activated protein kinase (MAPK) (Pataer et al., 2002). Inhibition of PKR with the inhibitor 2-aminopurine (2-AP) prevents Ad.*mda-7*-induced apoptosis, eIF-2 α phosphorylation, and inhibition of protein synthesis. In this context, PKR activation appears to be crucial for Ad.*mda-7* induction of apoptosis in lung cancer cells. Additionally, induction of programmed cell death by Ad.*mda-7* in mouse embryo fibroblasts (MEF) was dependent on an active PKR locus because MEFs from PKR^{-/-} animals were unable to undergo apoptosis, whereas wild-type PKR^{+/+} MEFs were sensitive to *mda-7/IL-24* (Pataer et al., 2002). This latter observation is puzzling because Ad.*mda-7* does not appear to induce apoptosis in normal rat cells, whereas it does induce this effect in transformed and tumor-derived rat cells. In their proposed model, *mda-7/IL-24* and PKR act upstream of caspases and the pro-apoptotic Bak gene, where *mda-7/IL-24* induces PKR up-regulation with subsequent activation of cellular pathways leading to caspase activation and apoptosis induction (Pataer et al., 2002). It should be noted that activation of the PKR pathway may not be a general method of apoptosis induction by *mda-7/IL-24* because a recent paper studying the bystander activity of secreted MDA-7/IL-24 indicated apoptosis induction in human melanoma cells by a PKR-independent death pathway (Chada et al., 2004a, 2004b).

In a recent report, Pataer et al. (2005) investigated potential interactions between MDA-7/IL-24 and PKR proteins in the context of human lung cancer cells. Infection of A549 and H1299 NSCLC cell lines with Ad.*mda-7* resulted in a dose- and time-dependent induction of PKR protein and apoptosis. RT-PCR failed to detect an increase in PKR mRNA following infection with Ad.*mda-7*, suggesting a post-transcriptional regulation of PKR by MDA-7/IL-24 protein. To determine if exogenously applied MDA-7/IL-24 could induce PKR or apoptosis, purified MDA-7 protein was administered extracellularly to the lung cancer cell lines. Under these experimental conditions, PKR was not induced and no apoptosis ensued. In contrast, treatment of A549 cells with a GST-MDA-7/IL-24 fusion protein (Sauane et al., 2004a), which internalizes in cells, induced growth suppression and apoptosis (Sauane & Fisher, unpublished data). These studies, combined with experiments using plasmid transfection approaches or an adenovirus to deliver *mda-7/IL-24* lacking a signal peptide, thereby preventing secretion from cells, provide further support for a novel mode of killing by *mda-7/IL-24* that involves intracellular action without the requirement for secretion from cancer cells (Sauane et al., 2004b; Sieger et al., 2004). Immunofluorescence and co-immunoprecipitation techniques suggest that MDA-7/IL-24 protein physically interacts with PKR (Pataer et al., 2005). Employing mouse embryo fibroblasts containing PKR (PKR^{+/+}) or lacking PKR (PKR^{-/-}) indicated phosphorylation of MDA-7/IL-24 and PKR proteins in the lysates of PKR^{+/+} but not

in PKR^{-/-} cells. These studies suggest that in certain cellular contexts, such as lung cancer cells, Ad.*mda-7* can induce PKR and MDA-7/IL-24 (on threonine and serine residues) phosphorylation and MDA-7/IL-24 can physically interact with PKR (Pataer et al., 2005). Further studies are required to determine if this induction of PKR by Ad.*mda-7* is restricted to non-small cell lung carcinoma cells, or if it can also occur in additional cancer cell models.

9. Role of p38 MAP kinase and growth arrest and DNA damage-inducible genes in melanoma differentiation associated gene-7/interleukin-24-induced apoptosis in cancer cells

p38 MAPK, which is induced in response to stress and during growth signaling, is known to play a crucial role in apoptosis (Xia et al., 1995; Juo et al., 1997; Kummer et al., 1997; Schwenger et al., 1997; Dent et al., 2003). Sarkar et al. (2002a, 2000b) examined the role of the p38 MAPK pathway in response to Ad.*mda-7*-mediated growth suppression and apoptosis induction in melanoma cells. Ad.*mda-7* infection induced expression of the growth arrest and DNA damage (GADD)-inducible gene family. GADD genes are stress-induced genes that are up-regulated in response to agents/conditions such as UV radiation, chemical carcinogens, starvation, oxidative stress, and TNF- α . The GADD gene family comprises 5 gene members, GADD34, GADD45 α , GADD45 β , GADD45 γ , and GADD153 (Zhan et al., 1994; Hollander et al., 1997, 2001; Connor et al., 2001). These GADD family members are believed to play a crucial role in transcriptional regulation and apoptosis. GADD153 acts by regulating the activity of the B-cell CLL/lymphoma 2 (Bcl-2) promoter (Ubeda et al., 1996, 1999). Overexpression of GADD genes promoted growth inhibition/apoptosis and combined expressions of GADD genes lead to synergistic or cooperative anti-proliferative effects. Activating the p38 MAPK pathway regulated induction of these GADD genes by Ad.*mda-7*. Blocking the p38 pathway using a specific inhibitor SB203580 suppressed the induction of the GADD genes and apoptosis (Fig. 6) (Sarkar et al., 2002b). Antisense inhibition of GADD genes also blocked induction of apoptosis and inhibition was greatest when the various antisense constructs were used in combination (Sarkar et al., 2002b). Apart from the GADD genes, p38 MAPK also acted on the downstream target heat shock protein (HSP27), which initiates apoptosis (Sarkar et al., 2002b).

Infection of lung cancer cells with Ad.*mda-7* results in phosphorylation of PKR and also its downstream targets, such as eIF-2 α , Tyk2, Stat1, Stat3, and p38 MAPK (Pataer et al., 2002). Phosphorylation of eIF-2 α activates the transcription factor ATF4, which activates GADD153 (Fawcett et al., 1999). In this context, there is a significant level of crosstalk between the PKR and the p38 MAPK pathway (Sarkar et al., 2002b). Further studies are needed to comprehend the relevance of this crosstalk and to identify upstream molecules regulating the PKR and the p38 MAPK pathway.

10. Role of β -catenin and the phosphoinositide 3-kinase signaling pathway in melanoma differentiation associated gene-7/interleukin-24-induced apoptosis in cancer cells

In breast and lung tumor cells, an inverse relationship between expression of β -catenin and the phosphoinositide 3-kinase (PI3K) signaling pathway was observed (Mhashilkar et al., 2003). β -Catenin and PI3K are involved in up-regulation of apoptotic and survival pathways as well as cell-cell adhesion and metastasis. β -Catenin is a downstream effector of the Wnt signaling pathway and binds to and activates the transcription factors in the T-cell-specific transcription factor/lymphoid enhancer binding factor (TCF/LEF) family leading to induction of TCF/LEF responsive genes (McCormick, 1999). Elevated levels of β -catenin have been observed in many

tumors, such as colon and gastric carcinomas and adenocarcinoma of the breast. β -Catenin/TCF-responsive genes play a pivotal role in cell cycle progression and loss of cell differentiation properties and some of these gene products, for example, cyclin D1, matrilysin, and *c-myc*, are elevated in mammary tumors and cell lines expressing activated β -catenin (McCormick, 1999). PI3K plays a crucial role in the regulation of signal transduction, cytoskeletal rearrangement, and membrane trafficking. The PI3K family members are known to play a role in development of human cancers, cell growth, differentiation, and survival (Berrie, 2001; Fry, 2001; Katso et al., 2001). Constitutive expression of the PI3K pathway is seen in many human tumors and it is believed to play a crucial role in increased tumor cell survival and resistance to chemotherapy and radiotherapy (Berrie, 2001; Fry, 2001; Katso et al., 2001). In these contexts, inhibition of the PI3K pathway by *Ad.mda-7* is proposed as a mechanism by which this gene could inhibit cancer cell proliferation.

Mhashilkar et al. (2003) investigated the effects of *Ad.mda-7* infection on β -catenin and PI3K signaling pathways in non-small cell lung carcinoma (NSCLC) cells and found that *Ad.mda-7* negatively regulates both the β -catenin and PI3K pathways by increasing steady-state levels of tumor-suppressive proteins, such as E-cadherin, adenomatous polyposis coli gene (APC), glycogen synthase kinase 3 beta (GSK-3 β), phosphatase and tensin homolog (PTEN), and decreasing expression of oncogenic proteins, such as PI3K, focal adhesion kinase (FAK), integrin-linked protein kinase 1 (ILK-1), phospholipase C gamma (PLC- γ), and protein kinase B (AKT), in breast and lung cancer cells. *Ad.mda-7* is proposed to function upstream of PLC γ and blocks the PLC- γ \rightarrow FAK \rightarrow PI3K \rightarrow AKT \rightarrow GSK3 pathway, although it is unclear whether regulation of downstream molecules is a direct or indirect action of *Ad.mda-7*. *Ad.mda-7* causes redistribution of β -catenin from the nucleus to the plasma membrane, reducing TCF/LCF transcriptional activity and increasing cell-cell adhesion. No direct binding of *mda-7/IL-24* to β -catenin was observed in the immunoprecipitation assays, suggesting that subcellular localization of *mda-7/IL-24* is different than β -catenin. *Ad.mda-7* is contained within the secretory granules, whereas β -catenin staining reveals its presence in both nucleus and cytoplasmic compartments. Further studies are necessary to determine the mechanism by which *mda-7/IL-24* modulates β -catenin/PI3K signaling pathways, how frequent these changes are in additional tumors, and what are the initiator signaling molecules that are activated. Moreover, it is essential to understand the role of protein-protein interactions to provide a better understanding of the molecules involved in regulating the β -catenin and PI3K signaling pathways.

11. Role of pro-apoptotic and anti-apoptotic proteins in *Ad.mda-7*-mediated killing of cancer cells: support for activation of the mitochondrial intrinsic pathway of apoptosis as a predominant mediator of apoptosis induction by melanoma differentiation associated gene-7/interleukin-24

The first observation that forced expression of *mda-7/IL-24* by means of a replication incompetent adenovirus (*Ad.mda-7*) could alter the ratio of apoptotic proteins in cancer cells resulting in apoptosis was provided by Su et al. (1998). *Ad.mda-7* induced apoptosis in human breast cancer cells with different p53 genotypes, indicating p53 independence in its mode of action as an antitumor gene in breast cancer. *Ad.mda-7* reduced colony formation in MCF-7 (wt p53), MDA-MB-157 (null p53), MDA-MB-231, MDA-MB-453, and T47D (mut p53) cells (Su et al., 1998). Programmed cell death reflects a balance between signaling events and molecules that either promote or inhibit apoptosis (Reed, 1995, 1997; Danial & Korsmeyer, 2004; Reed, 2004). Proteins such as Bcl-2, Bcl-X_L, McL-1, Bcl-W, and Ad-E1B protect cells from apoptosis whereas proteins such as Bax, Bad, Bak, and Bcl-X_s stimulate apoptosis in specific target cells (Reed, 1995, 1997; Danial & Korsmeyer, 2004). In human breast carcinoma cells, *Ad.mda-7* altered the ratio of specific pro- and anti-apoptotic proteins tipping the balance

from survival to death. Up-regulation of BAX protein after infection with Ad.*mda-7* was apparent in tumor cell lines containing wild-type or mutant p53 or that were devoid (null) of p53. Comparison of BAX to BCL-2 protein ratios revealed BAX/BCL-2 to be significantly higher in Ad.*mda-7*-infected breast cancer cells implicating BAX and a reduction in BCL-2 proteins as potential components in *mda-7*/IL-24-induced programmed cell death (Su et al., 1998). Ectopic over-expression of *bcl-2* or Ad E1B by transfection with expression vector constructs expressing these different gene products, in combination with Ad.*mda-7* infection, protected T47D and MCF-7 cells from inhibition of colony formation that normally results following infection with Ad.*mda-7*. These results provided initial evidence that *mda-7*/IL-24-induced growth suppression and apoptosis could be modified by anti-apoptotic proteins (Su et al., 1998). This protection from apoptosis is consistent with the prominent role played by *mda-7*/IL-24-induced up-regulation of BAX protein in the apoptosis-inducing mechanism employed by this gene. Moreover, although *bax* is considered a p53-dependent downstream gene, the ability of *mda-7*/IL-24 to up-regulate BAX protein independent of p53 suggests that alternative pathways are involved in BAX up-regulation after ectopic expression of this cancer-specific apoptosis-inducing gene in specific cancer cells. Further studies have confirmed that infection with Ad.*mda-7* results in a significant reduction in the levels of specific anti-apoptotic proteins in a cancer cell-type-specific context. A 3- to 9-fold reduction in the levels of BCL-X_L protein was evident in HO-1, FO-1, MeWo, and WM35 cells, whereas a 3-fold reduction in the levels of BCL-2 protein was observed in MeWo and FO-1 cells, suggesting that decreases in the levels of single or multiple anti-apoptotic proteins may be major determinants of induction of apoptosis in human melanoma cells following infection with Ad.*mda-7* (Lebedeva et al., 2002).

In human prostate cancer cells, Ad.*mda-7* infection induces apoptosis in LNCaP, DU-145, and PC-3 cells, whereas in HuPEC normal early passage human prostate epithelial cells, growth and survival are not affected (Lebedeva et al., 2003a). A recent report by Saito et al. (2005) supports this differential effect of Ad.*mda-7* in the context of cancer versus normal prostate epithelial cells. In these cell types, Ad.*mda-7* infection elevates the levels of BAX and/or BAK proteins while significantly reducing the levels of anti-apoptotic proteins, BCL-2 and BCL-X_L, again supporting the hypothesis that it is the change in the ratio of pro-apoptotic to anti-apoptotic proteins that may directly participate in *mda-7*/IL-24-induced apoptosis in prostate cancer cells (Lebedeva et al., 2003a). Stable overexpression of *bcl-2* and *bcl-X_L* differentially protect prostate cancer cells from Ad.*mda-7*-induced apoptosis (Lebedeva et al., 2003a). *bcl-X_L*, but not *bcl-2*, afforded protection from *mda-7*/IL-24-induced apoptosis in PC-3 and DU-145 cells, whereas in LNCaP cells *bcl-2*, but not *bcl-X_L*, protected these cells from *mda-7*/IL-24 (Lebedeva et al., 2003a). At present, it is not known why protection is not achieved in all the prostate carcinomas with a single functionally similar anti-apoptotic protein.

The complexity of potential mechanisms by which *mda-7*/IL-24 can selectively induce killing in specific cancer cell types is highlighted by a recent study by Gopalan et al. (2005). In a specific ovarian cancer cell line, MDAH 2774, but not in normal ovarian epithelial (NOE) cells, Ad.*mda-7* activated the Fas–Fas ligand (FasL) signaling pathway resulting in apoptosis. In this specific ovarian cancer cell line, Ad.*mda-7* induced activation of the transcription factors c-Jun and activating transcription factor 2 (ATF 2), which stimulated transcription of the death-inducer FasL and its cognate receptor Fas. This induction of FasL was associated with the activation of NF- κ B and Fas-associated factor 1, Fas-associated death domain, and caspase 8. A potential cause and effect relationship between these changes was suggested by the ability of siRNA inhibiting Fas to significantly decrease Ad.*mda-7*-mediated death in MDAH2774 cells. Similarly, blocking FasL with NOK-1 Fas ligand antibody inhibited Ad.*mda-7*-mediated killing of this cell line. Collectively, these studies indicate that in specific tumor contexts, Ad.*mda-7* can exploit the Fas-FasL signaling pathway to kill cancer cells. In additional ovarian cancer cells, a mitochondrial-mediated killing effect elicited by *mda-7*/IL-24 has been

demonstrated (Leath et al., 2004; I.V. Lebedeva, P. J. Mahasreshti, D.T. Curiel, & P.B. Fisher, unpublished data). Moreover, as reviewed recently by Lebedeva et al. (2005a), the studies by Gopalan et al. (2005) and Leath et al. (2004) provide additional examples of the ability of *mda-7/IL-24* to selectively induce apoptosis in histologically similar cancer cell types using different proapoptotic signaling mechanisms; that is, this novel cytokine finds and exploits specific weaknesses in cancer cells promoting their death.

12. Mitochondrial dysfunction promoted by melanoma differentiation associated gene-7/interleukin-24 selectively promotes cell death in prostate cancer cells

Up-regulation of pro-apoptotic and down-regulation of anti-apoptotic proteins suggest a role of mitochondria in the induction of apoptosis in response to Ad.*mda-7* infection in specific cancer cell types. Studies by Lebedeva et al. (2003c) provide insights into the relationship between mitochondrial function and cellular redox status in response to Ad.*mda-7* infection. Reactive oxygen species (ROS), including singlet oxygen, superoxide ions, hydroxide, and hydroxyl radicals, are known to regulate apoptosis and proliferation in response to various stimuli, including TNF- α , UV, and γ -radiation (Jacobson, 1996). A relationship between ROS induction by Ad.*mda-7* and apoptosis induction has now been established in prostate cancer cells (Lebedeva et al., 2003c). Antioxidants such as *N*-acetyl-L-cysteine (NAC) and Tiron, at non-cytotoxic doses, inhibited the killing effect of Ad.*mda-7* in DU-145, PC-3, and LNCaP cells, whereas addition of compounds such as As₂O₃ and NSC656240 (a dithiophene) that promote ROS production in combination with Ad.*mda-7* infection potentiated cell death in all 3 carcinoma cell lines, but not in normal P69 cells, suggesting that free radicals are involved in the process of killing by Ad.*mda-7* in prostate carcinoma cells (Lebedeva et al., 2003c). FACS analysis revealed a 3- to 5-fold increase in the levels of ROS in prostate carcinoma cells, but not in P69 cells. Because ROS is a modulator of mitochondrial membrane potential (ψ_M) (Zamzami et al., 1995; Kroemer & Reed, 2000), time course evaluations of mitochondrial changes with membrane apoptotic changes after Ad.*mda-7* infection were determined. It was established that the initial decrease in $\Delta\psi_m$ occurs before ROS production in Ad.*mda-7*-infected DU-145, LNCaP, and PC-3 carcinoma cells. The initial drop in $\Delta\psi_m$ occurs 6–7 hr followed by increased ROS production (10–20 hr) and the decline in $\Delta\psi_m$ continues up to 12 hr in LNCaP and up to 30 hr in DU-145 and PC-3 cells. At 45–50 hr, a secondary burst of ROS and concomitant final steep increase in $\Delta\psi_m$ are observed, indicating complete mitochondrial dysfunction (Lebedeva et al., 2003c). The decline in ψ_M and the increase in annexin V binding occurred, concomitantly suggesting that Ad.*mda-7*-mediated apoptosis correlates with changes in mitochondrial function. Further studies focused on mitochondrial potential transition (MPT). MPT is characterized by opening of mitochondrial mega channels to allow solutes and water to enter mitochondria. MPT is triggered by ROS and other agents resulting in a decrease of $\Delta\psi_m$ followed by depletion of ATP and activation of caspases/endonucleases (Jacobson, 1996). This process is controlled by a multiprotein complex found in the inner and outer membranes of mitochondria known as the permeability transition pore (PTP). Upon PTP opening, the mitochondria lose their $\Delta\psi_m$ across the inner membrane resulting in apoptosis along with shutdown of mitochondrial biosynthesis. Inhibitors of the PTP, such as cyclosporin A and bongkreikic acid that bind to different components of the PTP, prevented cell death and the decline in $\Delta\psi_m$, whereas pretreatment with the peripheral benzodiazepine receptor agonist (PK11195) potentiated the induction of MPT followed by apoptosis (Lebedeva et al., 2003c). These studies established the importance mitochondrial dysfunction and ROS production in Ad.*mda-7*-induced death in prostate carcinoma cells as overviewed in Fig. 7.

13. Secretion of melanoma differentiation associated gene-7/interleukin-24 is not mandatory for cancer-specific cell killing

The endoplasmic reticulum (ER) is a principal site for protein synthesis and folding, calcium signaling, and calcium storage (Berridge et al., 2000; Berridge, 2002). Alterations in calcium homeostasis and accumulation of misfolded protein in the ER cause “ER stress”. This ER stress response triggers specific signaling pathways including the unfolded protein response (UPR), the ER-overload response (EOR), and the ER-associated degradation (ERAD) pathway to enable cells to survive pro-apoptotic ER stress (Herr & Debatin, 2001). Prolonged activation of these pathways leads ultimately to apoptosis. After Ad.*mda-7* infection, the expressed protein was shown to localize in the ER/Golgi compartments by 2 independent studies, one utilizing an adenovirus vector expressing a non-secreted version of MDA-7/IL-24 protein generated via deletion of its signal peptide (Sauane et al., 2004b) and the other utilizing plasmid-based analyses (Sieger et al., 2004). Earlier findings from our group indicated that Ad.*mda-7*-induced GADD genes, classically associated with the stress response including ER stress pathways in human melanoma cells, but not in immortalized melanocytes (Sarkar et al., 2002b). This induction of GADD genes and further upstream events such as activation of p38 MAPK as well as downstream pathways such as HSP-27 was reproducibly induced in a transformed cell-specific manner after Ad.*mda-7* infection (Sarkar et al., 2002b). In addition, studies with the virus producing the non-secreted as well as secreted versions of *mda-7/IL-24*, only in the context of transformed cells, specifically activated the p44/42 MAPK pathway (Sauane et al., 2004b). Furthermore, Ad.*mda-7* infection produced an up-regulation in inositol 1,4,5-trisphosphate receptor (IP3R) in H1299 cells (Mhashilkar et al., 2003). IP3R is an intracellular calcium release channel implicated in apoptosis and localized in the ER. Activation of a series of heat-shock-related chaperones as well as various stress proteins [GADDs, protein phosphatase 2A (PP2A), X-box binding protein 1 (XBP-1), immunoglobulin binding protein (BiP), etc.] indicates that adenovirus infection with *mda-7/IL-24* induces ER stress and this might be the earliest contributor to the appearance of apoptosis in the different cancer cell lines after infection with Ad.*mda-7* (Sarkar et al., 2002b; Mhashilkar et al., 2003). Further investigation to determine the mechanism of specificity of MDA-7/IL-24-triggered ER stress is clearly needed to determine why cellular ER stress mechanisms are differentially activated in transformed cells by MDA-7/IL-24 and possibly other agents (Sauane et al., 2004b). From additional independent studies performed by our groups, we know that Ad.*mda-7* induces reactive oxygen species (ROS) in different cell lines (Lebedeva et al., 2003c, 2005b; Yacoub et al., 2003c). Nevertheless, it is not clear at the present time if the activation of mitochondrial-mediated events that trigger ROS and caspase-dependent and -independent pathways is temporally followed by ER stress, vice versa, or whether there is temporally coordinated co-stimulatory crosstalk between both pathways (Sauane et al., 2004a, 2004b).

Our observations are beginning to provide additional insights into the diverse pathways that are involved in selective *mda-7/IL-24*-induced apoptosis in tumor cells and identify ER- and mitochondrial-mediated events as important causative effectors of apoptosis by *mda-7/IL-24*. These experiments offer potential for developing ways of enhancing the clinical utility of this novel cancer-gene therapeutic for treating diverse human neoplasms.

14. Pancreatic cancer cells provide a unique model of melanoma differentiation associated gene-7/interleukin-24 action and highlight the potent “antitumor bystander” activity of this cytokine

Pancreatic cancer is an aggressive neoplastic disease where long-term survival of patients in which tumor spread has occurred outside the pancreas is only 4% (Perugini et al., 1998; Friess

et al., 1999; Hilgers & Kern, 1999; Lorenz et al., 2000; Rosenberg, 2000). In pancreatic cancer, multiple subsets of genes undergo genetic changes in a temporal manner resulting in specific oncogene activation and tumor suppressor gene inactivation during tumor progression (Perugini et al., 1998; Friess et al., 1999; Hilgers & Kern, 1999; Bardeesy & DePinho, 2002). Genes such as the Kirsten-*ras* (*K-ras*) oncogene (85–95%) are activated whereas inactivation of genes such as *p16/RB1* (>90%), *p53* (75%), and *DPC* (55%) have been reported (Perugini et al., 1998; Friess et al., 1999; Hilgers & Kern, 1999; Bardeesy & DePinho, 2002), reinforcing the complexity of this disease and providing a potential genetic basis underlying its aggressiveness and resistance to conventional therapies. Anti-sense (AS) targeting of *K-ras* using a plasmid or mutation-specific phosphorothioate oligodeoxynucleotide (PSODN) inhibits the growth of pancreatic cancer cells containing *K-ras* mutations, but not those having a wild-type *K-ras* gene, suggesting that a single approach of inhibiting this oncogene is not sufficient to eradicate pancreatic carcinoma cells (Su et al., 2001). From these observations, it is believed that mutations in *K-ras* change the physiology of the pancreatic cancer cell and similar biochemical changes may not be evident in pancreatic cancers containing a wild-type *K-ras* genotype.

Unlike virtually all other cancers studied to date, pancreatic carcinoma cells are inherently resistant to ectopic expression of *mda-7/IL-24* (Su et al., 2001; Lebedeva et al., 2005b, in press). Infection of human pancreatic tumor cells with 100 pfu/cell of Ad.*mda-7*, which promotes apoptosis and reduces colony formation in the vast majority of cancer cell types, does not significantly alter growth, inhibit colony formation, or induce apoptosis in this tumor model (Su et al., 2001; Lebedeva et al., 2005b, in press). In contrast, higher doses of *mda-7/IL-24* or vector modifications used to express *mda-7/IL-24* result in growth inhibition, a reduction in colony forming ability and/or apoptosis in several *K-ras* mutant pancreatic carcinoma cell lines (Chada et al., 2005; Lebedeva et al., 2005b, in press). This failure to respond to standard concentrations of *mda-7/IL-24* (effective in inducing apoptosis in virtually all other cancer cell types), combined with an understanding of potential changes induced by an activated *K-ras* gene, prompted us to propose that downstream signaling pathways may be altered in mutant *K-ras* pancreatic carcinoma cells rendering these cells resistant to *mda-7/IL-24*-induced growth suppression and apoptosis (Su et al., 2001). Studies were performed to directly test this hypothesis. Treatment of pancreatic tumor cells with AS *K-ras* PSODN or transfection with an AS *K-ras* expression plasmid and infection with Ad.*mda-7* had a profound synergistic growth inhibitory effect and decreased survival of MIA PaCa-2 cells, AsPc-1 and PANC-1 cells containing a mutant *K-ras* gene, but not in BxPC-3, which has a wild-type *K-ras* genotype (Fig. 8; data shown for MIA PaCa 2 cells) (Su et al., 2001). Suppression in tumor formation was also evident in athymic nude mice when MIA PaCa 2 cells were transfected with an AS *K-ras* plasmid and infected with Ad.*mda-7* prior to injection into animals (Su et al., 2001). This finding is worth commenting on because it provided the first definitive evidence for “antitumor bystander” activity of *mda-7/IL-24*. The combination of AS *K-ras* transfection plus infection with Ad.*mda-7* results in only ~8% of the cells receiving both agents; that is, ~3% to ~4% maximum delivery of the *K-ras* AS gene, ~100% delivery of *mda-7/IL-24* (by Ad.*mda-7*), and potentiation of transfection efficiency following adenovirus infection (hence ~8% transfection efficiency), yet tumor formation was completely inhibited when these combination-treated cells were injected into nude mice (Su et al., 2001). These observations are very provocative, highlighting an interesting and relevant phenotypic property of *mda-7/IL-24*; that is, an ability of this cytokine to promote potent “antitumor bystander” activity. Moreover, this novel combinatorial approach of inhibiting a dominant-acting oncogene and administering a cancer-specific tumor suppressor gene (such as *mda-7/IL-24*) (Gazdar & Minna, 2001; Su et al., 2001; Lebedeva et al., 2005b, in press) provides a rationale for developing a potentially effective therapy for this aggressive and invariably fatal cancer.

Infection of pancreatic cancer cells, containing both mutated and wild-type *K-ras*, results in high levels of *mda-7/IL-24* mRNA, but little if any of this mRNA is translated into protein (Su et al., 2001; Lebedeva et al., 2005b, in press). However, when expression of mutant *K-ras* is ablated, using an AS-based strategy (either AS PSODN or an AS *K-ras* expression vector) or by using a bipartite adenovirus (expressing *mda-7/IL-24* and AS *K-ras*, Ad.m7/KAS), this “mRNA translational block” is reversed, large amounts of MDA-7/IL-24 protein are produced, and mutant pancreatic cancer cells undergo apoptosis. Although the mechanism involved in this altered translation is not currently known, it may result from inhibition of *K-ras* signaling through MAPK because studies by Rajasekhar et al. (2003) indicate that inhibiting this pathway facilitates the translation of specific mRNAs into protein. This occurs by enhancing the association of defined mRNAs with polysomes, thereby promoting their translation into protein. We have tested this hypothesis and shown that when mutant *K-ras* expression is extinguished in pancreatic carcinoma cells infected with Ad.*mda-7*, there is an increase in *mda-7/IL-24* mRNA associated with polysomes (Lebedeva et al., in press). Additionally, we have now demonstrated that combining *mda-7/IL-24* with inhibition of the *K-ras*-activated extracellular-regulated kinase 1/2 (ERK1/2) also results in reversal of the “translational block” culminating in MDA-7/IL-24 protein and apoptosis (Lebedeva et al., in press).

Recent studies highlight an additional means of abrogating the *mda-7/IL-24* “mRNA translational block” in pancreatic cancer cells (Lebedeva et al., 2005b). Treatment of both mutant and wild-type *K-ras* pancreatic carcinoma cells with compounds that induce reactive oxygen species (ROS), including arsenic trioxide, *N*-(4-hydroxyphenyl) retinamide, or dithiophene (NSC656240), results in the production of MDA-7/IL-24 protein and induction of apoptosis (Lebedeva et al., 2005b). Confirmation of a role for ROS induction in this process was provided by studies employing 2 ROS inhibitors, *N*-acetyl-L-cysteine and Tiron, which prevented a reversal of the “mRNA translational block,” production of MDA-7/IL-24 protein, and induction of apoptosis when pancreatic carcinoma cells were subsequently treated with a ROS inducer and infected with Ad.*mda-7*. These effects were not associated with a reduction in the levels of *K-ras* protein in these cells, which supports a novel mode of action of this combinatorial approach. Because this dual treatment did not induce apoptosis in normal cells, these findings provide support for potentially using a ROS inducer with Ad.*mda-7* as a treatment modality for pancreatic cancer (Lebedeva et al., 2005b).

15. Further insights into the mechanism underlying the potent “antitumor bystander” activity of melanoma differentiation associated gene-7/interleukin-24

A limitation frequently encountered using tumor suppressor gene replacement therapy is an inability to transduce a significant portion of tumor cells with a bioactive suppressor gene (Lebedeva et al., 2003a; Su et al., 2005b). This impediment profoundly limits the effectiveness of this strategy of tumor suppressor gene replacement as a therapy for cancer. An approach for ameliorating this situation would be to exploit a tumor suppressor gene that not only affects cells that directly receive this genetic agent, but also exerts activity on tumor cells at a distance (Fisher et al., 2003; Fisher, 2005; Lebedeva et al., 2005a). As discussed above, the potential for *mda-7/IL-24* to induce a potent “antitumor bystander” effect was first uncovered in the context of pancreatic cancer (Su et al., 2001). This effect has now been substantiated in a Phase I clinical trial involving intratumor injections into advanced carcinomas and melanomas (Fisher et al., 2003; Cunningham et al., 2005; Fisher, 2005; Lebedeva et al., 2005a) and the mechanism of this “antitumor bystander” activity in vitro has been investigated in 2 recent studies (Chada et al., 2004a; Su et al., 2005c). Chada et al. (2004a) determined the effect of secreted glycosylated, tumor-cell-produced MDA-7/IL-24 protein (re-leaked by a stable 293 cell clone expressing a transfected *mda-7/IL-24* gene) on human melanoma cells. This form of MDA-7/

IL-24 protein produced a dose-dependent induction of programmed cell death in human melanoma cells. Moreover, the apoptosis-inducing effect of secreted glycosylated tumor-derived MDA-7/IL-24 protein on melanoma cells was dependent on the presence of functional IL-20/IL-22 receptors in these cells. These studies also indicated that glycosylated MDA-7/IL-24 protein induced phosphorylation and nuclear translocation of STAT3 in melanoma cells and resulted in up-regulation of BAX protein and subsequent apoptosis. In contrast, additional IL-10 family members, including IL-10, -9, -20, and -22, which also activate STAT3, did not promote programmed cell death in melanoma cells. Additionally, in the context of normal cells, MDA-7/IL-24 was found to bind to its cognate (IL-20/IL-22) receptors and induce phosphorylation of STAT3 without initiating apoptosis. Experiments to define the role of PKR in this process indicated a lack of dependence on this signaling pathway in melanoma cells for MDA-7/IL-24 to induce apoptosis. These experiments provide further insight into MDA-7/IL-24 “antitumor bystander” activity and suggest that, at least in human melanoma cells, this process occurs by a receptor-mediated process and by pathways that are STAT3 and PKR independent.

In patients, *mda-7/IL-24* is currently administered by intratumoral injections using a conventional type 5 adenovirus vector that is replication incompetent, *Ad.mda-7* (INGN 241) (Fisher et al., 2003; Cunningham et al., 2005; Fisher, 2005; Lebedeva et al., 2005a; Tong et al., 2005). In this adenovirus, the cytomegalovirus promoter controls *mda-7/IL-24* gene expression. This will result after *Ad.mda-7* infection of both normal and tumor cells with secretion of MDA-7/IL-24 protein, which would be predicted to be self-limiting in cancer cells (because they will undergo apoptosis) but continuous in normal cells [as long as the *Ad.mda-7* (INGN 241) persists]. Su et al. (2005c) used several experimental protocols to investigate the role of MDA-7/IL-24 protein secreted by normal and cancer cells in the “antitumor bystander” activity of this novel cytokine. (1) Agar diffusion overlay assays were employed to define the effect of infecting normal cells with *Ad.mda-7* on the anchorage-independent growth of tumor cells. (2) Matrigel invasion assays were used to determine the ability of *Ad.mda-7* infection of normal cells to impact on the invasiveness of co-cultivated tumor cells. (3) The effect of co-cultivation of normal–cancer and cancer–cancer cells on cell survival following infection of one of the co-cultivating pair with *Ad.mda-7* was used to determine the role of normal versus tumor cell secreted MDA-7/IL-24 on tumor cell survival. Human cervical cancer (HeLa) cells, engineered to produce green fluorescence protein (GFP), facilitated these assays permitting the fate, that is, induction of apoptosis, of these tumor cells to be monitored by FACS analyses. (4) The effect of secreted MDA-7/IL-24 on agar (anchorage-independent) growth of *mda-7/IL-24*- and radiation-sensitive and -resistant prostate cancer cells in the presence or absence of radiation were evaluated using the agar diffusion overlay assay. These studies, in combination with assays designed to determine mRNA levels of the IL-20R1, IL-20R2, and IL-22R1 receptor subunits and STAT3 activation, confirm a role for functional IL-20/IL-22 receptor complexes in mediating the various “antitumor bystander” effects of MDA-7/IL-24 (Su et al., 2005c). Additionally, these experiments document and confirm several relevant aspects of the “antitumor bystander” effect of MDA-7/IL-24, including the following: (1) demonstrating a self-limiting role of MDA-7/IL-24 produced by cancer cells (which undergo apoptosis) and a more protracted role of MDA-7/IL-24 produced by normal cells (which do not undergo apoptosis) in inducing apoptosis in co-cultivation experiments; (2) an ability of the combination of secreted MDA-7/IL-24 and radiation to promote antitumor activity not only in *mda-7/IL-24*- and radiation-sensitive cancer cells, but also in prostate tumor cells overexpressing the anti-apoptotic proteins, *bcl-2* or *bcl-X_L* (Lebedeva et al., 2003a), and displaying resistance to either agent alone; and (3) an ability to use a cell-type-specific promoter, in our studies the excitatory amino acid transporter 2 (EAAT2) promoter (Su et al., 2003b; Rothstein et al., 2005; Sitcheran et al., 2005), to target expression of *mda-7/IL-24* in astrocytes resulting in the secretion of MDA-7/IL-24 that affects agar growth and sensitivity to radiation of malignant human glioma cells (Su

et al., 2005c). These innovative studies support a novel approach for using *mda-7/IL-24*, by targeting expression in normal target cells, to produce a constant supply of MDA-7/IL-24 protein in a local organ environment, as well as systemically, to enhance the therapeutic applications of this novel cytokine not only in the context of organ-defined disease, but also for treating metastases.

16. Melanoma differentiation associated gene-7/interleukin-24 inhibits invasion and migration of cancer cells

Tumor development and metastasis are complex processes mediated by changes in cancer cell physiology and biochemistry that frequently occur in a temporal manner during the process of tumor progression (Fisher, 1984; Fidler, 2002; Fidler et al., 2002; Onn & Fidler, 2002). Key components of tumor progression that contribute to the metastatic phenotype are tumor cell invasion and migration (Fidler, 2002; Fidler et al., 2002; Onn & Fidler, 2002). The ability of *mda-7/IL-24* to affect tumor cell invasion has been evaluated in the context of direct viral administration of this cytokine gene to tumor cells (Ramesh et al., 2004; Sauane et al., 2004b) and in experiments analyzing the putative “antitumor bystander” role of MDA-7/IL-24 secreted by normal cells (Su et al., 2005c). In the case of C8161 metastatic human melanoma cells, *mda-7/IL-24* administered by adenovirus (*Ad.mda-7*) inhibited invasion through Matrigel without altering cell viability (Fig. 9) (Sauane et al., 2004b). This effect did not require secretion of *mda-7/IL-24* because administering *mda-7/IL-24* by means of an adenovirus expressing a signal peptide-deleted gene construct (*Ad.SP-mda-7*) was as effective as the native gene product in blocking invasion of C8161 cells (Fig. 10) (Sauane et al., 2004b). In the case of human A549 (non-small cell lung carcinoma) and H1299 (large cell lung carcinoma) cell lines, infection with *Ad.mda-7* inhibited invasion and migration prior to inducing cell death (Ramesh et al., 2004). This anti-invasion and anti-migration effect was comparable in these 2 cancer cell lines to that observed when these cells were treated with the phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 or with a matrix metalloproteinase II inhibitor. Analysis of gene expression changes indicated down-regulation of specific genes associated with invasion by *Ad.mda-7*, including PI3K/protein kinase B, focal adhesion kinase, and matrix metalloproteinase-2 (MMP-2) and metalloproteinase-9 (MMP-9). Decreased MMP-2 and MMP-9 expressions following *Ad.mda-7* infection of A549 and H1299 cells were confirmed by zymography and Western blot analyses and a decrease in metastasis was observed following injection of A549 cells infected with *Ad.mda-7* into nude mice (Ramesh et al., 2004).

Studies performed to evaluate the “antitumor bystander” effect of MDA-7/IL-24 secreted by normal cells indicated that functional IL-20/IL-22 receptors were necessary for this cytokine to inhibit tumor cell invasion (Su et al., 2005c). Co-cultivation of immortal normal P69 prostate epithelial cells infected with *Ad.mda-7* with DU-145 (prostate carcinoma cells) or BxPC-3 (pancreatic carcinoma cells), which have a full-repertoire of IL-20/IL-22 receptors on their surface, but not A549 cells, which lack complete receptors for MDA-7/IL-24, inhibited invasion through Matrigel. These results argue that the ability of *mda-7/IL-24* to affect distant tumor cells, by means of its “antitumor bystander” effect, will be dependent on the presence of canonical receptors for MDA-7/IL-24 on secondary tumor cells. This raises the question as to whether the cytokine antitumor effects of MDA-7/IL-24 will be diminished when using this gene in the context of specific patient cancers, should their primary or secondary tumor cells lack appropriate receptors for MDA-7/IL-24. This will most likely not be the case because the vast majority of cancer cells, as well as normal cells, contain functional IL-22/IL-24 receptors (Sauane et al., 2003a; Chada et al., 2004a, 2004b; Su et al., 2005c). Moreover, the ability of *mda-7/IL-24* when administered directly to cancer cells by virus and the use of a GST-MDA-7 fusion protein can still induce tumor-specific cell killing by an intracellular mechanism (Sauane et al., 2004a, 2004b).

17. Melanoma differentiation associated gene-7/interleukin-24 enhances the sensitivity of cancer cells to radiation, chemotherapy and monoclonal antibody therapies

Infection of human lung cancer cell lines, including A549 (wild-type p53 and wild-type RB1) and H1299 (deleted p53 and wild-type RB1), with Ad.*mda-7* increases their sensitivity to radiation (Kawabe et al., 2002). In contrast, a similar effect of radiation was not observed in normal lung fibroblast cell lines, CCD-16 and MRC-9 (Kawabe et al., 2002). The combination of Ad.*mda-7* and radiation resulted in ~ 38% TUNEL-positive cells, whereas radiation and Ad.*mda-7* infection alone resulted in ~ 10% and ~ 18% TUNEL-positive cells, respectively. These results confirm that Ad.*mda-7* increases the radiosensitivity of non-small cell lung and large cell lung carcinoma cells resulting in enhanced apoptosis (Kawabe et al., 2002). Radiation activates the c-jun N-terminal MAP kinase (JNK) pathway and the levels of c-jun and JNK proteins raising the possibility that Ad.*mda-7* mediates radiosensitivity and enhances apoptosis through activation of JNK kinase and subsequent activation of c-jun protein (Kawabe et al., 2002). This observation is seen predominantly in the context of radiosensitization studies because induction of JNK by *mda-7*/IL-24 is not readily observed in most cells in the absence of radiation. This enhancement of radiosensitization is independent of the p53, Fas, and BAX status in lung cancer cell lines (Kawabe et al., 2002).

Recent studies by our groups have revealed that treatment of malignant gliomas, both rat and human, with purified GST-MDA-7 fusion protein or infection with Ad.*mda-7* also sensitizes these tumor cells to radiation induced growth suppression and apoptosis (Su et al., 2003a; Yacoub et al., 2003b, 2003c, 2004; Sauane et al., 2004a; Dent et al., 2005). Ad.*mda-7* infection induced growth suppression and apoptosis in human malignant gliomas expressing both mutant and wild-type p53, and these effects correlated with enhanced expression of members of the GADD gene family (Su et al., 2003a). This effect differed from that observed using an adenovirus to deliver wild-type p53 (Ad.*wtp53*), in which biological (growth suppression and induction of apoptosis) and GADD gene family induction effects were restricted to malignant gliomas containing a mutant p53 gene. In the context of normal human primary fetal astrocytes, Ad.*mda-7* and Ad.*wtp53* were significantly less growth inhibitory than in malignant gliomas and no toxicity was apparent. When malignant gliomas (containing a wild-type or mutated p53 gene) were infected with Ad.*mda-7* or treated with a GST MDA-7/IL-24 fusion protein, GST-MDA-7, sensitivity to ionizing radiation's growth inhibitory and antisurvival effects correlated with increased expression of specific members of the GADD gene family. Based on the observation that heterogeneity of p53 expression is a common event in evolving gliomas, these findings suggest that in specific contexts *mda-7*/IL-24 may prove superior as a gene-based therapy for malignant gliomas, both with and without irradiation, than administration of wild-type p53.

Studies by Yacoub et al. (2003b, 2004) extended the findings of Su et al. (2003a), confirming that both Ad.*mda-7* and GST-MDA-7 reduce proliferation and decrease survival of human and rat malignant gliomas and these effects were increased in a greater than additive fashion. These cellular changes, which were not observed in cultures of non-transformed primary astrocytes, correlated with enhanced numbers of cells in the G₁/G₀ and G₂/M phases of the cell cycle, implying that Ad.*mda-7* radiosensitizes glioma cells in a cell-cycle-independent manner. Decreased growth correlated with increased necrosis and DNA degradation, suggesting that the combination of agents alters survival of malignant glioma cells by mechanisms involving both apoptosis and necrosis. A single infection with Ad.*mda-7* enhanced p38 and ERK 1/2 activity without altering JNK or Akt activity. When cells containing *mda-7*/IL-24 were irradiated, ERK 1/2 activity was suppressed while JNK 1/2 activity was enhanced, without altering either Akt or p38 activity. Moreover, abolishing JNK 1/2, but not p38, signaling

extinguished the radiosensitizing properties of MDA-7/IL-24. In contrast, inhibition of neither ERK 1/2 nor PI3K signaling enhanced the anti-proliferative effects of Ad.*mda-7*, whereas combined inhibition of both pathways enhanced cell killing, suggesting that ERK and PI3K signaling can be protective against MDA-7/IL-24 lethality in specific cell contexts.

Ad.*mda-7* as well as GST-MDA-7 synergizes with radiation-induced free radicals, which further reduces the expression of the anti-apoptotic protein BCL-X_L and enhances expression of BAX protein leading to enhanced radiosensitivity in vitro (Yacoub et al., 2003b). This effect, induction of BAX protein following combination treatment with MDA-7/IL-24 and radiation in malignant rat and human glioma cells, was not evident in NSCLC cell lines (Kawabe et al., 2002). Forced expression of Bcl-X_L (administered by adenovirus transduction) protected RT2 (rat malignant glioma) cells from growth suppression and loss of viability induced by the combination of Ad.*mda-7* and ionizing radiation (Yacoub et al., 2003c). *N*-acetyl-L-cysteine (NAC), an antioxidant, inhibited the anti-proliferative interaction between *mda-7/IL-24* and radiation in RT2 cells, suggesting that radiation-induced free radicals cooperates with changes induced by *mda-7/IL-24* to enhance mitochondrial dysfunction thereby contributing to apoptosis (Yacoub et al., 2003b, 2003c). Infection of RT2 cells with Ad.*mda-7* prior to intracranial injection into the brains of Fischer 344 rats increased survival following 6 Gy of ionizing radiation in comparison with animals receiving only radiation or animals infected with Ad.*vec* (lacking the *mda-7/IL-24* gene) or Ad.*mda-7* (which by itself enhanced survival, above Ad.*vec* or radiation treatment alone) (Yacoub et al., 2003c). Additional studies are required to understand the mechanism by which JNK and c-jun proteins are activated by Ad.*mda-7* or GST-MDA-7 and how this gene causes radiosensitization of malignant glioma cells thereby facilitating killing of these cancer cells both in vitro and in vivo.

To further evaluate the effects of GST-MDA-7 and ionizing radiation on malignant glioma cells, experiments were performed using primary (non-established) human glioblastoma multiforme (GBM) cells (Yacoub et al., 2004). For this study, a spectrum of primary GBM cells were chosen, including ones expressing mutated PTEN and p53 proteins, activated ERBB VIII, overexpressing wild-type ERBB1, or without receptor overexpression. GST-MDA-7 produced a dose-dependent decrease in proliferation of primary glioma cells, whereas viability was only decreased at high concentrations of this purified protein. Irradiation enhanced these effects in a greater than additive manner, which was also dependent on JNK 1/2/3 activation. As observed in RT2 cells, the enhancement of killing by radiation and GST-MDA-7 was blocked by NAC (a ROS scavenger), a JNK 1/2/3 inhibitor (SP600125), a pancaspase inhibitor (zVAD), and an inhibitor of caspase 9 (LEHD), but not by an inhibitor of caspase 8 (IETD). The combination of low concentrations of irradiation or GST-MDA-7 also decreased clonogenic survival of GBM cells, which was enhanced when both agents were employed together and blocked by inhibition of caspase 9 functions. In concordance with activation of the intrinsic caspase pathway, cell death correlated with reduced Bcl-X_L expression and with elevated levels of the pro-apoptotic proteins BAD and BAX. Inhibition of caspase 9 after combination treatment blunted neither JNK 1/2/3 activation nor the enhanced expression of BAD and BAX expression. These findings support an hypothesis that after combination treatment JNK 1/2/3 activation is a primary pro-apoptotic event and loss of BCL-X_L expression and ERK 1/2 activity are secondary caspase-dependent processes. These data also argue that GST-MDA-7 induces 2 overlapping pro-apoptotic pathways via ROS-dependent and -independent mechanisms. In total, these findings demonstrate that MDA-7/IL-24 reduces proliferation and enhances the radiosensitivity of non-established human GBM cell in vitro and that sensitization occurs independently of basal EGFR/ERK1/2/AKT activity or the functions of PTEN or p53.

Studies were performed to determine the effect of GST-MDA-7 alone and in combination with radiation on the growth and viability of human breast cancer cells (Sauane et al., 2004a). When

applied to MDA-MB-231 (mutant p53) breast carcinoma cells, GST-MDA-7 induced a dose-dependent decrease in viability as determined by MTT assays. Additionally, growth suppression by GST-MDA-7 was enhanced in a greater than additive fashion when combined with radiation. These studies were extended to include additional breast cancer and normal immortal breast epithelial (HBL-100) cells. Whereas no significant change in viability or growth was apparent in HBL-100 cells treated with GST-MDA-7, decreased growth and viability was observed in breast carcinoma cells that were independent of their p53 status; that is, in MCF-7 (wild-type p53), T47D (mutant p53), and MDA-MB-157 (null for p53) cells.

In human prostate cancer cells, a role for mitochondrial dysfunction and induction of reactive oxygen species in the apoptotic process has been documented. Ectopic overexpression of *bcl-X_L* and *bcl-2* prevents these changes including apoptosis induction in prostate tumor cells by Ad.*mda-7* (Lebedeva et al., 2002, 2003a, 2003c). Recent studies document that resistance to apoptosis can be reversed by treating *bcl-2* family overexpressing prostate tumor cells with ionizing radiation in combination with Ad.*mda-7* or purified GST-MDA-7 protein (Su et al., in press). Additionally, radiation augments apoptosis induction by *mda-7/IL-24* in parental neomycin-resistant prostate tumor cells. Radiosensitization to *mda-7/IL-24* is dependent on JNK signaling because treatment with the JNK 1/2/3 inhibitor SP600125 abolishes this effect. Because elevated expression of *bcl-X_L* and *bcl-2* are frequent events in prostate cancer development and progression, these studies support the use of ionizing radiation in combination with *mda-7/IL-24* as a means of augmenting the therapeutic benefit of this gene in prostate cancer, particularly in the context of tumors displaying resistance to radiation therapy due to *bcl-2* family member overexpression.

The studies described briefly above indicate that MDA-7/IL-24 promotes radiation sensitivity in a wide spectrum of human cancers, including NSCLC, malignant gliomas, breast carcinomas, and prostate carcinomas. This diversity of targets suggests that employing MDA-7/IL-24 with ionizing radiation may provide a means of enhancing the therapeutic benefit of this multifunctional cytokine. It would be of immense clinical importance to better understand the mechanism of *mda-7/IL-24*-induced radiosensitization to treat cancers where radiotherapy may only provide minimum benefit, whereas the combination of *mda-7/IL-24* and radiation may provoke a significant therapeutic response.

A recent study by Nishikawa et al. (2004) demonstrated that a combination of ionizing radiation and Ad.*mda-7* resulted in a substantial and long-lasting suppression of A549 NSCLC tumor growth in nude mice. This represents an interesting model because A549 cells do not contain a full complement of IL-20/IL-22 receptors (Chada et al., 2004a; Su et al., 2005c), making them resistant to “antitumor bystander” activity of secreted MDA-7/IL-24 (Chada et al., 2004a; Su et al., 2005c). Suppression of tumor growth by administering MDA-7/IL-24 by Ad.*mda-7* intratumorally into A549 xenograft tumors in animals in combination with ionizing radiation correlated with a reduction of angiogenic factors [basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF)] and microvessel density, which exceeded that observed with Ad.*mda-7* or radiation alone. Using soluble MDA-7/IL-24 (sMDA-7/IL-24) from 293 cells and in vitro assays, HUVECs were sensitized to radiation, whereas A549 cells and normal human lung fibroblasts were not affected. Similarly, infection of normal human cells with Ad.*mda-7* resulted in secretion of MDA-7/IL-24 that can inhibit tumor cell invasion and synergize with radiation in decreasing tumor cell growth in agar in cells that contain a complete set of functional IL-20/IL-22 receptors, but not in A549 tumor cells lacking appropriate receptors for MDA-7/IL-24 (Su et al., 2005c). These studies confirm that Ad.*mda-7* in combination with radiation can enhance apoptosis and sMDA-7/IL-24 can inhibit angiogenesis by sensitizing endothelial cells to ionizing radiation without affecting normal cells. These observations are interesting and provide further support for the use of a combination of MDA-7/IL-24 with radiation, which would in principle override resistance in

tumor cells that lack complete IL-20/IL-22 receptors by targeting their tumor vasculature and inhibiting angiogenesis.

Recent studies document that the non-steroidal anti-inflammatory drug sulindac can augment the antitumor activity of Ad.*mda-7* in the context of human non-small cell lung carcinoma cells (Oida et al., 2005). The combination of sulindac and Ad.*mda-7* promoted growth suppression and apoptosis in A549 and H1299 human lung cancer cells. This enhancement effect of Ad.*mda-7* was dose dependent for sulindac in the cancer cells, whereas no growth inhibitory or apoptotic effect was evident in normal human lung fibroblasts (CCD-16). The mechanism underlying this synergy was intriguing, in that sulindac increased expression of ectopic MDA-7/IL-24 protein in tumor cells, thereby elevating downstream targets of this cytokine in lung cells, including PKR, p38 MAPK, caspase-9, and caspase-3. Pulse-chase studies suggested that the increase in MDA-7/IL-24 protein in sulindac-treated cells was a consequence of elevated half-life of this protein. The combination of sulindac and Ad.*mda-7* also resulted in enhanced suppression in human lung tumor growth in nude mice as compared to a single treatment with either agent. This process also reflected an increased half-life of MDA-7/IL-24 protein. This study by Oida et al. (2005) supports the use of *mda-7/IL-24* with other agents, in this case the drug sulindac, to enhance its therapeutic activity.

McKenzie et al. (2004) investigated the effect of Ad.*mda-7* in combination with Herceptin (Trastuzumab), an anti-p185^{ErbB2} murine monoclonal antibody (4D5) that binds to the extracellular domain of ErbB2 and down-regulates expression of cell surface ErbB2 proteins, on Her-2/neu-overexpressing breast cancer cells. This combination treatment resulted in decreased levels of β -catenin, Akt, and phosphorylated Akt as compared with single treatment with Ad.*mda-7* or Herceptin. Additionally, in vivo studies in nude mice injected with MCF-7-Her-18 cells, in their thoracic mammary fat pads, indicated that the combination of Ad.*mda-7* plus Herceptin enhanced suppression in tumor growth of established Her-2/neu-overexpressing tumors to a greater extent than treatment with only Ad.*mda-7* or Herceptin. These studies suggest that a combination of Ad.*mda-7* plus Herceptin would be more efficacious for the therapy of Her-2/neu-overexpressing breast cancer than a single treatment modality. Although further studies are needed, this combinatorial effect could be a consequence of targeted inhibition of β -catenin and Akt pathways that are important in breast cancer cell growth.

18. Anti-angiogenic activity of melanoma differentiation associated gene-7/interleukin-24

A critical component of cancer development and tumor progression involves development of an adequate blood supply to insure survival of primary and secondary (metastatic) tumors (Folkman, 1996, 2002, 2003; Fidler et al., 2002). This process is dependent on the generation of new blood vessels, angiogenesis, which contributes to many pathological conditions (Folkman, 1996, 2001, 2002, 2003; Fidler et al., 2002). Key components of angiogenesis involve secreted factors, including primarily vascular endothelial growth factor (VEGF) as well as basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), and interleukin-8 (Fidler et al., 2002; Folkman, 2002, 2003). Defining ways of inhibiting this process, including approaches targeting the angiogenic factor itself or its receptors as well as the tumor vasculature, has now become a major focus for anticancer therapy, which has recently culminated in numerous clinical studies (Hegeman et al., 2004; Purow & Fine, 2004; Hoff, 2005). In this context, agents that can impact on the angiogenic process offer potential as significant therapeutic modalities for treating both primary and metastatic tumors.

Studies by Ramesh et al. (Saeki et al., 2002; Ramesh et al., 2003, 2004; Nishikawa et al., 2004) have demonstrated a unique aspect of *mda-7/IL-24* action, an ability to inhibit

angiogenesis. Initial studies in the context of human vascular endothelial cells (HUVECs) and A549 (NSCLC) and H1299 (large cell lung carcinoma) lung cancer cells demonstrated that *Ad.mda-7* had anti-angiogenic properties (Saeki et al., 2002). Infection of HUVEC cells, involved in new blood vessel formation, with *Ad.mda-7* inhibited endothelial tube formation, without affecting cell viability. When tested in in vivo animal models, containing injected A549 or H1299 cells, *Ad.mda-7* infection decreased tumor formation and this effect correlated with a decrease in CD31 expression, a marker of neoangiogenesis. These provocative studies supported the possibility that in addition to the ability of *Ad.mda-7* to induce apoptosis in tumor cells in animals, it also had the capacity to alter blood vessel formation, that is, it was an inhibitor of angiogenesis.

A more detailed follow-up study by Ramesh et al. (2003) confirmed that MDA-7/IL-24 secreted from a 293 cell clone transformed with a full-length *mda-7/IL-24* cDNA, sMDA-7/IL-24, could regulate angiogenesis and this effect was dependent on the IL-22 receptor (Ramesh et al., 2003). Application of sMDA-7/IL-24 to endothelial cells in vitro inhibited their differentiation into tubes and their migration when exposed to VEGF or bFGF. This inhibitory effect surpassed that of endostatin, gamma interferon and IP-10 (interferon-inducible protein 10). Interferon alpha and beta that exhibit strong anti-angiogenic activity (Fidler et al., 2002; Folkman, 2002; Pestka, 2003) were not compared with MDA-7/IL-24. This activity of MDA-7/IL-24 was shown to involve the IL-22 receptor because blocking antibody to IL-22 receptor combined with sMDA-7/IL-24 inhibited VEGF-induced angiogenesis as shown by reduced vascularization and hemoglobin content in an in vivo Matrigel plug assay. When A549 cells were mixed with 293 cells producing sMDA-7/IL-24, tumor growth in vivo in nude mice was inhibited. Similarly, systemic administration of sMDA-7/IL-24 also inhibited lung tumor growth in a mouse xenograft model. This reduction in tumor growth correlated with a decrease in tumor microvessel density and hemoglobin content, supporting the concept of anti-angiogenic activity of MDA-7/IL-24. In this context, one would anticipate that *mda-7/IL-24* may exert its antitumor properties in vivo by evoking multiple pathways, including direct cancer cell apoptosis, inhibition of angiogenesis, and as discussed previously modulation of immune responses (Fig. 10).

19. Phase I clinical studies with *Ad.mda-7* (INGN-241) indicate safety and clinical efficacy

mda-7/IL-24 has now reached a critical juncture relative to its evolution as a potential gene therapy for cancer. This novel cytokine, administered by a replication incompetent adenovirus (*Ad.mda-7*; INGN 241), has been injected intratumorally in patients with advanced carcinomas and melanomas (Fisher et al., 2003; Cunningham et al., 2005; Lebedeva et al., 2005a; Tong et al., 2005). These studies, which will be expanded on below, indicate that *mda-7/IL-24* is safe and provides evidence of clinically significant activity. Moreover, much of the responses observed in vitro and in animal tumor models have now been recapitulated in the context of patients. These early successes in patients suggest that *mda-7/IL-24* has considerable potential as an effective gene therapy for multiple cancers.

Preclinical animal modeling studies confirmed that *Ad.mda-7* had potent growth inhibiting and apoptosis-inducing properties in various tumor models (Su et al., 1998; Madireddi et al., 2000c; Gopalkrishnan, 2002; Ramesh et al., 2003), supporting its potential as a gene therapeutic for cancer. In comparison with existing anticancer drugs, *Ad.mda-7* has distinct advantages, including the following: (1) robust activity toward a spectrum of genetically diverse cancers; (2) a defined dose–response pharmacologic relationship; (3) no apparent toxicity toward a wide array of normal human or rat cells; (4) novel mechanism of action that exploits multiple defects in cancer cell physiology resulting in induction of programmed cell death; and (5) ability to generate a profound “antitumor bystander” effect (reviewed in Fisher et al., 2003; Fisher,

2005; Lebedeva et al., 2005a). Additionally, *mda-7/IL-24* is a potent inhibitor of angiogenesis, a profound stimulator of radiation sensitivity and an immune modulator, all of which may contribute further to its significant *in vivo* therapeutic properties.

To define safety and biologic activity of *mda-7/IL-24*, a Phase I clinical trial was conducted using intratumoral injections of Ad.*mda-7* (IL-24; INGN 241) in 28 patients with resectable solid tumors (Cunningham et al., 2005). In all cases, injected lesions demonstrated Ad.*mda-7* vector transduction, *mda-7/IL-24* mRNA, MDA-7/IL-24 protein, and apoptosis induction, with greatest concentration and activity near the injection site (Fig. 11 and data not shown) (Cunningham et al., 2005; Lebedeva et al., 2005a). Ad.*mda-7* (INGN 241) vector DNA and mRNA were readily detected more than 1 cm from the injection site (Fig. 11), whereas MDA-7 protein and bioactivity were more widely disseminated. Minimal and mild self-limiting toxicity, attributable to the injections, were apparent in most patients. Of relevance, evidence of clinical activity was apparent in 44% of lesions receiving repeated injections, including complete and partial responses in 2 melanoma patients. Thus, intratumoral administration of Ad.*mda-7* (INGN 241) is apparently well tolerated, induces programmed cell death (apoptosis) in a predominant percentage of tumor cells and provides clinically noteworthy activity (Fisher et al., 2003; Cunningham et al., 2005; Lebedeva et al., 2005a).

Additional results of a Phase I dose-escalation clinical trial using Ad.*mda-7* (INGN 241) in 22 patients with advanced cancer was presented by Tong et al. (2005). Tumors injected with Ad.*mda-7* (INGN 241) were excised and evaluated for vector-specific DNA and RNA, MDA-7/IL-24 expression and biologic effects. Effective gene transfer was demonstrated in 100% of patients evaluated using DNA- and RT-PCR. These studies confirmed a dose-dependent penetration of Ad.*mda-7* (INGN 241) with parallel dispersion of vector DNA and RNA, MDA-7/IL-24 protein, and apoptosis induction in all tumors, with signals diminishing from the initial injection site. Support for bioactivity of injected *mda-7/IL-24* was provided by documentation of elevated expression of putative MDA-7/IL-24 target genes, including β -catenin, iNOS, and CD31. Moreover, transient increases (up to 20-fold) were also apparent in the serum levels of IL-6, IL-10, and TNF- α . In the context of IL-6 and TNF- α induction, a direct relationship between the levels of these cytokines and clinical response to *mda-7/IL-24* was indicated. Patients injected with Ad.*mda-7* (INGN 241) also displayed significant increases in CD3⁺CD8⁺ T-cells, supporting the suggestion that this treatment increased systemic T_H1 cytokine production and activated CD8⁺ T-cells. These observations are consistent with preclinical features of MDA-7/IL-24 and support the potential immune modulatory as well as direct antitumor apoptosis properties of this cytokine in patients.

Taken together, the initial clinical studies using Ad.*mda-7* are exciting and provide optimism of the clinical utility of MDA-7/IL-24 for cancer therapy. However, very few if any therapeutic agents have been found to elicit a complete cancer cure. Only further studies will indicate if *mda-7/IL-24* is an exception to this rule. Of import, a number of studies have documented that combining *mda-7/IL-24* with other therapeutic modalities or agents can further augment its antitumor properties. This includes radiation (Kawabe et al., 2002; Su et al., 2003a; Yacoub et al., 2003b, 2003c, 2004; Nishikawa et al., 2004) and recent reports indicating cooperativity between *mda-7/IL-24* and monoclonal antibody therapy (McKenzie et al., 2004) and treatment with sulindac (Oida et al., 2005). Further studies are clearly necessary to determine if these and other agents will also result in enhanced therapeutic efficacy in patients, thereby improving the ability of *mda-7/IL-24* to eradicate diverse cancers. Additionally, employing cancer-selective conditionally replicating adenoviruses to deliver *mda-7/IL-24* has proven more active *in vivo* in animal models in inducing an antitumor response than using replication incompetent adenoviruses to deliver this cytokine (Sarkar et al., 2005; Zhao et al., 2005).

20. Concluding perspectives and future directions

In a short time frame, *mda-7/IL-24* has progressed from a laboratory discovery to a potential therapy for cancer (Fisher, 2005). In this context, this distinctive molecule has become the focus of increasing scientific scrutiny. This intensive analysis has provided significant new insights into the multitude of properties of this molecule, confirming its selective antitumor apoptosis-inducing ability in vitro and in vivo in human tumor xenograft nude mouse tumor models, demonstrating anti-angiogenic properties, elucidating key signal transduction pathways and molecules mediating activity in specific cancers (including p38 MAPK, PKR, GADD gene induction, changes in the ratio of pro-apoptotic to anti-apoptotic proteins), demonstrating a unique ability to radiosensitize tumor cells and finally documenting safety and potential clinical efficacy in patients with advanced carcinomas and melanomas (specific aspects of *mda-7/IL-24* have been reviewed in Sarkar et al., 2002a; Fisher et al., 2003; Sauane et al., 2003b; Chada et al., 2004b; Gopalkrishnan et al., 2004; Dent et al., 2005; Fisher, 2005; Lebedeva et al., 2005a). If anything, this rapid evolution in our knowledge has taught us to appreciate the complexity of action of *mda-7/IL-24*, culminating in the recent discovery that this gene can selectively kill cancer cells by a mechanism potentially independent of its cytokine properties, that is, by intracellular targeting and the stress response (Fig. 12). Although further studies are mandatory, it appears that *mda-7/IL-24* is able to find kinks in the armor of most tumor cells and exploit inherent weaknesses to destroy these cancer cells (Fisher et al., 2003; Lebedeva et al., 2005a). Additionally, it can attack the fundamental lifeline of the cancer, blood vessel formation (angiogenesis) (Folkman, 1996, 2001, 2002, 2003; Fidler et al., 2002), providing an additional means of thwarting tumor growth and progression. Adding further to its potential clinical utility, *mda-7/IL-24* is a potent sensitizer of cancer cells to radiation and chemotherapeutic agents (including specific drugs and monoclonal antibodies) (Kawabe et al., 2002; Lebedeva et al., 2003c, 2005b; Su et al., 2003a; Yacoub et al., 2003b, 2003c, 2004; McKenzie et al., 2004; Oida et al., 2005; Su et al., in press) and this secreted cytokine exerts potent “bystander” antitumor activity (increasing its range of action) (Su et al., 2001; Chada et al., 2004a; Su et al., 2005c). Finally, although not well understood, *mda-7/IL-24* appears to embody potent immune modulating properties (Caudell et al., 2002; Fisher et al., 2003; Tong et al., 2005), providing still another method to quell tumor cell growth and spread. In these contexts, if one hoped to design the ideal anticancer gene therapy, a strong candidate would be *mda-7/IL-24*, which can obstruct cancer on many levels and by attacking it in so many ways may provide a means of developing a long-term effective therapy for this pervasive and debilitating malady of mankind. We are optimistic that the future will provide additional insights into the mechanism of action of this intriguing cytokine and with this knowledge will come ways of further enhancing the utility and efficacy of *mda-7/IL-24* as a cancer gene therapeutic.

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Abbreviations

AKT, protein kinase B

APC, adenomatous polyposis coli gene
 Bcl-2, B-cell CLL/lymphoma 2
 bFGF, basic fibroblast growth factor
 BiP, immunoglobulin binding protein
 DISH, differentiation induction subtraction hybridization
 ER, endoplasmic reticulum
 Fak, focal adhesion kinase
 GADD genes, growth arrest and DNA damage inducible genes
 GSK-3 β , glycogen synthase kinase 3 beta
 IFN- β , fibroblast interferon
 IL, interleukin
 ILK-1, integrin-linked protein kinase 1
 JNK, c-jun N-terminal MAP kinase
 LPS, lipopolysaccharide
 MAPK, mitogen-activated protein kinase
 mda, melanoma differentiation associated genes
mda-7/IL-24, melanoma differentiation associated gene-7/interleukin-24
 MEZ, mezerein
 MPT, mitochondrial potential transition
 NAC, *N*-acetyl-L-cysteine
 NSCLC, non-small cell lung carcinoma
 PBMC, peripheral blood leukocytes
 PDGF, platelet-derived growth factor
 PI3K, phosphoinositide 3-kinase
 PLC- γ , phospholipase C gamma
 PP2A, protein phosphatase 2A
 PTEN, phosphatase and tensin homolog
 PTP, permeability transition pore
 RGP, radial growth phase primary melanoma
 ROS, reactive oxygen species
 TCF/LEF, T-cell-specific transcription factor/lymphoid enhancer binding factor
 Th1, Th2, helper T-lymphocytes, type 1 and type 2, respectively
 TNF- α , tumor necrosis factor alpha
 VEGF, vascular endothelial growth factor
 VGP, vertical growth phase primary melanoma
 XBP-1, X-box binding protein 1

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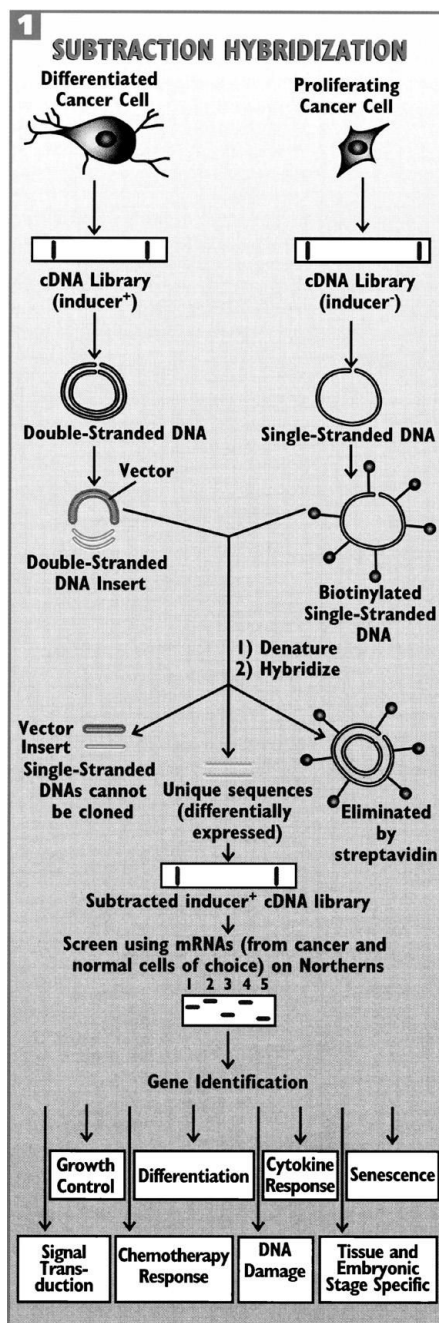


Fig. 1. Schematic of the differentiation induction subtraction hybridization (*DISH*) approach. This procedure has been used to identify and clone genes displaying differential expression as a function of induction of irreversible growth arrest, terminal differentiation, and loss of tumorigenic potential in HO-1 human melanoma cells. Temporally spaced libraries are constructed from actively growing HO-1 cells and from HO-1 cells treated with combination of IFN- β plus MEZ. The actively growing HO-1 cDNA library is then subtracted from the IFN- β plus MEZ-treated HO-1 library resulting in a subtracted cDNA library enriched for differentially expressed genes that associate with a multitude of processes, some of which are indicated in this figure. Further details of the subtraction approach and its application with

reverse Northern blotting of cDNAs and high throughput cDNA microarrays can be found in Jiang and Fisher (1993) and Huang et al. (1999a, 1999b) (reproduced, by permission of the publisher, from Fisher et al., 2003).

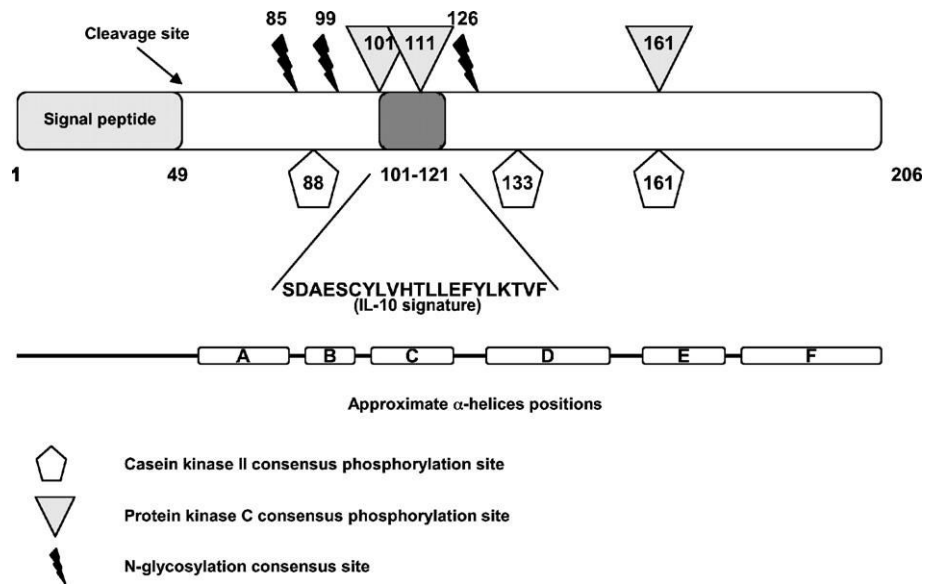


Fig. 2. Schematic representation of the MDA-7/IL-24 polypeptide showing various predicted and established protein motifs (reproduced in modified form, by permission of the publisher, from Sauane et al., 2003b).

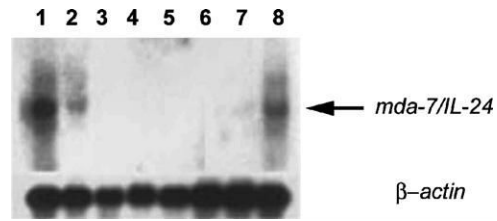


Fig. 3. Expression of *mda-7/IL-24* message in the human immune system. Human multiple tissue Northern blot consisting of poly(A)⁺ mRNA from different tissues shows tissue-specific expression of *mda-7/IL-24*. The mRNAs immobilized on the blot are from spleen (1), thymus (2), prostate (3), testis (4), ovary (5), small intestine (6), colon (7), and peripheral blood leukocytes (8) (reproduced, by permission of the publisher, from Huang et al., 2001).

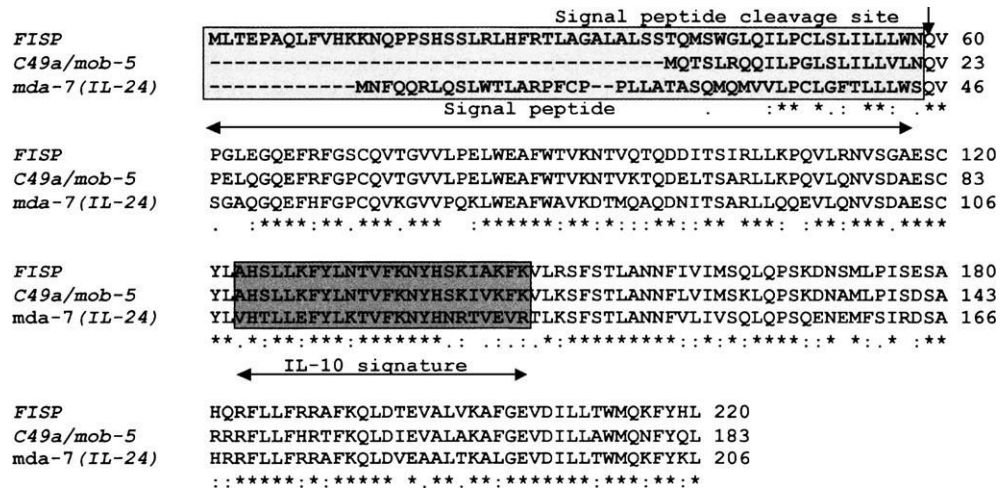


Fig. 4.

MDA-7/IL-24-related molecules: alignment of mouse (FISP), rat (c49a/MOB-5), and human protein sequences showing identical (*) conserved (:) and similar (.) amino acid residues (reproduced, by permission of the publisher, from Sauane et al., 2003b).



Fig. 5. Reduction in *mda-7/IL-24* mRNA expression as a consequence of human melanoma progression. Quantitative RT-PCR-based analysis of *mda-7/IL-24* mRNA expression versus GAPDH expression in independent normal melanocytes, primary melanoma, radial or early vertical growth phase (RGP and VGP, respectively), and metastatic melanoma cell cultures and patient-derived samples. Results are expressed as the ratio of *mda-7/IL-24* mRNA to GAPDH mRNA. These data indicate progressive reduction or complete loss of *mda-7/IL-24* expression in association with melanoma progression.

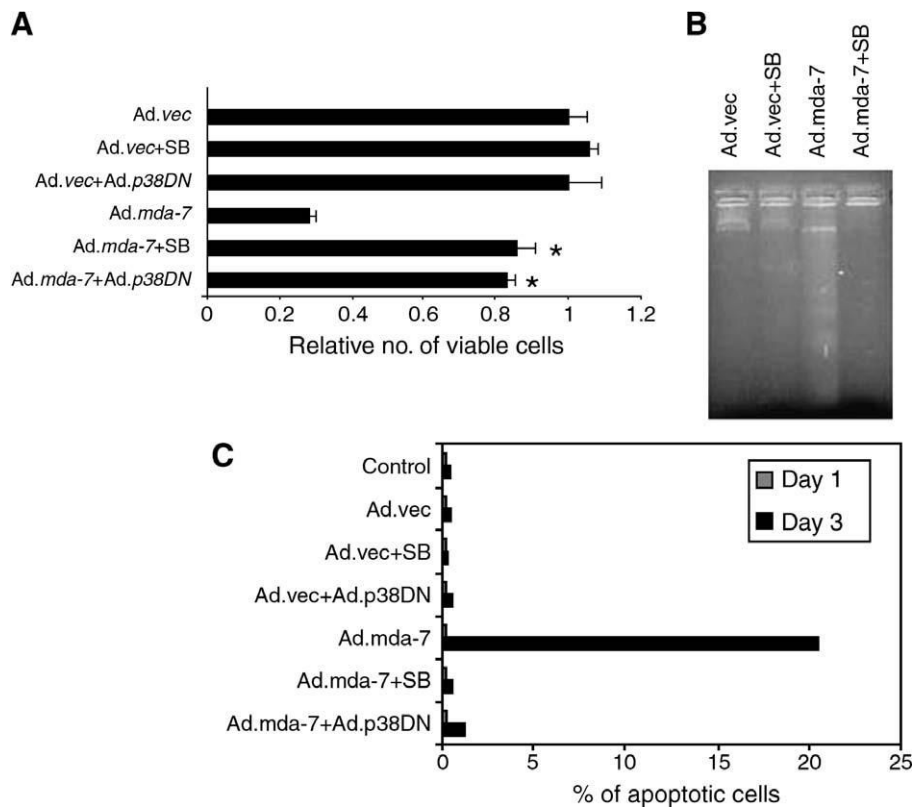


Fig. 6. Inhibition of the p38 MAPK pathway protects FO-1 melanoma cells from Ad.mda-7-mediated cell death. **(A)** FO-1 cells were infected with either Ad.vec or Ad.mda-7 (100 pfu/cell) and treated with 1 μ M SB203580 or infected with Ad.p38DN (an adenovirus expressing a dominant-negative p38 mutant gene) (100 pfu/cell). Cell viability was measured by MTT assay after 4 days. Cell viability of Ad.vec-treated cells was regarded as 1. *Significant differences from Ad.mda-7 ($P < 0.0001$). **(B)** FO-1 cells were infected with either Ad.vec or Ad.mda-7 (100 pfu/cell) and treated with 1 μ M SB203580 for 3 days. DNA was isolated from the cells and fragmentation was analyzed as described in Sarkar et al. (2002b). **(C)** FO-1 cells were infected with either Ad.vec or with Ad.mda-7 (100 pfu/cell) and treated with 1 μ M SB203580 or infected with Ad.p38DN (100 pfu/cell). Percentage of apoptotic cells at days 1 and 3 after infection in each group were plotted (reproduced, by permission of the publisher, from Sarkar et al., 2002b).

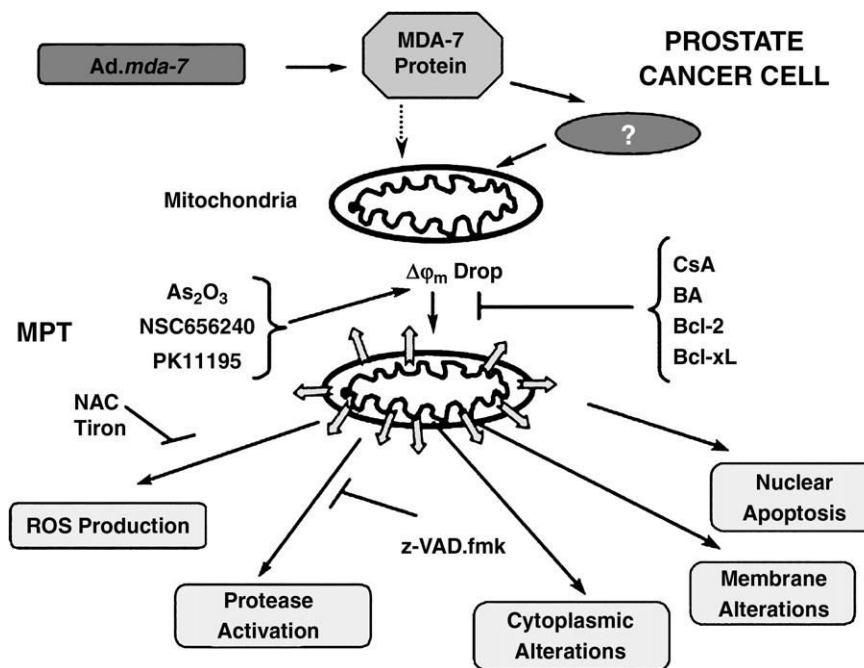
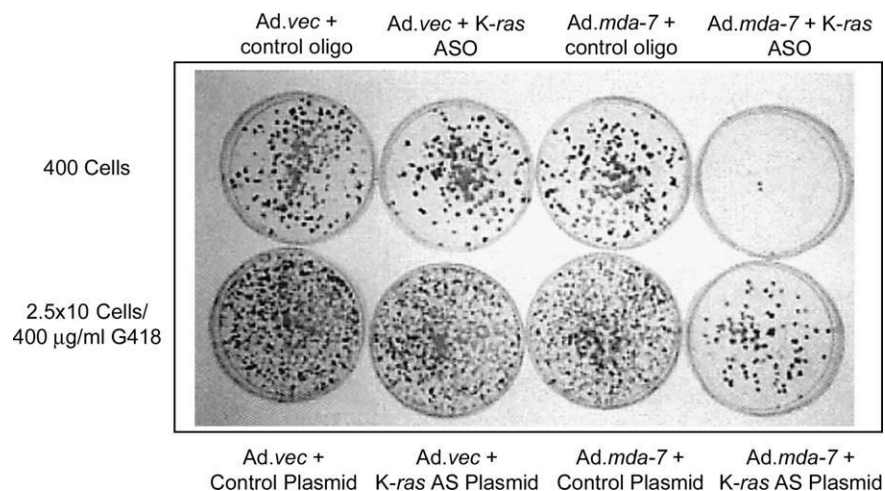
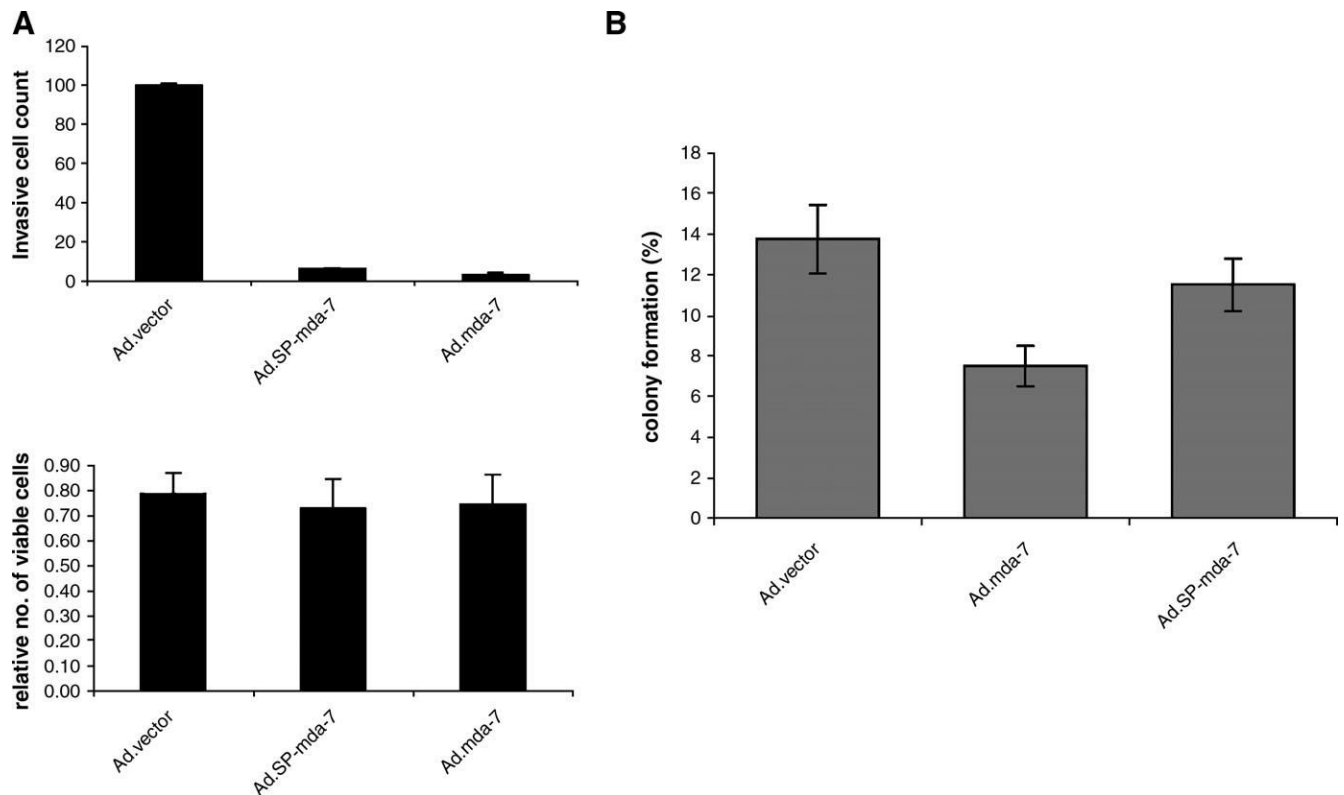


Fig. 7.

Proposed model for Ad.mda-7-induced apoptosis in prostate cancer cell lines. Following Ad.mda-7 infection, MDA-7 protein affects mitochondria directly or indirectly, causing alterations in mitochondrial function (decrease in $\Delta\psi_m$ and MPT) and ROS production. The reductions in $\Delta\psi_m$ and MPT are caspase-independent because they are not inhibited by the general caspase inhibitor z-VAD.fmk. Moreover, MPT can be blocked by inhibitors of MPT, such as CsA and BA, and can be promoted by activators of MPT, such as PK11195, a PBzR agonist. ROS inhibitors (NAC and Tiron) block Ad.mda-7-induced apoptosis, whereas ROS producers (As_2O_3 and NSC656240) enhance apoptosis only in the context of prostate cancer cells. Abbreviations: $\Delta\psi_m$, mitochondrial transmembrane potential; MPT, mitochondrial permeability transition; ROS, reactive oxygen species; z-VAD.fmk, *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone; CsA, cyclosporin A; BA, bongkreikic acid; PK11195, 1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinolinecarboxamide; PBzR, peripheral benzodiazepine receptors; As_2O_3 , arsenic trioxide; NSC656240, dithiophene (reproduced, by permission of the publisher, from Lebedeva et al., 2003c).

**Fig. 8.**

The combination of *Ad.mda-7* plus AS *K-ras* PS ODN or AS *K-ras* plasmids synergistically inhibits colony formation in mutant *K-ras* MIA PaCa-2 pancreatic carcinoma cells. (Upper) Effect of *Ad.mda-7* plus AS *K-ras* PS ODN on MIA PaCa-2 colony formation. Cells were infected with 100 pfu/cell of *Ad.vec* or *Ad.mda-7*, treated with 0.5 µM AS *K-ras* PS ODN plus 10 µl of Lipofectamine, reseeded at a density of 400 cells per plate, and fixed and stained with Giemsa after 3 weeks. (Lower) Effect of *Ad.mda-7* plus AS *K-ras* plasmid transfection on MIA PaCa-2 G418-resistant colony formation. Cells were infected with 100 pfu/cell of *Ad.vec* or *Ad.mda-7*, transfected with 10 µg of plasmid (either control pcDNA3.1 lacking insert or the pcDNA3.1 vector containing a 346-nt AS *K-ras* fragment), reseeded at a density of 2.5×10^5 cells per plate, and selected in 400 µg/mL G418, and G418-resistant colonies were fixed and stained with Giemsa after 3 weeks (reproduced, by permission of the publisher, from Su et al., 2001).

**Fig. 9.**

Comparative mechanism of *mda-7*/IL-24 action after infection of various cell lines with Ad.vec, Ad.*mda-7*, and Ad.SP-*mda-7* (adenovirus expressing an *mda-7*/IL-24 cDNA lacking the signal peptide). (A) *mda-7*/IL-24 inhibits C8161 Matrigel invasiveness without altering C8161 viability. C8161 cells were infected with 100 pfu/cell of Ad.vec, Ad.*mda-7*, or Ad.SP-*mda-7*. After 24 hr, 1×10^6 cells were allowed to invade for 48 hr through transwell inserts (8- μ m pores) coated with Matrigel. The cells that invaded through the Matrigel-coated inserts were stained, counted, and photographed under a light microscope at $\times 20$ magnification. Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay in parallel to ascertain whether the inhibition of invasion was associated with a decrease in growth or viability of infected C8161 cells, bars, \pm SD. Direct cell counts were performed on all surviving, attached cells in the lower chamber to quantitate the relative efficiency of invasiveness. (B) “Bystander” suppression of anchorage-independent growth of DU-145 cells after adenovirus infection of P69 cells. P69 cells were seeded at 2×10^5 /6-cm plate, infected 24 hr later with 25 pfu/cell of Ad.vec, Ad.*mda-7*, or Ad.SP-*mda-7* and overlaid with 1×10^5 DU-145 cells suspended in 0.4% agar. Fourteen days later, with agar medium feeding every 4 days, the number of anchorage-independent DU-145 colonies >2 mm was enumerated microscopically. Average number of colonies \pm SD from triplicate plates. Qualitatively similar results were obtained in 2 additional studies. pfu, plaque-forming unit; IL, interleukin (reproduced, by permission of the publisher, from Sauane et al., 2004b).

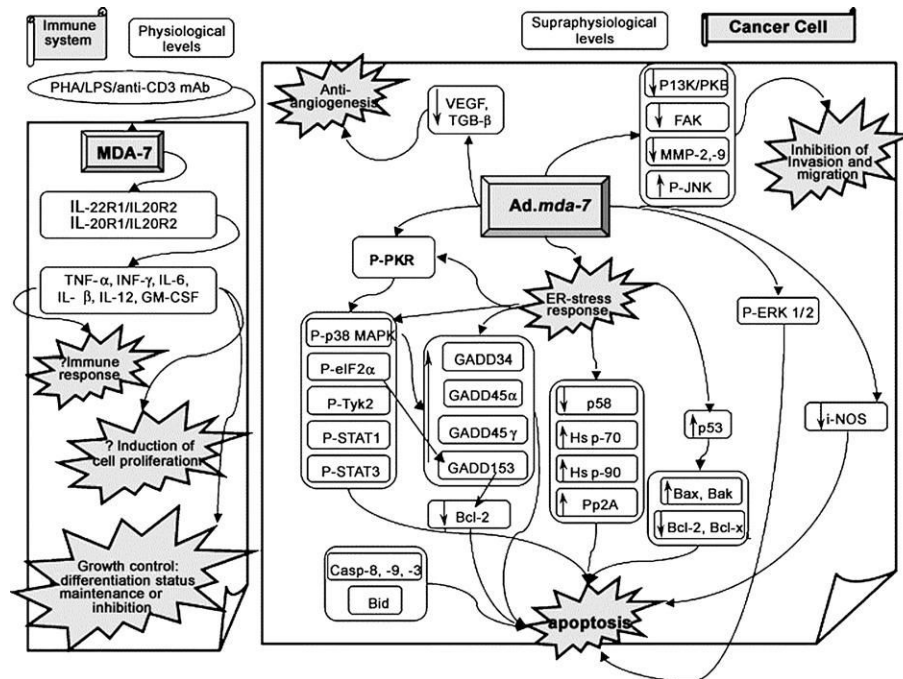
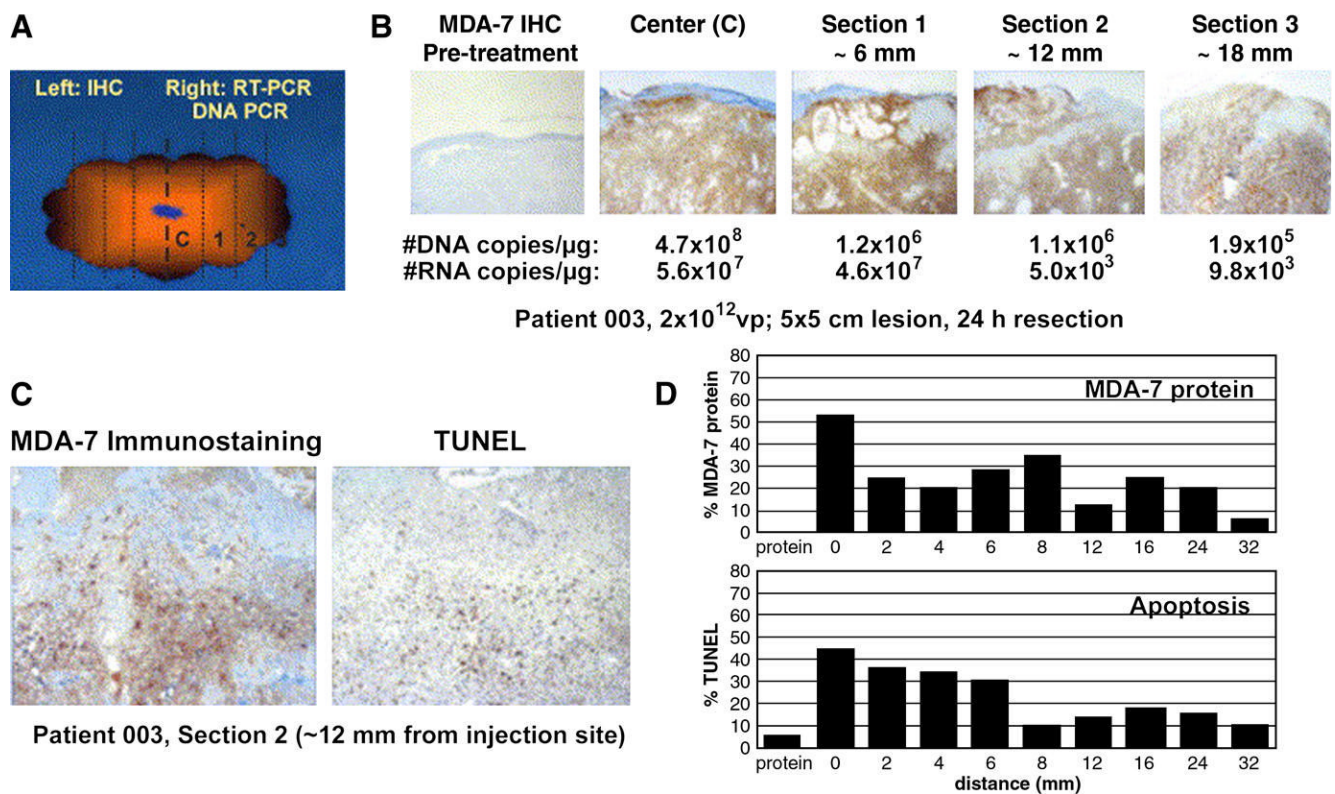


Fig. 10.

Overview of the signaling pathways associated with *Ad.mda-7* and MDA-7/IL-24 activity in cancer cells and in the immune system. Abbreviations: P, phosphorylation; PHA, phytohemagglutinin; LPS, lipopolysaccharide; IL, interleukin; TNF- α , tumor necrosis factor alpha; IFN- γ , interferon- γ ; GM-CSF, granulocyte macrophage-colony stimulating factor; VEGF, vascular endothelial growth factor; TGF- β , transforming growth factor- β ; PI3K/PKB, phosphatidylinositol 3-kinase/protein kinase B; FAK, focal adhesion kinase; MMP, matrix metalloproteinase; PKR, double-stranded RNA-dependent protein kinase R; MAPK, mitogen-activated protein kinase; eIF2 α , eukaryotic translation initiation factor-2 α ; Tyk2, tyrosine kinase-2; STAT, signal transducer and activator of transcription; GADD, growth-arrest and DNA-damage inducible; Hsp, heat shock protein; Pp2A, protein phosphatase-2A; iNOS, inducible nitric oxide synthase (reproduced, by permission of the publisher, from Lebedeva et al., 2005a).

**Fig. 11.**

Spread of *mda-7/IL-24* RNA, DNA, and protein and biological effects (apoptosis) 24 hr after intratumoral injection. **(A)** Schematic representation of serial sections of tumor. **(B)** Decay of INGN 241 (*Ad.mda-7*) vector at the injection site. Immunohistochemical staining of different tumor sections and the median numbers of DNA and RNA copies determined by PCR and RT-PCR, respectively, are shown for each section. **(C)** Spread of MDA-7/IL-24 protein and biological effect (apoptosis) at the injection site. Protein expression correlates with apoptosis. Serial sections from each tumor were evaluated for MDA-7/IL-24 expression and TUNEL reactivity using immunohistochemistry. **(D)** Data from TUNEL assay and immunohistochemistry are plotted to indicate signals compared to distance from injection site (reproduced, by permission of the publisher, from Lebedeva et al., 2005a).

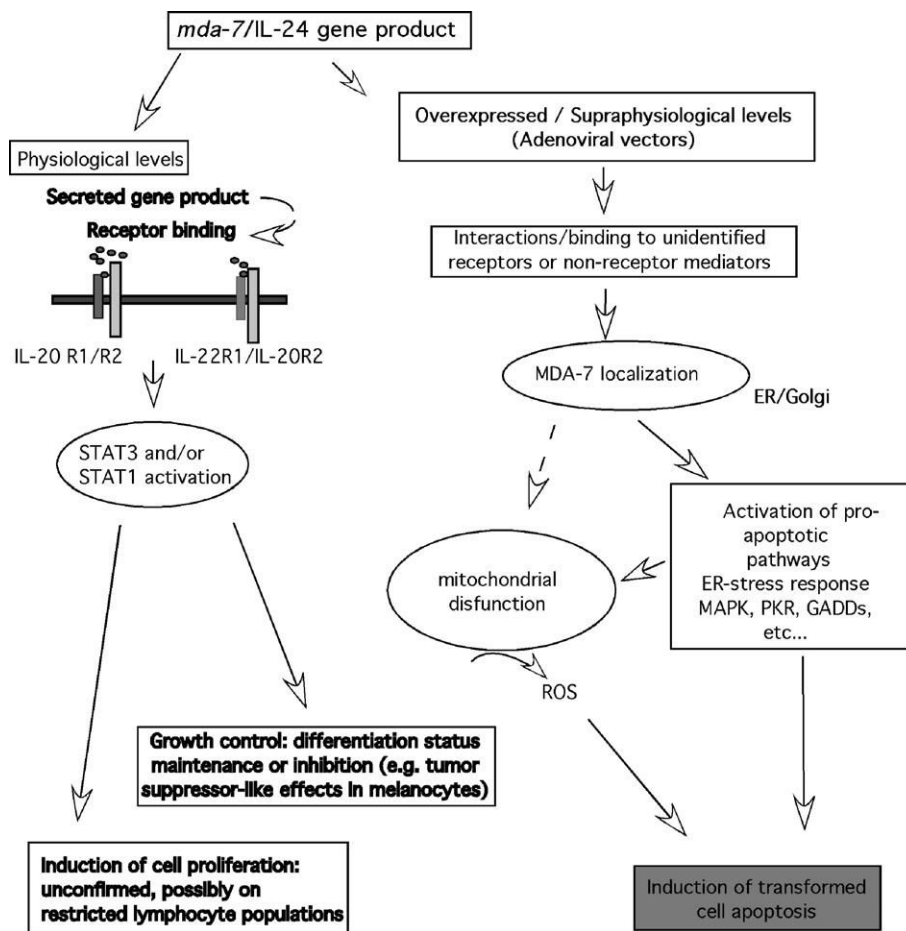


Fig. 12.

Model illustrating the possible molecular basis of *mda-7/IL-24* cancer cell-mediated apoptosis. The effects of known physiological and ectopic overexpression of *mda-7/IL-24* are shown on left and right sides of the figure, respectively. Normally, *mda-7/IL-24* binds to cognate receptors and activates STAT-1 and -3 transcription factors to mediate pathways affecting cell growth. Because *mda-7/IL-24* mRNA and protein are normally seen in subpopulations of immune cells and melanocytes, effects are likely initiated in these cell types but might also affect neighboring non-producing cells because the protein is secreted. When normally or ectopically overexpressed, current findings indicate localization to the ER/Golgi compartments, whether or not the protein contains a secretory signal. Accumulation of MDA-7/IL-24 protein in this compartment triggers apoptosis that could apparently involve induction of pathways described currently as ER stress. However, MDA-7/IL-24 additionally acts indirectly on mitochondria to generate reactive oxygen species. A combination of pathways triggered by *mda-7/IL-24* results in transformed cell-specific apoptosis. IL, interleukin; ER, endoplasmic reticulum; STAT, signal transducers and activators of transcription; ROS, reactive oxygen species (reproduced, by permission of the publisher, from Sauane et al., 2004b).

Table 1

Ad.mda-7 selectively inhibits growth in a wide spectrum of cancer cells, without affecting normal cells

Growth not affected	Growth inhibited
Mammary epithelial cells (HuMEC)	Breast carcinoma
Prostate epithelial cells (HuPEC)	Prostate carcinoma
Melanocytes (NHuMel)	Melanoma
Bronchial epithelial cells (HNBE)	Lung cancer
Fetal astrocytes (PHFA)	Glioblastoma multiforme
Skin fibroblasts (MJ90)	Osteosarcoma
Skin fibroblasts (HF)	Colon carcinoma
Lung fibroblasts (NHLF)	Nasopharyngeal carcinoma
Endothelial cells (HuVEC)	Pancreatic carcinoma*
Renal epithelial cells	Cervical carcinoma
Mesothelial cells	Ovarian carcinoma

* In pancreatic carcinoma cells containing a mutant *K-ras* gene, the combination of *Ad.mda-7* and inhibition of mutant *K-ras* induces growth inhibition. *Ad.mda-7* alone or in combination with inhibition of mutant *K-ras* fails to induce growth inhibition or apoptosis in BxPC-3 pancreatic carcinoma cells, which contain a wild-type *K-ras* gene.