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Loss of Novel *mda-7* Splice Variant (*mda-7s*) Expression is Associated with Metastatic Melanoma[1]

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Expression of melanoma differentiation associated gene-7 (*mda-7*) also known as interleukin 24 (IL-24) decreases during melanoma cell differentiation and induces apoptosis in melanoma cells but not in melanocytes. Here we identify a novel splice variant of the cancer growth suppressor gene *mda-7*/IL-24 (*mda-7s*) that is differentially expressed in RNA preparations from normal human melanocytes, transformed melanocytes, nevi, subcutaneous metastasis, lymph node metastasis, and melanoma cell lines. The 450 bp *mda-7s* mRNA encodes a protein of 63 residues with a molecular weight of 12 kDa. *mda-7s* lacks exons 3 and 5 of the full-length transcript and contains only 14 amino acids of homology to MDA-7 located within the signal peptide region of the wild-type sequence. Despite minimal homology, MDA-7S coprecipitates full length MDA-7 and reduces secretion of cotransfected MDA-7. *mda-7* and *mda-7s* are coexpressed in all RNA preparations other than subcutaneous and lymph node metastasis where *mda-7s* expression is lacking. *mda-7s* expression is therefore linked to a non-metastatic phenotype.

Key words: alternative splicing/interleukin-24/neoplasm metastasis
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Melanoma currently affects one person in 70 with an increasing lifetime risk and represents, in the advanced stages of disease, a paradigm of oncological treatment resistance (Soengas and Lowe, 2003). In the progression of melanocytic transformation within normal nevi to radial and the vertical growth phase melanoma eventually leading to metastatic melanoma, cells of the melanocytic lineage lose contact with keratinocytes and become resistant to apoptosis induction by chemotherapeutic agents with a corresponding reduction of median survival of patients (Houghton and Polsky, 2002).

The melanoma differentiation-associated gene 7 (*mda-7*), also known as interleukin 24 (IL-24), contains seven exons and is transcribed from a locus at 1q 34.2–41 which contains several members of the IL-10 family of cytokines (Huang *et al*, 2001). The full-length human *mda-7* 1718 nt cDNA encodes a protein of 206 amino acids in the major open-reading frame, with a predicted molecular weight of 23.8 kDa (Jiang *et al*, 1996), which, due to glycosylation, migrates with an apparent molecular weight of 35 kDa (Wang *et al*, 2002). Originally cloned as a gene upregulated during terminal differentiation of melanoma cells induced by recombinant human fibroblast interferon (IFN-β) and the protein kinase C activator mezerein (Jiang *et al*, 1995), MDA-7 expression levels progressively decrease during the transformation of melanocytic cells to metastatic melanoma (Ekmekcioglu *et al*, 2001). At supraphysiological levels, MDA-7 selectively induces growth suppression and apoptosis in diverse human cancers including melanoma, breast, colon, prostate, small cell lung, and pancreatic carcinoma whereas no quantitatively significant effect is found in normal human cells such as melanocytes and endothelial cells (Sarkar *et al* 2002). MDA-7 overexpression in a replication-defective adenovirus (Ad.*mda-7*) in melanoma cells induces activation of the growth arrest and DNA damage (GADD) family genes (GADD153, GADD34, GADD45α, and GADD45γ) via p38 MAPK. This is associated with a reduction of BCL2 protein levels leading to induction of apoptosis in melanoma cells but not in normal melanocytes (Sarkar *et al*, 2002). It has also been reported that Ad.*mda-7* transfection not only confers growth suppression, but also arrests the cell cycle at G2/M in melanoma cells (Ekmekcioglu *et al*, 2001). Ad.*mda-7* (INGN 241) has entered clinical trials for solid tumors (Fisher *et al*, 2003).

As an IL-10 family cytokine, MDA-7 is secreted by concanavalin A-activated peripheral blood mononuclear cells (Wang *et al*, 2002) and demonstrates immunostimulatory activity (Caudell *et al*, 2002). Secreted MDA-7 binds two functional heterodimeric receptors IL-20R1/IL-20R2 and IL-22R1/IL-20R2 on target cells such as keratinocytes, which leads to stat activation (Dumoutier *et al*, 2001; Wang *et al*, 2002).

Although *mda-7* mRNA is found in normal human melanocytes and metastatic melanoma cell lines at lower levels, MDA-7 protein levels decrease in more advanced

Abbreviations: IL, interleukin; *mda-7*, melanoma differentiation-associated gene-7
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melanoma and metastatic disease (Su *et al*, 1998; Ekmekcioglu *et al*, 2001).

In recent years, the identification of tissue-specific differential splicing of key gene products has greatly increased the complexity of the transcriptome and led to further understanding of the control of functional protein expression (Sorek and Amitai, 2001). During a search of GenBank-expressed sequenced tags, we identified examples of *mda-7* splicing of exons 1–4 in prostate and colon tissue. To examine the role of differential *mda-7* splicing in the melanocytic lineage, we amplified *mda-7* sequences by PCR and identified a splice variant of *mda-7* (*mda-7s*) in normal human melanocytes, which lacks both exons 3 and 5. MDA-7S has a molecular weight of 12 kDa, contains homologies to wild-type MDA-7 derived only from exon 2, but heterodimerizes with wild-type MDA-7 and reduces secretion of the wild-type protein. Subsequent screening of melanoma RNA samples identified a complete lack of *mda-7s* expression in subcutaneous metastatic tumor material, with lymph node metastases demonstrating positive association with a non-metastatic phenotype.

Results

Differential splicing of *mda-7* and isolation of *mda-7s* The full-length 1718 bp *mda-7* mRNA (accession NM_006850.1) transcript (Jiang *et al*, 1995) is spliced from a 5532 bp locus at chromosome 1q 34.2–41 (Huang *et al*, 2001) on GenBank clone RP11-462N18 (accession AC023534.3). Seven exons are spliced to form the mature transcript in which the reading frame extends from exons 2 to 7. To investigate the possible role of differential splicing in the control of *mda-7* expression and function, we conducted BLAST searches of expressed sequence tag homologies to the wild-type *mda-7*/IL-24 sequence. Two clones (accession numbers AW949784.1 and AW949792.1) isolated from colon tumor material and a sequence isolated from normal prostate gland tissue (AA370518.1) that showed direct splicing of *mda-7* exons 1–4 were identified. RT-PCR is a highly sensitive method for detection of even single molecules of cDNA. To investigate the possibility of differential splicing in the melanocytic lineage, cDNA preparations from normal human melanocytes were PCR amplified by primer sets spanning the coding region of *mda-7*. In addition to the full-length *mda-7* transcript, an additional smaller amplificant was also seen in all melanocytic preparations (Fig 3*a*, lanes 1–4). Isolation, cloning, and sequencing of this transcript demonstrated the presence of a novel splice variant (*mda-7s*) where exon 2 sequences, which contain the *mda-7* start codon, are spliced directly to exon 4, and exon 4 sequences are spliced directly to exon 6 (Fig 1*a* and *b*). The coding sequence of *mda-7s* and positions of inter-exonic splicing are shown in Fig 1*a*. The predicted open reading frame of *mda-7s* has 63 amino acid residues derived from sequences located in exons 2, 4, 6, and 7 (Fig 1*a*). Protein homology to wild-type MDA-7 is limited to 14 amino acids derived from exon 2 (Fig 1*a*), since the exon 2 to exon 4 transition results in a frame shift, which is maintained after the splicing of exon 4 to exon 6. These 14 amino acids represent the initial residues of the 49 residue wild-type N-

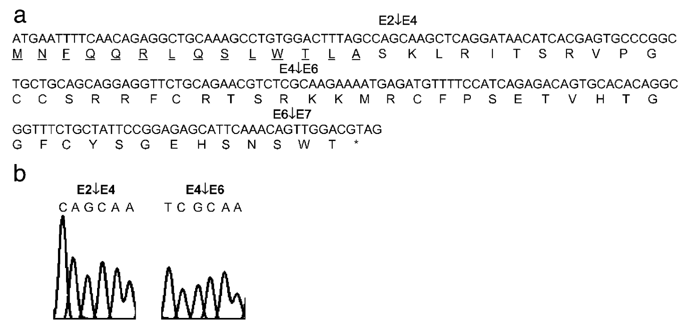

Figure 1
Differential splicing of *mda-7* RNA. The *mda-7* splice variant (*mda-7s*) was amplified and cloned from normal human melanocytes. (*a*) *mda-7s* is spliced (↓) aberrantly between exons (E) 2 and 4, and exons 4 and 6 shown in the sequence traces (*b*) generating a reading frame between exons 2 and 7. Homologies between the 206 residue wild-type MDA-7 and the 63 amino acid MDA-7S are restricted to 14 residues (underlined) derived from exon 2.

terminal amino acid signal peptide sequence but lack the signal peptidase cleavage sites between residues 49 and 50 of wild-type MDA-7 (Sauane *et al*, 2003). MDA-7S also contains no predicted cleavage sites suggesting MDA-7S is not secreted. Due to the low sequence homologies, MDA-7S also lacks the IL-10 signature motif, N-glycosylation sites, and the protein kinase c and casein kinase II phosphorylation motifs present in the wild-type sequence (Sauane *et al*, 2003).

MDA-7S heterodimerizes with MDA-7 The molecular mass of C-terminal HA-tagged MDA-7S as determined by western blotting of transfected extracts is 12 kDa (data not shown) approximating to the predicted MDA-7S molecular mass (7.3 kDa) which increases to 9.2 kDa incorporating the HA peptide. It is unlikely that this slight increase in observed molecular weight is due to glycosylation since no N-linked sequences are found and low probilities of O-linked glycosylation are predicted with bioinformatics (data not shown). The transfected MDA-7CTGFP fusion protein migrates as predicted with a molecular weight of 62 kDa equaling the sum of MDA-7 (35 kDa) and cycle 3 GFP (27 kDa) molecular masses (data not shown).

Since a common property of IL-10 family cytokines is homodimerization (Fickenscher *et al*, 2002), we investigated whether MDA-7S interacts *in vitro* with wild-type MDA-7. HEK293 cells were cotransfected with the pMH*mda-7s*-HA, pMH-HA, p*mda-7*CTGFP and pCTGFP plasmids. Following immunoprecipitation with either anti-HA or anti-GFP antibodies, associated proteins were detected by western blotting with antibodies directed against GFP or HA, respectively. The 12 kDa HA tagged MDA-7S associated with the wild-type 62 kDa MDA-7CTGFP fusion protein when precipitated with either the anti-HA (Fig 2*a*) or anti-GFP antibodies (Fig 2*b*). Cotransfected pMH*mda-7s*-HA and pCTGFP (Fig 2*a*) or pMH-HA and p*mda-7*CTGFP (Fig 2*b*) showed no interaction.

To examine whether *mda-7s* interaction affects the *mda-7*-mediated induction of apoptosis in melanoma cells, MelJUSO cells were first transfected with p*mda-7*CTGFP and monitored by flow cytometry. Twenty-four hours post transfection, 1%–2% of cells were green fluorescent and

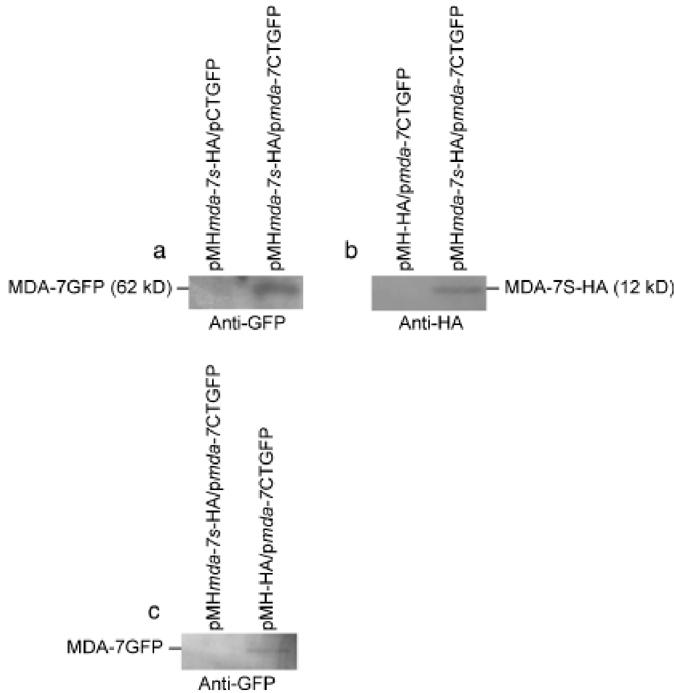

Figure 2
Functional interaction between MDA-7 and MDA-7S. HEK293 cells were transiently cotransfected with HA-tagged MDA-7S (pMH*mda-7s*HA), wild-type *mda-7* as a C-terminal GFP fusion protein (p*mda-7*CTGFP), or the empty vectors pCTGFP and pMH-HA. Cell lysates were precipitated either on anti-HA agarose (*a*) or with anti-GFP antibodies on protein G (*b*) and supernatants were western blotted and probed with monoclonal antibodies against GFP (*a*) or HA (*b*). MDA-7S-HA coprecipitated the 62 kDa MDA-7CTGFP protein (*a*) and MDA-7CTGFP, the 12 kDa MDA-7S-HA polypeptide (*b*), demonstrating functional interaction between the proteins. (*c*) HEK293 cells were transfected with p*mda-7*CTGFP and either pMH*mda-7s*HA or pMH-HA and the levels of secreted MDA-7CTGFP were assessed in the medium by western blotting. Coexpression of MDA-7CTGFP and MDA-7S-HA significantly reduced secretion of MDA-7CTGFP.

all cells were permeable to the vital dye propidium iodide after 48 h. Cotransfection with *mda-7s* did not influence the kinetics of cell death induction.

Since IL-10 family cytokines are known to homodimerize and MDA-7/MDA-7S homologies are confined to the leader peptide sequence, we investigated the role of MDA-7S on the secretion of MDA-7CTGFP. As previously reported (Caudell *et al*, 2002), secreted MDA-7 accumulates in the culture media of transfected HEK293 cells. In cells cotransfected with p*mda-7*CTGFP and pMH*mda-7s*-HA, reduced secretion of wild-type MDA-7CTGFP is observed (Fig 2*c*).

Association between *mda-7s* expression and metastatic melanoma To evaluate the expression of *mda-7s* during the transformation of melanocytes to metastatic melanoma, *mda-7* expression was analyzed by PCR. Normal human melanocytes coexpress *mda-7* and *mda-7s* (Fig 3*a*, lanes 1–4). RNA isolated from spontaneously transformed melanocyte cultures (Fig 3*a*, lanes 5–7) which are cultured in normal medium and correspond to the original melanocyte culture RNA in lanes 2–4 (Fig 3*a*), respectively, also express both *mda-7* and *mda-7s*. To examine possible changes in *mda-7s* expression during melanoma progression, samples were compared from normal nevi, subcutaneous metastasis

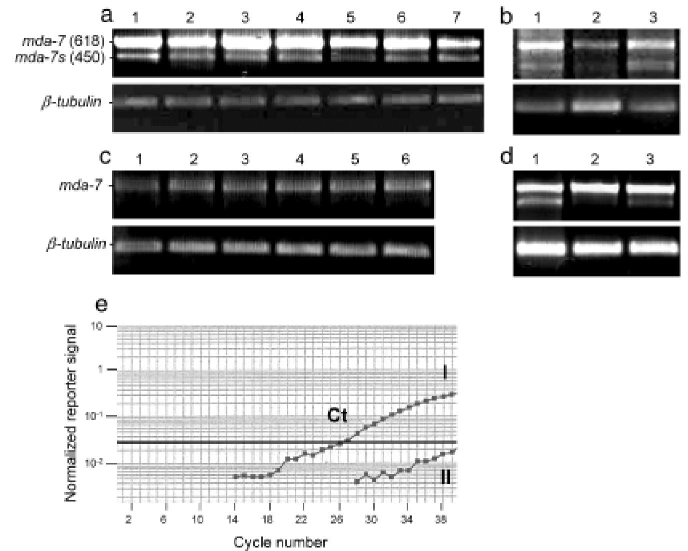

Figure 3
Expression of *mda-7s* during melanoma development. Expression of *mda-7*, *mda-7s*, and β-*tubulin* mRNA expression was analyzed by PCR in normal human melanocytes (*a*, lanes 1–4), spontaneously transformed melanocytes (*a*, lanes 5–7), nevi (*b*, lanes 1–3), lymph node metastasis (*c*, lanes 1–3), subcutaneous metastasis (*c*, lanes 4–6) and the melanoma cell lines A375, 607B and MelJUSO (d, lanes 1–3). In real-time PCR amplification of *mda-7s* (*e*), representative graphs show expression levels in normal melanocytes (I) decreasing to undetectable levels in metastatic melanoma samples within 40 cycles of amplification (II). Normalized reporter signal represents fluorescence changes adjusted to baseline before amplification and (Ct) the threshold cycle of amplified products.

(stage IV) and lymph node metastasis (stage IV). Similar to melanocyte patterns, coexpression of *mda-7* and *mda-7s* was seen in normal nevi (Fig 3*b*, lanes 1–3) mirroring the results obtained from spontaneously transformed melanocyte cultures showing no association between *mda-7s* expression and cellular transformation. Analysis of subcutaneous (Fig 3*c*, lanes 1–3) and lymph node metastatic (Fig 3*c*, lanes 4–6) tumor material, however, demonstrated no expression of *mda7-s* in independent patient samples summarized in Table I, indicating an association between loss of *mda-7s* expression and metastatic development. With regard to *mda-7* expression, we cannot exclude contamination of melanoma samples with other cells such as lymphocytes, which may express *mda-7*. Differential *mda-7s* expression patterns were seen in melanoma cell lines. In A375 and MelJUSO cells, coexpression of *mda-7* and *mda-7s* is seen (Fig 3*d*, lanes 1 and 3) whereas in the 607B cell line, no expression of *mda-7s* was detected (Fig 3*d*, lane 2). Expression levels of *mda-7s* were also assessed in a real time PCR assay. Confirming the RT-PCR data, *mda-7s* is expressed in melanocytes with normalized threshold cycle values of 28.3 ± 2.4 (mean three experiments) but is not detected in metastatic melanoma samples within 40 cycles of amplification (Fig 3*e*).

Discussion

Great interest in *mda-7* as a clinically relevant, candidate tumor suppressor gene has been generated due to its growth arrest and apoptosis-inducing properties in

Table I. Summary of *mda-7s* expression status during metastatic melanoma progression

Patient sample	Tissue type	Disease stage	Characterization	*mda-7s* profile
1	Nevi	–	Normal	Positive
2	Nevi	–	Normal	Positive
3	Nevi	–	Normal	Positive
4	Subcutaneous	III/IV	Metastatic	Negative
5	Subcutaneous	III/IV	Metastatic	Negative
6	Subcutaneous	III/IV	Metastatic	Negative
7	Lymph node	III/IV	Metastatic	Negative
8	Lymph node	IV	Metastatic	Negative
9	Lymph node	IV	Metastatic	Negative

mda-7s expression is seen in nevi and primary stage I/II melanoma tumors. In subcutaneous stage III/IV disease, lymph node metastatic stage III/IV disease and terminal disease stages, no expression of *mda-7s* is seen.

melanoma cells which are not seen in normal human melanocytes subjected to ectopic overexpression (Ekmekcioglu *et al*, 2001; Ellerhorst *et al*, 2002). Both *mda-7* RNA and MDA-7 expression are reduced during the progression of melanoma, and significant differences in MDA-7 expression between primary tumors and metastases have been reported (Ellerhorst *et al*, 2002). Expression levels may therefore be regarded as a diagnostic marker for disease progression and adenoviral vectors encoding *mda-7* (INGN 241) have entered clinical trials (Fisher *et al*, 2003). During a BLAST search of GenBank expressed sequence tag homologies to the wild-type *mda-7*/IL-24 sequence, we identified two clones from colon tumor material and a sequence isolated from normal prostate gland tissue that showed direct splicing of *mda-7* exons 1–4. To investigate the possible role of differential splicing in the control of MDA-7 expression and function in the melanocytic lineage, we subsequently isolated the *mda-7s* transcript, which is produced by direct splicing of exons 2, 4, and 6. As common to the other sequences found in databanks, our data clearly show aberrant splicing to exon 4 within the *mda-7* gene. *Mda-7s* RNA also contains the polyadenylation signal and the AU-rich mRNA destabilization sequences in exon 7 that are essential for protein-mediated post-transcriptional regulation of wild-type *mda-7* transcripts (Madireddi *et al*, 2000).

In general, alternative splicing often results in functional protein expression (Rinfret and Anderson, 1993) and considerably increases the transcriptome. Splice variant protein expression may also lead to a reduction in functional transcript expression leading to altered pathology (Hau *et al*, 2002). This finding has been reported by a number of groups and include the tumor suppressor gene *men1* (Mutch *et al*, 1999), *pten* (Butler *et al*, 1999), *nf-2* (Jacoby *et al*, 1994), and the transcription factor *pax-2* (Tavassoli *et al*, 1997). In recent years, the human-expressed sequence tag (EST) database has been analyzed by a number of different groups to investigate differential splicing (Sorek *et al*, 2001) and it has been estimated that up to 59% of human genes have at least two tissue-specific splice variants.

Since evidence of splice variants conferring dominant negative effects has recently been reported (Korkalainen *et al*, 2003), we investigated the effects of *mda-7s* on the apoptosis-promoting properties of the wild-type protein. In MelJuso melanoma cells, wild-type *mda-7* rapidly induced cell death, which was not inhibited by cotransfection with an *mda-7s* expression plasmid demonstrating that heterodimeric MDA-7/MDA-7S expression has no effect on the apoptosis-inducing properties of the wild-type protein. In addition, MDA-7S expression levels were not significantly decreased during spontaneously transformed melanocyte cultures described previously (Selzer *et al*, 1998), indicating that MDA-7S expression is not directly associated with melanocyte transformation, nor by culture in melanocyte growth medium which contains additional growth factors and phorbol esters.

Predicted amino acid sequence homologies between MDA-7S and MDA-7 are limited to the N-terminal 14 amino acids derived from exon 2. MDA-7S, however, lacks the signal peptidase cleavage sites present in wild type MDA-7 and no evidence of secretion is detected in transfected HEK293 supernatants (data not shown). In contrast to other IL-10 family cytokines, MDA-7 (IL-24) has an extended signal peptide sequence, the functional significance of which has not been addressed. We show that MDA-7S has homology to MDA-7 only within this signal peptide sequence and although homodimerization of wild-type MDA-7 has not been reported, MDA-7S and MDA-7 proteins functionally interact *in vitro* and secretion of transfected MDA-7 is inhibited. Future investigations of the MDA-7S residues responsible for interaction with wild-type MDA-7 should also reveal whether MDA-7 homodimerizes similar to other IL-10 family cytokines. Studies to determine the precise subcellular localization of MDA-7S are in progress and should lead to a fuller understanding of the role of this heterodimerization during the MDA-7 secretory process within melanocytic cells.

During the rapid development of malignant melanoma, transformed melanocytic lineage cells develop through a vertical growth phase and become highly metastatic. The exact expression patterns conferring a metastatic phenotype have not been elucidated. Although *mda-7s* expression is seen in melanocytes, transformed melanocytes and normal nevi, a complete absence of expression is associated with both local (subcutaneous) and distant (lymph node) metastasis. Since secreted MDA-7 binds IL-20R1/IL-20R2 and IL-22R1/IL-20R2 on target cells lead to stat activation (Wang *et al*, 2002), the decreases in *mda-7* expression seen during the development of metastatic disease may represent a previously unknown mechanism for MDA-7 secretion in the activation of accessory cells. Although the precise role of MDA-7S in this process is unclear, this association demonstrates that MDA-7S expression levels could potentially play a role in advances of diagnosis when determining the progression of a localized lesion and assessing the disease stage of melanoma tumors.

In summary, this study has identified a novel *mda-7* splice variant (*mda-7s*) in human melanocytes. The splice variant was determined to have molecular weight of 12 kDa and lacked exons 3 and 5 of *mda-7* but functionally interacted with and influenced the secretion of the wild-type

protein *in vitro*. Subsequently, a screen of melanoma patient samples was performed and lymph node metastatic and subcutaneous metastatic tumors were identified as being deficient of *mda-7s* message when assessed via both RT-PCR and quantitative PCR.

Material and Methods

Cell culture MelJUSO, A375 (ATCC, Manassas, Virginia) and 607B melanoma cells, transformed melanocytes (Selzer *et al*, 1998), and the HEK293 cell line were maintained in Dulbecco's modified Eagle's culture medium containing 4.5 g per liter glucose (DMEM), supplemented with 10% fetal calf serum (Gibco, Paisley, Scotland) and an antibiotic-antimycotic mix containing 100 U per mL of penicillin, 100 μg per mL of streptomycin and 0.25 μg per mL of amphotericin B (Gibco) in a fully humidified 5% CO_2–95% ambient air atmosphere at 37°C. Normal human melanocytes (Clonetics, Remagen, Germany) were cultured in serum-free melanocyte growth medium (MGM-2) containing 10 μg per mL phorbol 12-myristate-13-acetate, 0.5 μg per mL hydrocortisone, 5 μg per mL insulin, 5 μg per mL human fibroblast growth factor, 50 μg per mL gentamycin-sulfate, 50 μg per mL amphothericin-B supplemented with bovine pituitary extract as recommended by the suppliers.

Subjects and RNA isolation Tumor material was collected at the Department of Dermatology at the University of Vienna following approval by the University of Vienna ethics committee. Tumor samples were isolated after routine surgical removal and placed immediately in RNAlater (Qiagen, Hilden, Germany). Melanocyte and melanoma cell line pellets were resuspended and tissue samples homogenized in TRI Reagent (Sigma, St Louis, Mo.) and total RNA was reverse transcribed with Oligo $(dT)_{12–18}$ and SuperScript II (Gibco) according to the manufacturer's instructions. Institutional Review Board approval was granted for this work.

PCR and vector construction Primer positions are based on the *mda-7*/IL-24 sequence GenBank accession number NM_006850.1. RT-PCR was independently performed with *mda-7* forward (272 5′-GAGATGAATTTTCAACAGAGGC-3′ 293) and reverse (895 5′-CGAGCTTGTAGAATTTCTGCATCC-3′ 870) primers, and the tubulin forward (1263 5′-CATCCAGGAGCTCTTCAAGC-3′ 1282) and reverse (1442 5′-CTCCTCACCGAAATCCTCCT-3′ 1461) primers based on the β-*tubulin* RNA sequence (AF141349.1). cDNA (1 μL) was amplified in a 100 μL reaction containing 200 μM dNTPs, 1.5 mM $MgCl_2$, 20 mM Tris-HCl (pH 8), 50 mM KCl, and 1 U Platinum Taq polymerase (Invitrogen, San Diego, California) in a GenAmp thermocycler 2400 (Applied Biosystems, Perkin-Elmer, Foster City, California) by initial denaturation at 95°C for 2 min, 35 cycles of amplification at 95°C, denaturation for 30 s, annealing at 60°C for 30 s, extension at 72°C for 1 min and end extension at 72°C for 7 min. PCR products were separated on 1% agarose gels containing 0.5 μg per mL ethidium bromide at 1 V/cm in TBE buffer. *mda-7s* fragments were gel purified with the QIAquick PCR Purification Kit (Qiagen) and sequenced on both strands with the amplification primers on an ABI-Prism 3100 Genetic Analyzer (Applied Biosystems). The coding sequence of full length *mda-7* was amplified from melanocyte cDNA and gel purified as before and cloned into the pcDNA3.1/CT-GFP-TOPO vector (Invitrogen) as a C-terminal GFP fusion (*mda-7*-GFP) with the forward (272 5′-GAGATGAATTTTCAACAGAGGC-3′ 293) and reverse (5′-CGAGC TTGTAGAATTTCTGCATCC-3′ 868) primers, the reverse primer deleting the stop codon and inserting a 5′ terminal G residue to maintain the reading frame for GFP, according to the manufacturer's instructions. The reading frame of *mda-7s* was amplified from melanocyte cDNA using the forward (5′-GGGGTACCCCGAGAT GAATTTTCAACAG-3′ 289) and reverse (5′-CGGAATTCCGCGTC CAACTGTTTGAATG-3′ 801) primers incorporating KpnI and EcoRI restriction sites, respectively. The PCR product was gel purified, restriction digested and cloned as a C-terminal hemagglutinin (HA)-tagged protein into the pMH vector (Roche, Basel, Switzerland) by standard procedures (pMH*mda-7s*-HA) and sequenced on both strands with a ABI-Prism 3100 Genetic Analyzer (Applied Biosystems).

Transfection and conditioned media HEK293 and MelJUSO cells were transiently transfected with optimally diluted endotoxin-free p*mda-7*-GFP and pMH*mda-7s*-HA plasmid preparations in six-well plates in the presence of Fugene (Roche) at a DNA to lipid ratio of 3:1. As assessed by flow cytometry in the presence of 5 μg per mL propidium iodide, transfection efficiencies were 20%–30% and 1%–2% for HEK293 and MelJUSO cells, respectively. Conditioned media were harvested after 48 h.

Western blotting and immunoprecipitation Cells were harvested by trypsinization and washed first in cold complete medium and subsequently in phosphate-buffered saline (PBS). Cells from cultures in logarithmic growth, where cell viabilities were estimated at greater than 99% by trypan blue exclusion, were lysed in hypotonic lysis buffer (1% Triton X-100, 150 mM NaCl, 25 mM Tris, pH 7.4, 5 μg per mL leupeptin, aprotinin, and pepstatin, 1 μg per mL benzamidine HCl, 1 mM sodium orthovanadate and 1 mM PMSF) on ice at a density of approximately 10^6 cells per mL. Protein concentrations were determined by a modified Bradford method (Bio-Rad, Hercules, California). For immunoprecipitations, HEK293 cells were harvested in PBS and incubated in 1 mL of cell lysis buffer (PBS containing 5 mM EDTA, 0.5% Triton X-100, 0.1 mM PMSF, 5 μg per mL leupeptin, aprotinin, and pepstatin) for 30 min on a rotary shaker at 4°C. After centrifugation at 15,000 × *g* for 30 min at 4°C, supernatants were incubated with 100 μL anti-HA affinity matrix (clone 3F10; Roche) overnight or sequentially with protein G-Agarose (Roche) for 1 h before addition of mouse monoclonal anti-GFP (clone ab1218; Abcam, Cambridge, UK). The matrix was then washed three times in cell lysis buffer at 4°C. Western blotting was performed essentially as described (Lucas *et al*, 2001). Protein loading was routinely controlled by staining of gels and membranes (Gelcode blue; Pierce, Rockford, Illinois). Briefly, cell extracts, immunoprecipitates or conditioned medium were mixed with loading buffer (62.5 mM Tris, pH 6.8%, 10% glycerol, 2% sodium dodecyl sulphate (SDS), 5% β-mercaptoethanol and 0.003% bromophenol blue) at a ratio of 1:1, boiled for 7 min at 95°C and centrifuged at 15,000 × *g* for 30 s. Proteins (15 μg per lane) were separated by SDS polyacrylamide gel electrophoresis (PAGE) at 100 V on 12% gels and transferred onto PVDF membranes (Tropix, Foster City, California) at 100 V for 1.5 h. Membranes were blocked for 1 h in 0.2% I-block (Tropix) in PBS and then incubated with monoclonal mouse anti-GFP or rat anti-HA. Membranes were then incubated with alkaline phosphatase conjugated anti mouse immunoglobulins (Tropix) or sequentially with biotinylated monoclonal anti-rat IgG_1 (clone RG11/39.4; Pharmingen, San Diego, California) and alkaline phosphatase-conjugated streptavidin (Pharmingen) in 0.2% I-block. Blots were then washed twice in 0.2% I-block and developed using CSDP (Tropix).

Real-time PCR To detect *mda-7s*, a forward primer in exon 2 (281 5′-TTTCAACAGAGGCTGCAAAGC-3′ 301) was combined with a reverse primer in exon 4 (546 5′-AGCCGGGCACTCGTGAT-3′ 530) and a fluoresent (303′ 5′-TGTGGACTTTAGCCAGCAAGCTCAGGA-3′ 525) probe spanning the exon 2/4 boundary. Primer positions are based on the wild-type *mda-7*/IL-24 sequence (NM_006850.1). PCR amplifications were performed on 2 μL of melanocytic or melanoma cDNA samples with 5 pmol of FAM-labeled probe (VBC-genomics, Vienna, Austria) and 5 pmol of primers in a 25 μL reaction containing TaqMan Universal PCR master mix (Applied Biosystems). Real-time PCR was initiated at 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min and threshold cycle values were analyzed with Sequence Detector v1.7 software (Applied Biosystems) in a PRISM 7700 sequence detector (Applied Biosystems) as described previously (Lucas *et al*, 2004).

Sequence analysis Molecular mass predictions were calculated with the Compute Mw tool (http://us.expasy.org/tools/pi_tool.html), signal peptide recognition at SignalP V1.1 (http://www.cbs.dtu.dk/services/SignalP) and homologies were determined at the ncbi.nlm.nih.gov/blast interface.

Accession numbers The *mda-7s* sequence has the GenBank accession number AY237723.

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