



Genomic structure, chromosomal localization and expression profile of a novel melanoma differentiation associated (*mda-7*) gene with cancer specific growth suppressing and apoptosis inducing properties

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Abnormalities in cellular differentiation are frequent occurrences in human cancers. Treatment of human melanoma cells with recombinant fibroblast interferon (IFN- β) and the protein kinase C activator mezerein (MEZ) results in an irreversible loss in growth potential, suppression of tumorigenic properties and induction of terminal cell differentiation. Subtraction hybridization identified melanoma differentiation associated gene-7 (*mda-7*), as a gene induced during these physiological changes in human melanoma cells. Ectopic expression of *mda-7* by means of a replication defective adenovirus results in growth suppression and induction of apoptosis in a broad spectrum of additional cancers, including melanoma, glioblastoma multiforme, osteosarcoma and carcinomas of the breast, cervix, colon, lung, nasopharynx and prostate. In contrast, no apparent harmful effects occur when *mda-7* is expressed in normal epithelial or fibroblast cells. Human clones of *mda-7* were isolated and its organization resolved in terms of intron/exon structure and chromosomal localization. Hu-*mda-7* encompasses seven exons and six introns and encodes a protein with a predicted size of 23.8 kDa, consisting of 206 amino acids. Hu-*mda-7* mRNA is stably expressed in the thymus, spleen and peripheral blood leukocytes. *De novo mda-7* mRNA expression is

also detected in human melanocytes and expression is inducible in cells of melanocyte/melanoma lineage and in certain normal and cancer cell types following treatment with a combination of IFN- β plus MEZ. *Mda-7* expression is also induced during megakaryocyte differentiation induced in human hematopoietic cells by treatment with TPA (12-O-tetradecanoyl phorbol-13-acetate). In contrast, *de novo* expression of *mda-7* is not detected nor is it inducible by IFN- β +MEZ in a spectrum of additional normal and cancer cells. No correlation was observed between induction of *mda-7* mRNA expression and growth suppression following treatment with IFN- β +MEZ and induction of endogenous *mda-7* mRNA by combination treatment did not result in significant intracellular *MDA-7* protein. Radiation hybrid mapping assigned the *mda-7* gene to human chromosome 1q, at 1q 32.2 to 1q41, an area containing a cluster of genes associated with the IL-10 family of cytokines. *Mda-7* represents a differentiation, growth and apoptosis associated gene with potential utility for the gene-based therapy of diverse human cancers. *Oncogene* (2001) 20, 7051–7063.

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Introduction

Cancer is a progressive disease in which evolving tumor cells exhibit profound changes in gene expression that occur in a temporal manner (DeRisi *et al.*, 1996; Schena *et al.*, 1996; Ross *et al.*, 2000). These include, activation of genes serving as positive regulators of the cancer phenotype, oncogenes; inactivation of genes functioning as inhibitors of the cancerous state, tumor

suppressor genes; and altered expression of genes that act at a later stage in cancer development resulting in increased cancer aggressiveness which can culminate in metastasis, progression modulating genes (Fisher, 1984; Bishop, 1991; Vogelstein and Kinzler, 1991; Su *et al.*, 1997, 1999; Kang *et al.*, 1998). Extensive effort is being directed toward identifying and characterizing the genetic elements that contribute to cancer development and evolution. Achieving this goal offers promise for identifying genomic targets for improved cancer diagnosis and therapy.

A failure to display normal patterns of differentiation is common in many cancer subtypes (Jiang *et al.*, 1994; Waxman, 1996). Recent experimental approaches have targeted this defect in cancer cells by using agent(s) that modify tumor growth by inducing terminal differentiation, a process termed 'differentiation therapy' (Waxman, 1996). Although several models exist to explain how metastatic melanoma cells develop, a widely accepted scheme involves a linear set of changes from melanocyte to nevus to primary melanoma [radial growth phase (RGP) to vertical growth phase (VGP)] to metastatic melanoma (Clark, 1991; Armstrong and Krickler, 1994; Herlyn *et al.*, 2000). This process is associated with profound changes in cellular physiology and gene expression suggesting temporal modifications in the differentiation of cells as this tumor evolves (Jiang *et al.*, 1995a; Huang *et al.*, 1999a,b; Herlyn *et al.*, 2000). In human melanoma cells, the combination of recombinant human IFN- β plus the protein kinase C activator MEZ results in an irreversible loss in proliferative ability concluding in terminal cell differentiation (Fisher *et al.*, 1985; Jiang *et al.*, 1993). Combining this model system with subtraction hybridization resulted in the identification and cloning of several genes regulated during the process of growth arrest and terminal differentiation, i.e., melanoma differentiation associated (*mda*) and differentiation induction subtraction hybridization (DISH) genes (Jiang and Fisher, 1993; Jiang *et al.*, 1995a,b, 2000; Huang *et al.*, 1999a,b; Kang *et al.*, 2001).

A specific *mda* gene, *mda-7*, displays elevated mRNA expression in melanocytes and nevi, reduced mRNA expression in RGP and early VGP melanoma lesions and little or no mRNA expression in late VGP and metastatic melanoma (Jiang *et al.*, 1995b). Moreover, ectopic expression of *mda-7* in human melanoma cells results in growth suppression, without induction of terminal differentiation (Jiang *et al.*, 1995b). When expressed at high levels, by means of an adenovirus expression system, *mda-7* induces growth suppression and programmed cell death (apoptosis) in a broad spectrum of human cancers, including carcinomas of most tissue origins (Su *et al.*, 1998; Madireddi *et al.*, 2000c; Saeki *et al.*, 2000; Mhashilkar *et al.*, 2001). In contrast, *mda-7* has a negligible effect on growth and does not induce apoptosis in normal epithelial and fibroblast cells (Jiang *et al.*, 1996; Su *et al.*, 1998; Madireddi *et al.*, 2000c; Saeki *et al.*, 2000; Mhashilkar *et al.*, 2001). These findings indicate that this novel

cancer growth suppressing and apoptosis-inducing gene may have wide applications for the gene-based therapy of multiple human cancers (Jiang *et al.*, 1996; Su *et al.*, 1998; Madireddi *et al.*, 2000c; Saeki *et al.*, 2000; Mhashilkar *et al.*, 2001).

Studies designed to define the mechanism of *mda-7* gene expression regulation during human melanoma terminal differentiation document that the *mda-7* promoter is constitutively active in human melanoma cells and treatment with IFN- β +MEZ does not significantly alter promoter function (Madireddi *et al.*, 2000a,b). In contrast, *mda-7* mRNA levels during terminal differentiation are partly regulated by differential post-transcriptional-message stabilization dictated by the AU-rich (ARE) sequences present in the 3'-untranslated region of the *mda-7* cDNA (Madireddi *et al.*, 2000a). To further characterize this cancer suppressor gene we have isolated *mda-7* genomic DNA from human cells and defined its gene structure and analysed endogenous and inducible expression in melanocyte/melanoma and other lineage cells. Expression of *mda-7* is restricted to specific normal tissues, including thymus, spleen and blood (peripheral blood leukocytes), and certain cell types, including melanocytes, nevi and some early stage melanomas. Enhanced *mda-7* expression is also induced in melanoma cells following treatment with IFN- β +MEZ, in K562 erythroleukemia cells treated with TPA and in specific human normal and cancer cells following treatment with IFN- β +MEZ. These results demonstrate for the first time that appropriate treatment(s) can induce *mda-7* mRNA in a spectrum of normal and cancer cells, in addition to melanoma cells, which do not express detectable levels of *mda-7* mRNA *de novo*. The level of *mda-7* mRNA induced, as well as the level of MDA-7 protein, is significantly less following IFN- β +MEZ treatment than infection with a replication defective adenovirus expressing *mda-7*, Ad.*mda-7*. Moreover, IFN- β +MEZ inhibits growth in a panel of normal and cancer cells and this effect does not correlate with induction of *mda-7* mRNA, but rather corresponds with IFN- β treatment which does not induce *mda-7* mRNA. The chromosomal location of human *mda-7* was determined and found to reside within a region of human chromosome 1q, 1q 34.2 to 1q41, which corresponds with a genomic locus containing several members of the interleukin-10 family of cytokines.

Results

Genomic structure and chromosomal localization of mda-7 to a genomic region containing an interleukin-10 related gene cluster

Restriction fragment analyses of human genomic DNA demonstrate that the human *mda-7* gene is a single copy gene. Southern blot analyses reveal a single band upon hybridization with a *mda-7* cDNA probe (Jiang *et al.*, 1995b). Based on its unique presence in the human genome, a PCR-based method using *mda-7*

gene specific primers was used to identify and isolate its genomic DNA. Human genomic DNA from diploid fibroblasts (Clontech) was used as a template in a polymerase chain reaction amplification protocol with *mda-7* gene specific primers corresponding to the 5' and 3' untranslated regions. Agarose gel electrophoresis analyses identified a 5.5 Kbp amplification product, which was cloned into pBluescript, and the nucleotide sequence was obtained. In order to obtain genomic sequence information flanking the PCR generated fragment a human placental genomic library (Stratagene) was screened. Five clones were isolated from 2×10^6 plaques of human placental genomic library using an *mda-7* cDNA probe. Restriction enzyme digestion and probing with 5'- and 3'-UTR specific sequences revealed that one of the clones encompasses the entire reading frame of the *mda-7* gene. This phage clone was purified and sequenced using *mda-7* gene specific primers. Using the two methods described above a contiguous *mda-7* genomic sequence was obtained. The *mda-7* transcription unit is 6.33 Kbp, subsequent DNA walking resulted in the cloning of an additional 2.2 Kbp of the 5'-flanking region which contains the *mda-7* promoter (Madireddi et al., 2000a). The nucleotide sequence of human *mda-7* and exon/intron boundaries are shown in Figure 1. The *mda-7* cDNA deduced from the human exon sequence information corresponded exactly with the previously reported human *mda-7* cDNA (Jiang et al., 1995b).

The identified intron-exon boundaries conform to the consensus splicing signals (GT...AG). These dinucleotide sequences are putative splice sites implicated in primary transcript splicing (Breathnach et al., 1978; Breathnach and Chambon, 1981). The exons range in size from 64 to 889 bp while the introns range from 115 to 1443 bp. The transcriptional start site was mapped by 5' RACE using total RNA from terminally differentiated HO-1 human melanoma cells (Jiang et al., 1995b). Primer extension analysis produced similar results (data not shown), and the transcription start site was designated, and its position is shown in Figure 1. Analyses of the 5'-upstream nucleotide sequence reveals the presence of a TATA element at position -30 to -25 (Figure 1). Elimination of this TATA element resulted in a complete loss in promoter activity (Madireddi et al., 2000a).

A panel of rodent-human hybrid DNAs containing most human chromosome regions were tested for the presence of the *mda-7* locus by a PCR amplification based method. Hybrid DNAs were scored positive if they contained a 129 bp human *mda-7* specific product. Hybrids retaining chromosome region 1q showed a *mda-7* specific product (data not shown). To further refine the localization of *mda-7*, the Stanford and Genebridge radiation hybrid DNAs (Research Genetics) were tested. No linkage was found using the Stanford radiation hybrid DNAs. Using the Genebridge radiation hybrids and the WICGR mapping server (www.genome.wi.mit.edu/cgibin/contig/rhmapper.pl) *mda-7* displayed very close linkage to markers WI-9641 (D1S306) and D1S491, which map to the

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aggaagcat gaccaatttc cctttctcca cctccctctt tttctccac cctccccc
      PUTATIVE TATA-BOX +1 Transcription start
tcagccccca TATATAgcc caaatctcca caaagccttg CTGCTGCA AACCTTACT
EXON 1
TCGTAAATGA CTTCCACGGC TGGGACGGGA ACCTTCCACC CACAGCTAGC CCTCTGATG
GTGAATGGTG AAGGTGCTG TCTAACTTTT CTGTAAAAG AACCACTGTC CTCACGGCAG
CCAGCCCTCA AGCATCACTT ACAGGACCAG AGgagcat--- 115 bp intron ---
EXON 2
gatgtgGGGA CRAGACATGA CTGTGATGAG GAGCTGCTTT CGCCAATTTA ACACCAAGAA
MET
GAATTGAGGC TGCTTGGGAG GAAGGCCAGG AGGAACACGA GACTGAGAGA ATTAATTTCA
ACAGAGGCTG CAAGCCTGTG GGACTTTAGC CAGgaggggt--- 1443 bp intron ---
EXON 3
cagcACCCTTCCCTCC CTTTCTGGC GACAGCCTCT CAAATGACGA TGGTTGTGCT
CCCTTGCCCTG GGTTTTACC TGCTTCTCTG GAGCCAGGTA TCAGGGGCC AGGGCCAGA
ATTCCACTT GGGCCCTGCC AAGTGAAGGG GGTGTCTCC CAGAAGCTC GGGAAAGCCTT
CTGGGCTGTG AAAGACTA TGgagtaa --- 779 bp intron ---
EXON 4
tgtttgccaa CAAGCTCAG GATAACATCA CGAGTGCCCG GCTGCTGCAG CAGGAGGTT
TCCAGACGT CTCGgtaac --- 1136 bp intron ---
EXON 5
ccttccccgatgctgaga gctgttacct tgccacacc ctgctggagt tctacttgaa
aaactgtttc aaaaactacc acatagAAC agttgagctc aggactgata agcatcttc
actctggccc acaactttg ttctcatctg gtcacaactg caaccagctgagtagc
--- 343 bp intron ---
EXON 6
cccatgttat gtaCAAGAA AATGAGATGT TTTCATCAG AGACAGTGCA CACAGCGGGT
TTCTGTATT CCGGAGACCA TTCAACAGgaaagccaag
--- 903 bp intron --- gTTGGAGCT AGAACAGCT ATCCCAAAG CCCTTGGGGA
AGTGGACATT CTTCTGACCT GGATGACAAA ATTCTACAG CTCTGAATGT CTGACCAGG
ACCTCCCTCC CCCTGGCAGT GGTGTGTC CCCTGTCATT TCAACAGTC CCCTCTCTA
TGCTGTATC TGGACACTTC ACGCCCTTGG CCATGGGTC CATCTTGGC CCAGATTAT
TGTCAAAGAA GCATCTTTT AAGCAGCCCC AGTGACAGTC AGGGAAGGGT CCTCTGGATC
CTGTAAAGAG TCTACAGAGA AGATTCTGT ATTATTACA ACTTATTTA ATTAATGTCA
GATTTTCAAC TGAAGTCTA TTTATTGTG AGACTGTAA TTAATGAA GCAGCAGAA
ATTGTGCCCC ATGCTTCTT ACCCTCAC AATCTTCCA CAGTGTGGG CAGTGGATG
GTCTTAGTA AGTACTTAA AACCTGTGT GCTTTTTT GCTGTCTT GGATTGTAA
AAACAGAGA GGGATGCTG GATGAAAAC TGAATCTGAG AGCATGAAA TCACACTGC
TTCTGATTC TGACGGACA GAGCATTGGG GTGGGGTAA GGTGCATCTG TTTGAAAAT
AAACGATAAA ATGTGGATTA AAGTCCCGC CACAAAGCAG ATCTCAATA ACATTTTAT
TTCCACCACA CACTGCGCAG CTCACCCAT CATCCCTTC CCTGTGTC CTCCTTTT
TTTATCTTA GCATCTTTC CCTAATCTC CACTTGAGT TCARAGCTGAC CTGCTGATG
GTGACATTC
POLY-A SIGNAL
ACCTGGATG ACTATCCAAT CTGTGATGAC ATCCCTGCT AAGCAAGAC AACATAACT
Aagtctggca gactttcttc tctattcttg gatgaatgcc cagtgagact gtgtgtaca
gctagaaaag gccttcttcc caatagcaag gctgtgcatc t
    
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Figure 1 Human *mda-7* genomic structure. The nucleotide sequence of all exons including non-coding 5' and 3' UTR sequences and coding exons are shown in bold type. Sequences flanking the exon/intron junction including the splice consensus GT-AG sequences are indicated in normal type and shadow font respectively. Length of each intron and other landmark sequences including transcription start site (+1), putative TATA regulatory sequence, translation initiator codon (ATG) and polyadenylation consensus sequence are highlighted at the respective positions

region 1q32.2-q41 (data not shown). The genomic locus encoding *mda-7* appears to be within a IL-10 related gene cluster containing four genes including IL-10, IL-19, IL-20 and *mda-7* in linear order spanning 195 kb of genomic DNA (Blumberg et al., 2001). The functional implications of this arrangement are presently unclear.

Mda-7 displays restricted expression in normal tissue and inducible expression in human erythroleukemia cells

Multiple tissue Northern blot analyses of human tissue poly(A)⁺ RNA (Clontech^R) reveals that *mda-7* expression is restricted to those tissues associated with the immune system such as, spleen, thymus and peripheral blood leukocytes (Figure 2a). These findings are in agreement with the observations made by Soo et al. (1999) who reported significantly elevated (9–12-fold) levels of c49a (rat homologue of *mda-7*) mRNA during wound healing, specifically in areas surrounding the edge of the wound. To further analyse this relationship the incidence of *mda-7* expression in hematopoietic

(erythroid, myeloid and lymphoid) cell differentiation was analysed. Northern blotting analyses of total RNA from leukemic cell lines induced to differentiate with TPA were performed. The results of this study reveal an increase in *mda-7* mRNA levels in the leukemic cell line, K562 (erythroleukemia), when it is induced to differentiate into megakaryocytes upon treatment with TPA (Figure 2b). *Mda-7* mRNA expression in differentiated K562 cells (Figure 2b, lane 5) is similar to that found in terminally differentiated HO-1 human melanoma cells after correction for variations in total RNA based on the levels of rRNA (Figure 2b, lane 9). In contrast, HL-60 (human promyelocytic leukemia) induced to differentiate with TPA (monocyte/macrophage) or DMSO (granulocyte), CEM-C7 (human T-cell leukemia) treated with TPA and HL534 (TPA-resistant HL-60 cell variant) (Tonetti *et al.*, 1992) did not express *mda-7* in the absence or presence of inducer (Figure 2b).

De novo expression of mda-7 occurs in normal and immortalized human melanocytes and is readily inducible in most human melanoma cell lines

Experiments have been performed to examine *mda-7* expression profiles in normal and cancer-derived human cell types. In previous studies, expression of *mda-7* mRNA was detected using Northern blotting in an SV40-immortalized human melanocyte cell line (FM516-SV) and in one of six metastatic melanoma cell lines (Jiang *et al.*, 1995b). In addition, using patient-derived samples, including melanocytes (five samples), primary melanomas (seven RGP and early VGP samples) and metastatic melanomas (seven samples), an inverse correlation between melanoma progression and *mda-7* RNA expression was apparent using RT-PCR based approaches, with highest levels in melanocytes, decreasing levels in primary melanomas and lowest levels in metastatic melanomas (Jiang *et al.*, 1995b). Previous studies demonstrated that treatment of metastatic melanoma cells with IFN- β +MEZ for 24 h resulted in induction or enhanced *mda-7* mRNA expression in the six metastatic melanoma cell lines analysed (Jiang *et al.*, 1995b). These studies have now been extended to include normal early passage melanocytes and a series of additional melanoma cell lines, including WM35 (an early RGP melanoma), WM278 (an early VGP melanoma) and additional metastatic melanoma cell lines (SK-MEL p53 wt, SK-MEL p53 mt, MeWo, 3S5, 70W, WM239 and C8161) (Figure 3). In these cells, elevated *mda-7* mRNA was only apparent *de novo* in normal melanocytes, with lower levels of *de novo* expression in WM35 and FO-1 cells. However, 24 h treatment with IFN- β +MEZ resulted in a differential enhancement or induction of *mda-7* expression in all of the melanocyte/melanoma cell lines with the exception of SK-MEL p53 mt (containing a mutant p53 gene) (Figure 3). Additionally, the induction of *mda-7* in C8161 cells was significantly blunted in comparison with the other metastatic melanoma cell lines. It does not appear that

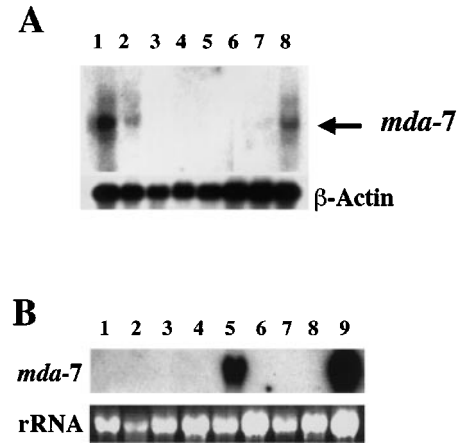


Figure 2 Expression of *mda-7* message in the human immune system and human leukemic cells. (a) Human multiple tissue Northern blot consisting of poly(A)⁺ mRNA from different tissues shows tissue specific expression of *mda-7*. The mRNAs immobilized on the blot are from spleen (1), thymus (2), prostate (3), testis (4), ovary (5), small intestine (6), colon (7) and peripheral blood leukocytes (8). (b) *Mda-7* expression in leukemic cells induced to undergo differentiation. HL-60 (lanes 1–3, human promyelocytic leukemia) uninduced (lane 1) or induced to differentiate by TPA (lane 2) or DMSO (lane 3), K562 (erythroleukemia) uninduced (lane 4), or induced to differentiate by TPA (lane 5), CEM-C7 (human T-cell leukemia) uninduced (lane 6) or induced to differentiate by TPA (lane 7), HL 534 (lane 8) and HO-1 (human melanoma) treated with IFN- β +MEZ

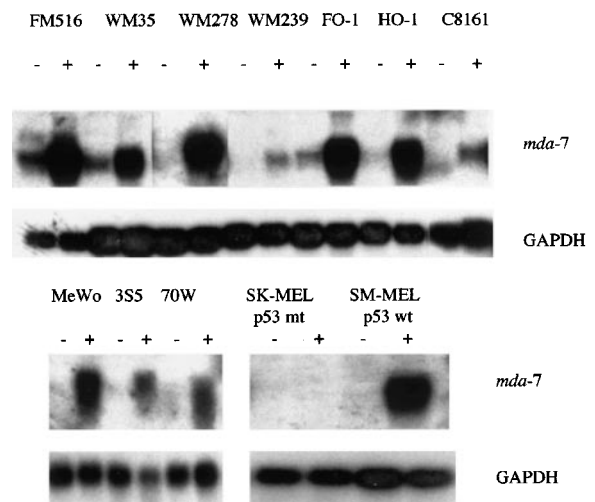


Figure 3 Expression of *mda-7* message in human melanocyte and melanoma cell lines with and without treatment with inducer. The indicated cell types were grown for 24 h in the presence of 2000 units/ml of IFN- β +10 ng/ml of MEZ, total RNA was isolated and analysed by Northern blotting and probing with a ³²P-labeled *mda-7* cDNA probe. The blot was stripped and then reprobated with a ³²P-labeled GAPDH probe. FM516 is an SV40-immortalized normal melanocyte cell line (FM516-SV); WM35 is an early RGP primary melanoma cell line; WM278 is an early VGP primary melanoma cell line; WM239, FO-1, C8161, MeWo, 3S5 and 70W are metastatic melanoma cell lines; SK-MEL p53 mt is a metastatic melanoma cell line with a confirmed mutant p53 genotype; and SK-MEL p53 wt is a metastatic melanoma cell line with a confirmed wild-type p53 genotype

having a mutant *p53* gene by itself can prevent *mda-7* expression, since MeWo and its two subclones, 3S5 displaying a reduction in metastatic competence and 70W exhibiting enhanced metastatic potential (Kerbel and Man, 1984; Graham et al., 1991), which contain one mutant and one wild-type *p53* allele are readily inducible for *mda-7* expression following IFN- β +MEZ treatment. These studies document *de novo* expression of *mda-7* in cultured normal human melanocytes, as observed with patient-derived melanocytes, and they provide additional support for the idea that *mda-7* may function as a negative regulator of melanoma progression (Jiang et al., 1995b).

Induction of mda-7 mRNA is not restricted to human melanoma cells, but can occur in various normal and cancer-derived human cell lines following treatment with IFN- β +MEZ

Previous studies and those described above document a clear association between *mda-7* mRNA expression and melanocyte/melanoma lineage cells. To determine if *mda-7* expression is restricted to this cell subtype or if *de novo* or inducible expression occurs in additional cell types, a panel of normal and cancer-derived cells were analysed for *mda-7* mRNA expression with and without a 24 h treatment with IFN- β +MEZ (Figure 4). In the case of normal human prostate epithelial and human prostate carcinoma cells no expression was apparent, with or without treatment with IFN- β +MEZ, in normal early passage prostate epithelial cells (HuPEC) or in two of three prostate carcinoma cell lines, i.e., LNCaP and PC-3 (Figure 4a). In contrast, although not expressed *de novo*, 24 h exposure to IFN- β +MEZ resulted in induction of *mda-7* mRNA expression in DU-145 human prostate carcinoma cells. Since DU-145 cells contain a mutant form of the tumor suppressor protein RB and they also contain a mutation in *p53*, induction of *mda-7* can occur in specific prostate cancer cells defective in these tumor suppressor proteins. In the case of breast-derived epithelial cells, *mda-7* mRNA expression was not detected *de novo* in six cell lines, but it was inducible by IFN- β +MEZ treatment in normal HBL-100 cells and in *p53* mutant MDA-MB-231 and *p53*-null MDA-MB-157 cells (Figure 4b). In contrast, expression of *mda-7* was not apparent in untreated or combination treated MCF-7 (wild-type *p53*), T47D (mutant *p53*) or MDA-MB-453 (mutant *p53*) breast carcinoma cell lines. Further analysis indicates that *mda-7* is also inducible by IFN- β +MEZ in normal human cerebellum cells (NC cell line), one of two human glioblastoma multiforme cell lines (GBM-18, but not T98G), a human cervical carcinoma cell line (HeLa), a human nasopharyngeal carcinoma cell line (HONE-1) and a human osteosarcoma cell line (Saos2, which is null for both *RB* and *p53*) (Figure 4c). In contrast, no expression, with or without combination treatment, was apparent in SW613 human colon carcinoma or BxPC-3, PANC-1, MIA PaCa-2 or AsPC-1 human pancreatic carcinoma cells (Figure 4c

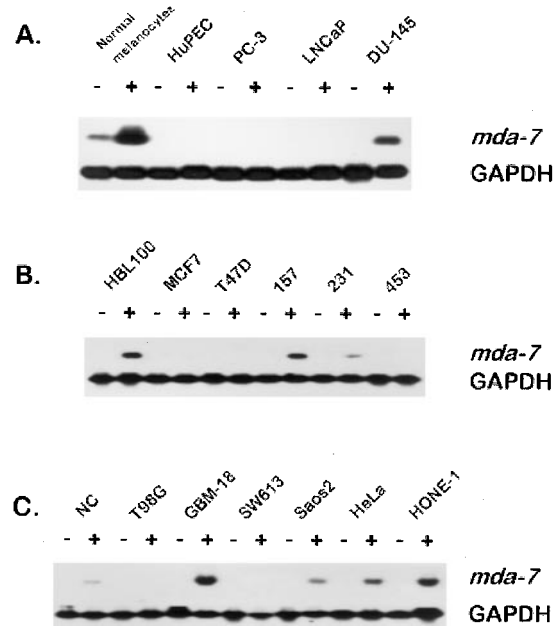


Figure 4 *De novo* and inducible expression of *mda-7* mRNA in normal and human cancer cell lines. (a) Expression of *mda-7* in normal early passage human foreskin melanocytes, early passage human prostate epithelial cells HuPEC and prostate carcinoma cell lines, PC-3, LNCaP and DU-145, grown for 24 h in the absence (–) or presence (+) of IFN- β (2000 units/ml) plus MEZ (10 ng/ml). (b) Expression of *mda-7* in a normal human breast epithelial cell line (HBL-100) and various breast carcinoma cell lines (MCF7, T47D, MDA-MB-157, MDA-MB-231 and MDA-MB-453) grown for 24 h in the absence (–) or presence (+) of IFN- β (2000 units/ml) plus MEZ (10 ng/ml). (c) Expression of *mda-7* in diverse human cell lines, including normal cerebellum (NC), glioblastoma multiforme (T98G or GBM-18), colon carcinoma (SW613), osteosarcoma (Saos2), cervical carcinoma (HeLa) and nasopharyngeal carcinoma (HONE-1), grown for 24 h in the absence (–) or presence (+) of IFN- β (2000 units/ml) plus MEZ (10 ng/ml)

and data not shown). These results demonstrate that *mda-7* is not expressed *de novo* in most normal and cancer cell types, but expression, at least at an mRNA level, can be induced by IFN- β +MEZ in a spectrum of normal and tumor cell types independent of alterations in *Rb* and/or *p53* genotypes.

Based on the ability of IFN- β +MEZ to induce *mda-7* mRNA expression in cells other than melanoma, experiments were performed to evaluate the functional significance of this treatment protocol in combination treated cells. Studies were performed to determine if induction of *mda-7* mRNA correlated with expression of *MDA-7* protein. To address this question FM516-SV (constitutively express *mda-7* mRNA) and HO-1 and DU-145 (which are inducible for *mda-7* mRNA) were either infected with 1, 10, 50 or 100 pfu/cell of Ad.*mda-7*, a replication incompetent adenovirus expressing the coding region of the *mda-7* gene (Su et al., 1998), or treated with 2000 units/ml IFN- β +10 ng/ml MEZ, and the amount of *mda-7* mRNA and *MDA-7* protein was determined by Northern and Western blotting, respectively (Figures 5 and 6). These studies

indicate quantitative differences in the levels of *mda-7* mRNA and/or *MDA-7* protein in the three different cell types with and without infection with *Ad.mda-7* or treatment with IFN- β +MEZ. In the case of DU-145, infection with *Ad.mda-7* results in a dose-dependent expression of *mda-7* mRNA and *MDA-7* protein, with intracellular protein readily detected 24 h following infection with 50 or 100 pfu/cell but not with 1 or 10 pfu/cell (Figures 5 and 6). In contrast, the level of *mda-7* mRNA following infection with 1 pfu/cell of *Ad.mda-7* is \sim twofold higher than observed after treatment of DU-145 with IFN- β +MEZ and no intracellular protein is detected in these cells after 24 h treatment (Figure 6). In the case of FM516-SV, *mda-7* mRNA is detectable *de novo* at a lower level than observed following infection with 1 pfu/cell of *Ad.mda-7* and treatment with IFN- β +MEZ elevates *mda-7* mRNA in these cells (Figure 5). With respect to protein, no *MDA-7* protein is detected in IFN-

β +MEZ treated cells and significantly less *MDA-7* protein than detected in *Ad.mda-7*-infected DU-145 cells is present in FM516-SV cells infected with 50 or 100 pfu/cell of *Ad.mda-7* (Figure 6). In HO-1, no *mda-7* mRNA is present *de novo* and treatment with IFN- β +MEZ results in induction of *mda-7* mRNA at levels that exceed those observed following infection with 1 pfu/cell of *Ad.mda-7* (Figure 5). In comparison with DU-145 and FM516-SV, infection of HO-1 cells with *Ad.mda-7* results in less *mda-7* mRNA and *MDA-7* protein, with intracellular protein only detected after infection with 100 pfu/cell of *Ad.mda-7* (Figures 5 and 6). These results indicate differences in the levels of *mda-7* mRNA and intracellular *MDA-7* protein following *Ad.mda-7* infection in the three different cell types, which may reflect differences in viral infectivity or kinetics of transgene expression. Moreover, the levels of intracellular *MDA-7* protein resulting from treatment with IFN- β +MEZ are minimal in all three cell types. Further studies are required to determine if the levels of secreted *MDA-7* protein differ in these

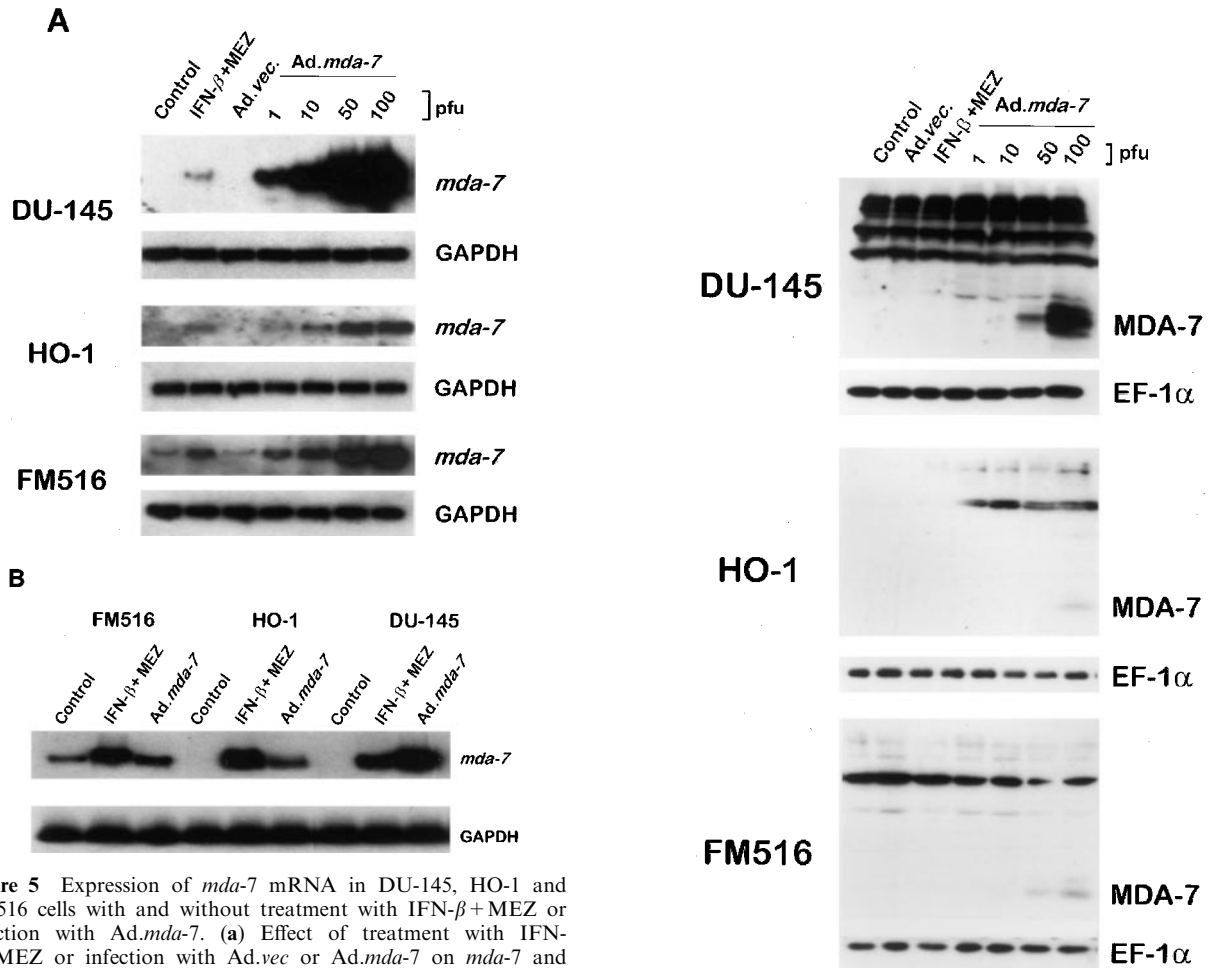


Figure 5 Expression of *mda-7* mRNA in DU-145, HO-1 and FM516 cells with and without treatment with IFN- β +MEZ or infection with *Ad.mda-7*. (a) Effect of treatment with IFN- β +MEZ or infection with *Ad.vec* or *Ad.mda-7* on *mda-7* and GAPDH mRNA. (b) Effect of treatment with IFN- β +MEZ or infection with 1 pfu/cell of *Ad.mda-7* on *mda-7* and GAPDH mRNA. The indicated cell type was untreated (Control), treated with 2000 units/ml of IFN- β plus 10 ng/ml of MEZ or infected with an *Ad.vec* (100 pfu/cell) or 1, 10, 50 or 100 pfu/cell of *Ad.mda-7* for 24 h. Total RNA was isolated and analysed by Northern blotting for *mda-7* and GAPDH mRNA expression

Figure 6 Expression of *MDA-7* protein in DU-145, HO-1 and FM516 cells with and without treatment with IFN- β +MEZ or infection with *Ad.mda-7*. The indicated cell type was treated as in Figure 5 for 24 h and levels of *MDA-7* and EF-1 α proteins in total cell lysates were determined by Western blotting using the appropriate polyclonal or monoclonal antibody, respectively

cells upon treatment with IFN- β +MEZ and/or following infection with Ad.*mda-7*.

Previous studies in HO-1 melanoma cells demonstrate a small induction of *mda-7* mRNA following treatment with MEZ and a major induction of *mda-7* mRNA following treatment with IFN- β +MEZ (Jiang *et al.*, 1995b; Madireddi *et al.*, 2000a,b). In contrast, IFN- β does not induce *mda-7* expression in HO-1 cells. To determine if a similar *mda-7* induction profile is apparent in non-melanoma cell types induced to express *mda-7* mRNA following treatment with IFN- β +MEZ the effect of treatment with IFN- β and MEZ on *mda-7* expression in DU-145 and HeLa cells was determined. The combination of IFN- β +MEZ induced *mda-7* mRNA in both cell types, whereas no induction of *mda-7* mRNA occurred after MEZ or IFN- β treatment (data not shown). These results provide additional evidence that the combination treatment with IFN- β +MEZ is a more effective inducer of *mda-7* mRNA than either agent used independently.

An important question is the physiological relevance of induction of *mda-7* by IFN- β +MEZ in specific cell types. To begin addressing this issue, we have compared the effect of IFN- β , MEZ and IFN- β +MEZ on 48 and 96 h growth and viability in cells displaying variable induction or no induction of *mda-7* after combination treatment (Figures 7 and 8). When administered at 10 ng/ml, MEZ did not inhibit the growth and in some cases even stimulated growth which was cell type specific. However, treatment with 2000 units/ml of IFN- β , alone or in combination with MEZ significantly inhibited growth, an effect that was apparent to different extents by 48 h in all the cell types tested (Figures 7 and 8). With many of the cell types, the combination of IFN- β +MEZ induced greater growth suppression, and in some cases a decrease in cell viability, than either agent used alone. Growth suppression and decreased viability was apparent in cells displaying inducible *mda-7* mRNA expression as well as in cells not showing inducible *mda-7* mRNA expression, such as MCF-7 and SK-MEL *p53* mt. These results, and studies demonstrating that IFN- β decreases growth without inducing *mda-7*, indicate that induction of *mda-7* by IFN- β +MEZ is not mandatory for growth suppression and decreasing viability in combination treated cells.

Discussion

Treatment of human melanoma cells with IFN- β +MEZ results in profound physiological changes, including an irreversible loss in proliferative potential, suppression of oncogenic potential in athymic nude mice, altered cell surface antigenicity, temporal alterations in gene expression and induction of terminal differentiation (Fisher *et al.*, 1985; Graham *et al.*, 1991; Jiang *et al.*, 1993, 1994, 1995a,b, 2000; Huang *et al.*, 1999a,b; Kang *et al.*, 2001; Leszczyniecka *et al.*, 2001). To obtain insights into this process we have begun to define the spectrum of gene

expression changes occurring as a consequence of this combination treatment in human melanoma cells using several molecular approaches. These include, construction of temporally spaced subtracted cDNA libraries from IFN- β +MEZ treated HO-1 melanoma cells combined with random clonal isolation, high density microarray analysis of subtracted cDNA clones and reverse Northern hybridization of randomly isolated subtracted cDNA clones (Jiang and Fisher, 1993; Jiang *et al.*, 1994; Huang *et al.*, 1999a,b). In addition, we have applied a new and highly efficient rapid subtraction hybridization, RaSH, protocol to address the question of temporal gene expression changes occurring during the induction of terminal differentiation in human melanoma cells, resulting in the cloning of additional previously identified and novel genes implicated in this process (Jiang *et al.*, 2000; Kang *et al.*, 2001). These studies have proven very informative and are providing a molecular snapshot of genes involved in cancer growth control, survival, apoptosis and differentiation (Huang *et al.*, 1999a,b; Jiang *et al.*, 2000; Leszczyniecka *et al.*, 2001).

A potentially relevant gene in melanoma progression, isolated by subtraction hybridization, is *mda-7* (Jiang *et al.*, 1995b). When originally cloned, it was proposed that *mda-7* might function as a tumor suppressor gene in the context of melanocyte/melanoma cells, displaying elevated expression in normal melanocytes but decreased expression in primary melanoma cells and a further diminution in expression as melanomas progress to a metastatic state (Jiang *et al.*, 1995b). It was further suggested that expression of *mda-7* in the context of normal melanocytes might contribute to the slower growth rate of these cells versus melanoma cells (Jiang *et al.*, 1995b). Two lines of evidence confirm an inverse relationship between growth rate and *mda-7* expression in specific cell types. Expression of an inducible *mda-7* construct in human melanoma cells decreases growth rate and antisense inhibition of *mda-7* expression in human cervical cancer (HeLa) cells engineered to express *mda-7* enhances their growth rate (Jiang *et al.*, 1995b, 1996). A hallmark of the terminal differentiation process is growth suppression. When *mda-7* is expressed at physiological levels in melanoma cells by DNA transfection growth is suppressed, whereas expression of *mda-7* at supraphysiological levels in melanoma cells following viral (Ad.*mda-7*) infection induces apoptosis (Madireddi *et al.*, 2000c). In contrast, neither of these treatment protocols with *mda-7* results in terminal differentiation in human melanoma cells. These observations suggest that additional genes working in combination with *mda-7* or operating independently of *mda-7* are involved in initiating and maintaining terminal differentiation in human melanoma cells following IFN- β +MEZ treatment.

The present study provides additional support for an inverse correlation between *mda-7* expression and human melanocyte to melanoma progression. Normal early passage human melanocytes and SV40-immorta-

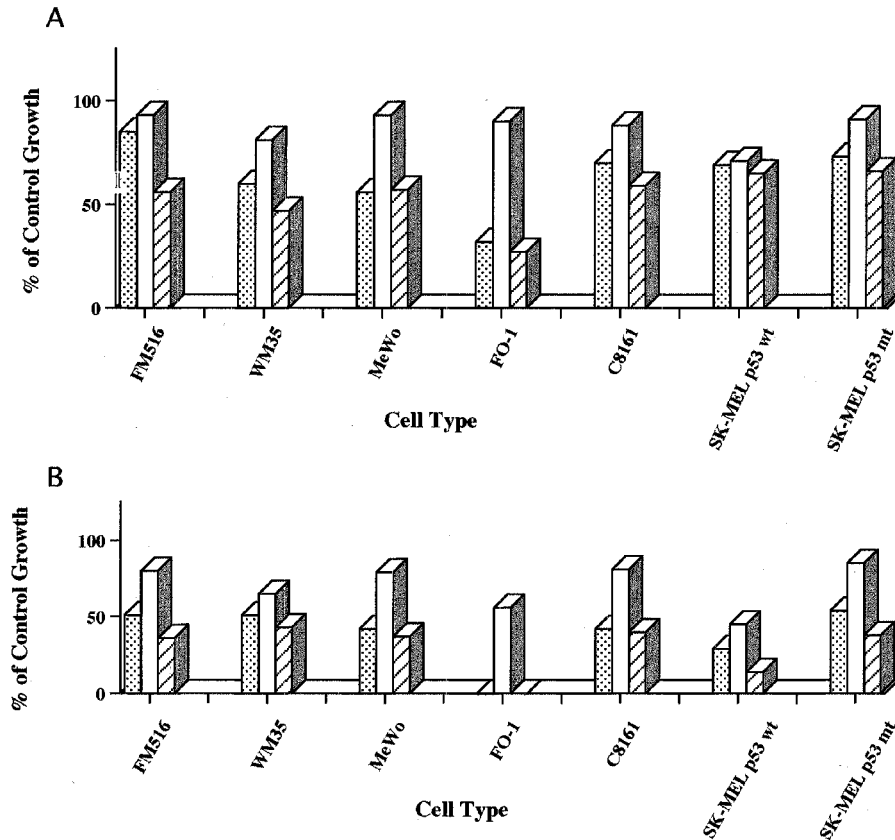


Figure 7 Effect of treatment with IFN- β , MEZ or IFN- β +MEZ on the growth of melanocyte/melanoma cell lines. The indicated cell type was treated for 48 h (a) or 96 h (b) with IFN- β (2000 units/ml), MEZ (10 ng/ml) or a combination of IFN- β +MEZ (2000 units/ml+10 ng/ml) and cell numbers were determined. Triplicate samples varied by $\leq 10\%$ and a replicate experiment varied by $\leq 15\%$. :IFN- β ; :MEZ; :IFN- β +MEZ

lized normal human melanocytes express *mda-7 de novo* as does WM35 cells (an early RGP primary melanoma cell line) (Figure 3). In contrast, only one of nine metastatic melanoma cells displayed endogenous *mda-7* expression, and in the one positive melanoma cell line, FO-1, expression was lower than observed in the normal melanocytes or WM35 cells (Figure 3). When treated with IFN- β +MEZ for 24 h, *mda-7* mRNA expression was elevated in normal early passage human melanocytes, SV40-transformed human melanocytes (FM516-SV) and WM35 cells and expression was induced in eight of the nine metastatic melanoma cells. In general, no correlation was found between the level of induction of *mda-7* by IFN- β +MEZ and the degree of growth-suppression in the melanocyte/melanoma cell lines, with the possible exceptions of C8161 and SK-MEL p53 mt cells which showed low-level or no induction of *mda-7*, respectively, and were more resistant to growth suppression and decreased viability than the other metastatic melanoma cell lines displaying elevated levels of *mda-7* expression following combination treatment (Figure 7). These findings support the hypothesis that *mda-7* may only partially contribute to the growth and differentiation changes observed in human melanoma cells after treatment with IFN- β +MEZ. Alternatively, *mda-7* may exert

effects on cellular physiology that are different in the context of a melanocyte versus a melanoma. This possibility is strengthened by the observation that Ad.*mda-7* does not significantly alter growth in normal melanocytes, whereas it induces growth suppression and apoptosis in metastatic human melanoma cells (unpublished data).

Analysis of *de novo mda-7* mRNA expression in a panel of normal and cancer cell types demonstrates limited expression in a normal cellular context, such as melanocyte, with little or no expression in cancer cells. The lack of expression in a cancer cell background supports the possibility that *mda-7* may function as a cancer growth suppressor and inactivation or decreased expression of this gene may contribute to the cancer phenotype. The lack of *de novo* expression of *mda-7* in most cancer cells could result because of permanent defects in the gene or pathways leading to stable mRNA expression, or alternatively could reflect a lack of expression based on cellular milieu or the physiological state of the cell. It does not appear that the lack of *mda-7* expression in cancer cells results from mutations in the *mda-7* gene since no changes have been observed in a spectrum of cancer and normal cells (Soo et al., 1999; Mhashilkar et al., 2001). At least a partial answer comes from previous studies in

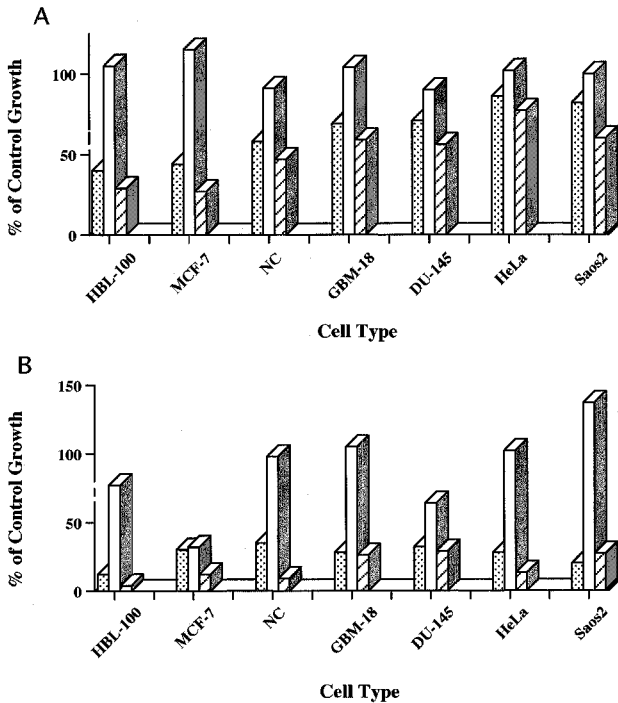


Figure 8 Effect of treatment with IFN- β , MEZ or IFN- β +MEZ on the growth of various normal and human cancer cell lines. The indicated cell type was treated for 48 h (a) or 96 h (b) as in Figure 7 and cell numbers were determined. Triplicate samples varied by $\leq 10\%$ and a replicate experiment varied by $\leq 15\%$. :IFN- β ; :MEZ; :IFN- β +MEZ

melanoma cells which suggest that the *mda-7* promoter is constitutively active in melanoma cells, whereas *mda-7* mRNA expression is restricted to melanoma cells treated with IFN- β +MEZ and induced to terminally differentiate (Madireddi *et al.*, 2000a,b). These findings suggest that post-transcriptional modifications may contribute to *mda-7* mRNA levels in human melanoma cells (Madireddi *et al.*, 2000a). To address the question of cell context specific expression of *mda-7* we have evaluated the effect of IFN- β +MEZ on *mda-7* mRNA expression in a panel of normal and cancer cell types (Figure 4). In addition to induction of *mda-7* expression in most melanomas and constitutive expression of *mda-7* in normal melanocytes and WM35 RGP cells, treatment with IFN- β +MEZ for 24 h induced *mda-7* expression in normal human cerebellum cells (NC), one of two human glioblastoma multiforme cell lines (GBM-18), a human nasopharyngeal carcinoma cell line (HONE-1), a human cervical carcinoma cell line (HeLa), one of three human prostate carcinoma cell lines (DU-145), a normal human breast epithelial cell line (HBL-100), two of five human breast carcinoma cell lines (MDA-MB-157 and MDA-MB-231) and a human osteosarcoma cell line (Saos-2) (Figure 4). To determine the functional significance of this mRNA induction, we determined levels of *MDA-7* protein following treatment of FM516-SV, HO-1 and DU-145 cells with IFN- β +MEZ. For comparison, the same cell types were infected with 1, 10, 50 or 100 pfu/

cell of Ad.*mda-7*, which contains the coding region of *mda-7* without the 3' or 5' regions of this gene (Su *et al.*, 1998). Infection with Ad.*mda-7* resulted in a dose-dependent induction of both *mda-7* mRNA and *MDA-7* protein, with the highest expression in DU-145 cells and reduced expression in FM516-SV and HO-1 cells (Figures 5 and 6). In contrast, treatment with IFN- β +MEZ efficiently induced *mda-7* mRNA, with the level of induction being comparable to low levels of Ad.*mda-7* infection (1 pfu/cell), which did not result in readily detectable intracellular *MDA-7* protein (Figure 6). These results suggest that the level of endogenous *MDA-7* protein resulting following treatment of specific cells with IFN- β +MEZ for 24 h is small and may not correlate with the increase or induction in *mda-7* mRNA. These results suggest a potential role for post-transcriptional processes in regulating endogenous *MDA-7* protein levels in cells. Further studies are also required to determine if there are differences in the stability and/or secretion of endogenously produced *MDA-7* protein versus virally generated *MDA-7* protein.

Evaluation of *mda-7* expression using multiple tissue Northern blots, containing poly(A)⁺ RNA from various tissues, demonstrate that *mda-7* expression is restricted to the thymus, spleen and peripheral blood leukocytes (Figure 2). Previous studies document a 9–12-fold elevation in mRNA expression of a gene *c49a* (a rat homologue of *mda-7*) during the process of wound healing (Soo *et al.*, 1999). Based on these observations, we evaluated a series of hematopoietic cells, representing erythroid, myeloid and lymphoid lineages, with and without induction of specific differentiation programs for expression of *mda-7*. No *de novo mda-7* expression was detected in HL-60 (human promyelocytic leukemia), K562 (human erythroleukemia), CEM-C7 (human T-cell leukemia) or a TPA-resistant variant of HL-60 (HL534) (Figure 2). Similarly, induction of HL-60 differentiation by treatment with TPA (monocyte/macrophage) or DMSO (granulocyte) or treatment of CEM-C7 cells with TPA did not induce *mda-7* expression. However, treatment of K562 cells with TPA, which induces these cells to differentiate into megakaryocytes, resulted in induction of *mda-7* mRNA at levels similar to that found in HO-1 cells treated with IFN- β +MEZ (Figure 2). If this increase in *mda-7* correlates with an elevation of functional *MDA-7* protein these results suggest that *mda-7* might have a biological role in megakaryocytic differentiation. Differentiation of K562 leukemia cells by TPA treatment to megakaryocytic cells is characterized by an increase in platelet peroxidase positivity, enhancement of thromboxane A2 receptors and increased cell volume and DNA ploidy (reviewed in Alitalo, 1990). There is also an increase in synthesis of platelet derived growth factor (PDGF) and transforming growth factor beta 1 (TGF- β 1) two of the cytokines that mark the wound-healing process (Chu *et al.*, 1999; Li *et al.*, 1999; Miller, 1999). The molecular function of *mda-7* during this program of differentia-

tion is unclear at this time, however, it is possible that *mda-7* may have multiple biological roles including an involvement in immune responses and wound healing. Of particular relevance, *mda-7* may have an indirect/direct role in platelet formation during erythroid cell differentiation.

At the time of initial isolation and characterization, computational analysis of *MDA-7* indicated that it was a unique cDNA, showing no homology to known sequences in the databases or containing easily identifiable structural attributes or motifs at the peptide level (Jiang *et al.*, 1994, 1995b, 1996). Among the reported protein domains present in the conceptually translated peptide sequence was an Interleukin-10 (IL-10) signature. Recent information (Chaiken and Williams, 1996; Kottenko *et al.*, 2001; and unpublished data) strongly suggest that *mda-7* belongs to the four-helix bundle family cytokine molecules (Chaiken and Williams, 1996; Kottenko *et al.*, 2001) most related to the IL-10 sub-family (Zhang *et al.*, 2000; Xie *et al.*, 2000; Kottenko *et al.*, 2001). In addition to IL-10, this protein family in humans presently includes IL-19, IL-TIF, AK-155 and IL-20. Although the extent of amino acid homology between members is not extensive (IL-10 and *mda-7* share <20% sequence identity), several similarly related molecules have been discovered and from the collectively available information there is now convincing evidence to classify *mda-7* as a member of the family (Gallagher *et al.*, 2000; Xie *et al.*, 2000; Zhang *et al.*, 2000; Kottenko *et al.*, 2001). Two features of *mda-7* in addition to the presence of an IL-10 family signature and predicted four-helix bundle protein conformation reinforce this idea. These features include a 49 amino acid N-terminal signal peptide classically present in secreted molecules and its location in the human genome on chromosome locus 1q32 where it shows tight linkage to other members of the family, including IL-10, IL-19 and IL-20 comprising, what appears to be a cytokine cluster. It seems from presently documented literature, even for those members of the IL-10 family whose discovery was reported within the last year (IL-19 and IL-20), that each member has a distinct set of functional attributes and tissue distribution. For example, IL-10 has a pleiotropic immunomodulatory effect and is produced in T-cell subsets, monocytes, keratinocytes and activated B-cells (Gallagher *et al.*, 2000; Saito, 2000). IL-19 is monocyte specific (Gallagher *et al.*, 2000) and IL-20 is expressed at low levels in skin and certain other tissues (Blumberg *et al.*, 2001) while *mda-7* appears to be primarily restricted to peripheral blood leukocytes, thymus and spleens of normal adult humans at the RNA level (Figure 2). It appears from the presently available information that each protein has distinct non-overlapping biological effects, not entirely unexpected given the limited homology of primary amino acid sequence. Of these effector functions, the transformed cell specific inhibitory activity appears to be a property unique to *mda-7*. Given its significant structural relatedness to IL-10 family cytokines and clustered location in the IL-10

genomic locus, studies directed toward understanding the cytokine related nature and properties of *mda-7* is likely to yield important information pertaining to biological activity.

Materials and methods

Cell cultures and growth assays

A normal SV40 immortalized human foreskin melanocyte cell line, FM516-SV (FM516), was provided by Dr L Diamond (Wistar Institute, PA, USA). WM35, WM278 and WM239 were obtained from Dr M Herlyn (Wistar Institute, PA, USA) (Jiang *et al.*, 1995a). Metastatic FO-1 and HO-1 melanoma cells were described previously (Fisher *et al.*, 1985). C8161 metastatic melanoma cells were obtained from Dr D Welch (University of Pennsylvania) (Jiang *et al.*, 1995a). Dr RS Kerbel provided the MeWo cell line and its reduced metastatic variant 3S5 and highly metastatic variant 70W (Kerbel and Man, 1984; Graham *et al.*, 1991). SK-MEL p53 mt and SK-MEL p53 wt were provided by Dr A Albino (American Health Foundation, NY, USA). HBL-100, MCF-7, T47D, MDA-MB-157, MDA-MB-231, MDA-MB-453, LNCaP, PC-3, DU-145, HeLa, Saos2, MIA PaCa-2, PANC-1, BxPC-3, AsPC-1, T98G and HONE-1 cells were obtained from the American Type Culture Collection. NC, a normal human cerebellum astrocyte cell line and the GBM-18 human glioblastoma multiforme cell line were established in culture from patient-derived samples (Vita *et al.*, 1988; Guarini *et al.*, 1990). Early passage normal human prostate epithelial cells (HuPEC) were obtained from Clonetics Inc. (CA, USA) and cultured using reagents and medium provided by the company. Most cell lines were grown in Dulbecco's modified Eagle's minimum essential medium supplemented with 5 or 10% fetal bovine serum and antibiotics. The pancreatic carcinoma cell lines were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum. All cells were cultured at 37°C in a humidified 5% CO₂ 95% air incubator. Growth assays were performed by seeding cells in complete growth medium at 1 × 10⁵ cells/35-mm plate and viable cell counts were determined by hemocytometer 48 and 96 h after a medium change without additions (control), 2000 units/ml of IFN-β, 10 ng/ml of MEZ or 2000 units/ml of IFN-β + 10 ng/ml MEZ. Data is presented as average of triplicate samples which varied by ≤10%. An additional study was performed which varied by ≤15%.

Virus construction and plaque assays

The recombinant replication-defective Ad.*mda-7* virus was created in two steps as described previously (Su *et al.*, 1998). Briefly, the coding region of the *mda-7* gene was cloned into a modified Ad expression vector pAd.CMV (Falck-Pedersen *et al.*, 1994). This vector contains, in order, the first 355 bp from the left end of the Ad genome, the cytomegalovirus immediate early promoter, DNA encoding splice donor and acceptor sites, the coding region of the *mda-7* cDNA, DNA encoding a poly(A) signal sequence from the β globin gene, and ~3 kbp of adenovirus sequence extending from within the E1B coding region. This arrangement allows high-level expression of the cloned sequence by the cytomegalovirus immediate early gene promoter, and appropriate RNA processing (Falck-Pedersen *et al.*, 1994). The recombinant virus was created *in vivo* in 293 cells (Graham *et al.*, 1977) by

homologous recombination between *mda-7*-containing vector and plasmid JM17, which contains the whole of the Ad genome cloned into a modified version of pBR322 (McGrory *et al.*, 1988). JM17 gives rise to Ad genomes *in vivo* but they are too large to package. This constraint is relieved by recombination with the vector to create a packageable genome (McGrory *et al.*, 1988), containing the *mda-7* gene. The recombinant virus is replication defective in human cells except 293 cells, which express adenovirus E1A and E1B. Following transfection of the two plasmids, infectious virus was recovered, the genomes were analysed to confirm the recombinant structure, and then virus was plaque purified, all by standard procedures (Volkert and Young, 1983).

Northern and Western blotting assays

Multiple-tissue Northern blots (Clontech) of poly(A)⁺ mRNA extracted from different human tissues were hybridized in ExpressHyb solution (Clontech) with the coding region of the *mda-7* cDNA. The normal tissue analysed included heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, colon and peripheral blood leukocyte. The *mda-7* probe was labeled with α -³²P-dCTP using a random primer labeling kit and membranes were washed according to manufacture's protocol (Amersham, IL, USA). Levels of *mda-7* and GAPDH mRNA in untreated, IFN- β +MEZ (2000 units/ml+10 ng/ml) treated or Ad.*mda-7* infected cells were determined by Northern blotting analysis of total cytoplasmic RNA as previously described (Su *et al.*, 1998). In brief, 10 μ g of total RNA from the different cell types were electrophoresed in 1% agarose gel, transferred to a nylon membrane, and hybridized with the different ³²P-labeled cDNA fragments. The membrane was stripped and hybridized with the indicated probes sequentially. Northern blots were washed in a 0.1% SDS, 1 \times SSC buffer at room temperature for 30 min followed by washing at 42°C for an additional 30 min in the same buffer. After hybridization, the nylon membranes were washed and exposed for autoradiography.

MDA-7 and elongation factor 1-alpha (EF-1 α) protein levels were determined by Western blotting as described previously (Su *et al.*, 1995). Cells were grown in 100-cm plates and after appropriate treatment were washed twice with cold PBS and lysed on ice for 30 min in 100 μ l of cold RIPA buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% SDS, 1% NP40, and 0.5% sodium deoxycholate] with freshly added 0.1 mg/ml phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 1 mg/ml aprotinin. Cell debris were removed by centrifugation at 14 000 *g* for 10 min at 4°C. Protein concentrations were determined using the Bio-Rad protein assay system (Bio-Rad Laboratories, Richmond, CA, USA). Aliquots of cell extracts containing 20–50 mg of total protein were resolved in 12% SDS-PAGE and transferred to Immobilon-P PVDF membranes (Millipore Corp., Bedford, MA, USA). Filters were blocked for 1 h at room temperature in Blotto A [5% nonfat milk powder in TBS-T; 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.05% Tween 20], and then incubated for 1 h at room temperature in Blotto A containing a 1:1000 dilution of rabbit anti-*MDA-7*, polyclonal antibody. After washing in TBS-T buffer (3 \times 5 min, room temperature), filters were incubated for 45 min at room temperature in Blotto A containing a 1:10 000 dilution of corresponding peroxidase conjugated anti-rabbit secondary antibody (Amersham, Arlington Heights, IL, USA). After washing

in TBS-T, ECL was performed according to the recommendation of the manufacturer.

Isolation of *mda-7* genomic clones

Polymerase chain reaction amplification Diploid human fibroblast DNA (Clontech) was used as a template with human *mda-7* gene specific primers, (5'-primer) 5'-ACAA-GACATGACTGTGAGGAG-3' and (3'-primer) 5'-AGACTGTTTCAAATGACACAG-3'. The proof reading and high efficiency Advantage Th DNA polymerase (Clontech) was used in all reactions. The PCR cycling conditions were 95°C/1 min, 60°C/1 min and 72°C/6 min. The reaction was processed for 30 cycles with an additional 72°C/10 min extension performed at the end. The PCR reaction product was analysed by agarose gel electrophoresis and sequencing by the ABI method.

Library screening A human placental genomic lambda Fix II library (Stratagene, La Jolla, CA, USA) was screened using the human *mda-7* cDNA. The probe was labeled with α -³²P-dCTP using a random primer labeling kit from Amersham according to manufacture's protocol. Plaque lift filters were hybridized overnight in hybridization buffer (ExpressHyb, Clontech) at 68°C. The filters were washed at 55°C for 20 min, twice in 2 \times SSC, 0.1% SDS buffer, and once in 0.5 \times SSC, 0.1% SDS buffer and exposed to X-ray (Kodak) film.

DNA sequencing of *mda-7* human genomic clone The isolated phage DNA clones were mapped by restriction enzyme analysis using standard procedures (Sambrook *et al.*, 1989). PCR generated products were cloned into pBluescript and sequenced using universal primers with an ABI automatic sequencer model 372 (Applied Biosystems). DNA and protein sequence alignment were determined using the GCG software package (Wisconsin Package Version 10.0, Genetics Computer Group (GCG), Madison, WI, USA). The percentage of DNA and Protein sequence homology was determined using the GenBank database and GCG homology algorithms including BLAST, FATSAs, BESTFIT and GAP.

Chromosomal mapping of the human *mda-7* gene

For chromosomal mapping studies, oligonucleotides for generating PCR products were designed using the computer program Oligo 4.0 (National Biosciences) based on the *mda-7* sequence (Jiang *et al.*, 1995b). The two primers used to amplify a 129 bp human *mda-7* specific gene product were: MDA7F, 5'GGTTTGTTCCTGTGTCATT3' and MDA7R, 5'GCGCTGCTTAAAGAATGACT3'. These primer sets were used with PCR to determine the presence or absence of the *mda-7* locus in a panel of 19 rodent-human hybrids. PCR reactions were conducted in a final volume of 12.5 μ l with 100 ng of template, 20 ng primers, 10 mM tris-HCL pH 8.3, 50 mM KCl, 0.1 mg/ml gelatin, 15 mM MgCl₂, 200 μ M dNTPs and 0.5 U Taq polymerase. Amplifications were performed in a Perkin-Elmer Cetus 9600 thermal cycler for 30 cycles at 94°C for 30 s, 58°C for 30 s and 72°C for 30 s. The PCR products were visualized in ethidium bromide stained 1.5% agarose gels. The amplification product was purified with Qiagen PCR purification kit, and 1 ng of DNA and 20 ng specific primer used with the Taq Dye Deoxy Terminator Cycle Sequencing Kit (ABI). The reaction products were electrophoresed and recorded on the 377 DNA sequencer (ABI).

Acknowledgments

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