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Epigenetic regulation of microRNA-375 and its role in melanoma development in humans

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ABSTRACT

To identify epigenetically regulated miRNAs in melanoma, we treated a stage 3 melanoma cell line WM1552C, with 5AzadC and/or 4-PBA. Several hypermethylated miRNAs were detected, one of which, miR-375, was highly methylated and was studied further. Minimal CpG island methylation was observed in melanocytes, keratinocytes, normal skin, and nevus but hypermethylation was observed in patient tissue samples from primary, regional, distant, and nodular metastatic melanoma. Ectopic expression of miR-375 inhibited melanoma cell proliferation, invasion, and cell motility, and induced cell shape changes, strongly suggesting that miR-375 may have an important function in the development and progression of human melanomas.

1. Introduction

Melanoma is the most lethal form of skin cancer in the United States and the risk of developing melanoma is increasing [1]. A number of protein-coding genes [2] have been identified as potential biomarkers and candidate drug targets for melanoma [3–9]. Several of these genes exhibit distinct expression signatures among a variety of malignant metastatic melanomas and their benign forms [2]. Recently, a group of small non-coding RNA molecules, especially microRNAs (miRNAs) have garnered increasing attention for their potential roles in tumorigenesis [10–14], including in melanoma [15–19]. miRNAs are thought to influence cancer development by regulating transcription and translation of tumor suppressor genes and oncogenes [20–26]. Several genome-wide expression studies, including our own study of Mazar et al. [27], have implicated a number of miRNAs that could be important players for melanoma development [9,19,28–30]. For example, miR-221/222 was found to down-regulate 27Kip1/CDKN1B and the c-KIT receptor, which controls the neoplastic transformation that leads to enhanced proliferation and reduced differentiation in melanoma cells [28]. Similarly, miR-137 down-regulates microphthalmia associated transcription factor (MITF) expression in melanoma cell lines. Thus, there is precedent for miRNA as regulators of melanocyte cell growth, maturation, apoptosis, and pigmentation [19]. In a recent study, the mechanistic role of miR-211 on melanoma development was reported [27,30].

Our results with melanoma cell lines and clinical samples indicate that several miRNA species are epigenetically regulated in melanomas and we report here for the first time the molecular mechanisms by which one such miRNA, miR-375, might affect melanoma development in humans. Though miR-375 has been previously reported as important miRNA in the pancreas and brain [31–33], its function is not known in melanoma. We believe that the finding that miRNA-375 is epigenetically modified contributes significantly to our understanding of the events regulating melanoma development, and will eventually lead to the development of novel diagnostic and therapeutic tools.

2. Materials and methods

2.1. Cell lines and clinical samples

The experimental studies in this manuscript utilized the human epidermal melanocyte cell line HEM-l (ScienceCell, Catalog #2200, grown in MelM media containing MelGS growth supplements, 0.5% FBS, and penn/strep solution), human epidermal keratinocytes (HEK, ScienCell, Catalog #2100, grown in Keratinocyte Medium, ScienCell, Catalog #2101), and the melanoma cell lines WM793B (stage 1, Wistar Institute), WM278 (stage 2, Wistar Institute), WM1552C (stage 3, ATCC® Number: CRL-2808), and A375 (stage 4, American Type Culture Collection). All melanoma cell lines were

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grown in Complete Tu Media containing a 4:1 mixture of MCDB-153 medium with 1.5 g/l sodium bicarbonate and Leibovitz's L-15 medium with 2 mM L-glutamine, 2% FBS, and 1.68 mM $CaCl_2$. All clinical samples were graciously donated by Dr. James Goydos, Robert Wood Johnson Medical School. Patient samples information listed [27].

2.2. Cell labeling and imaging

Live cells were imaged under normal culture conditions by light microscope (Olympus IX71). The morphology time course experiment was performed with cells growing exponentially, trypsinized and seeded with 2×10^4 cells into 4 well Lab-Tek II chamber slides (Thermo Fisher Scientific), with one slide per time point. At the designated time points (4, 24, 48, and 72 h), the slides were washed in PBS and fixed in 4% formaldehyde in PBS. The slides were then labeled with DAPI and fluorescence-labeled phalloidin, then imaged on a Nikon Eclipse microscope.

2.3. Genomic DNA isolation and bisulfite treatment

See Supplementary material.

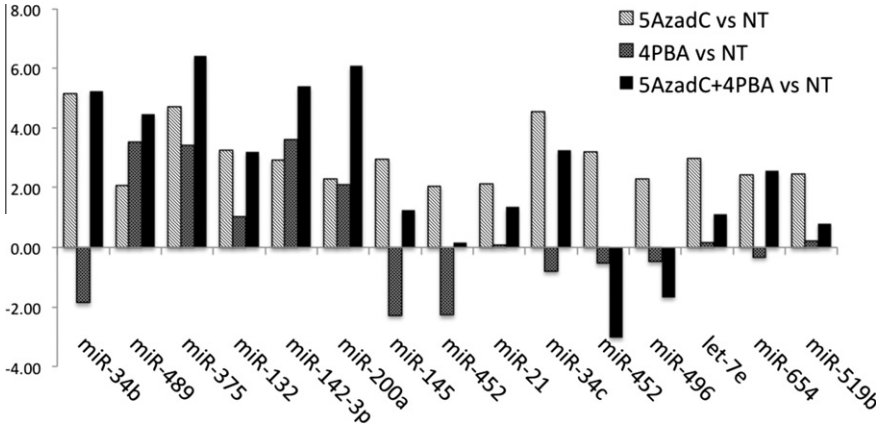

Fig. 1. Activation of epigenetically modified miRNAs in response to 5AzadC and 4-PBA. Differentially expressed miRNAs in melanoma cells treated with 5AzadC, 4-PBA, or both. Expression levels were measured by two miRNA profiling platforms (TILDA; ABI and NCode; Lifetech), and are presented as fold increase or decrease compared with expression in non-treated controls.

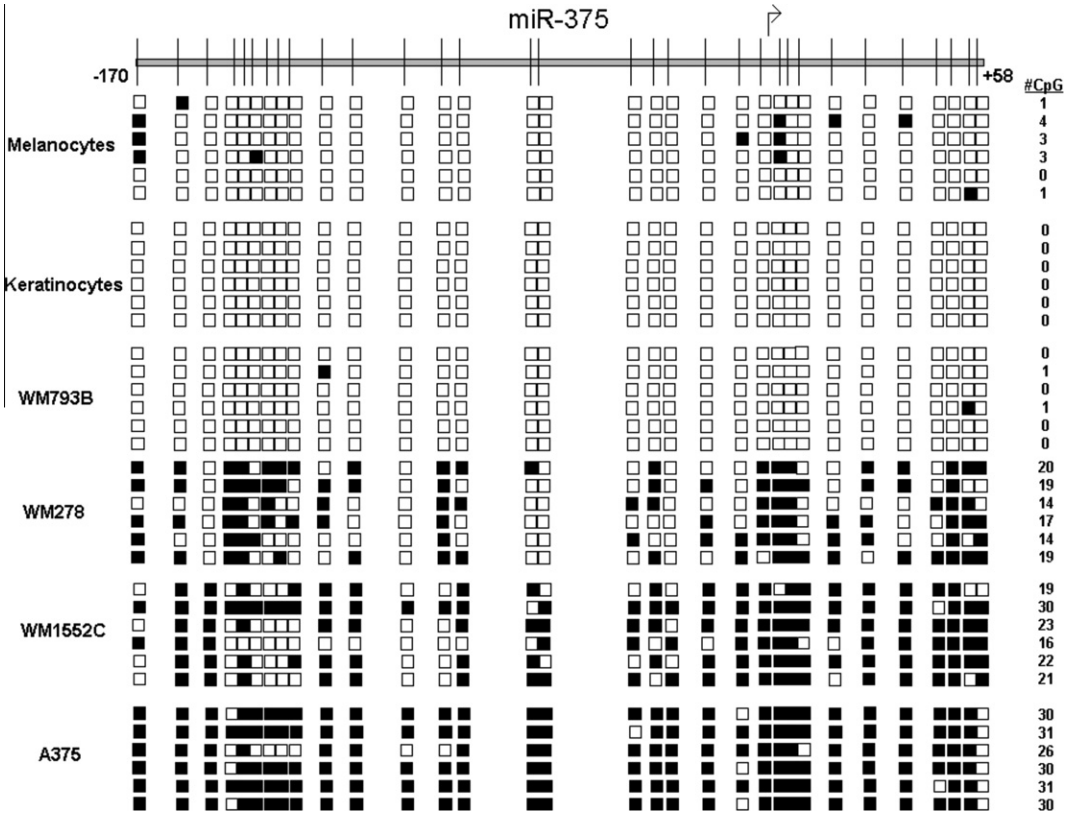

Fig. 2. miRNA-375 CpG island methylation in melanocytes, keratinocytes, and melanoma cells. CpG island methylation frequency in the *mir-375* loci as measured by bisulfite genomic sequencing. −170 to +58 represent nucleotides relative to the pri-miR-375 genomic sequence, with the arrow representing the pri-miR-375 start site. Vertical lines represent the positions of CpG sequences. Each cell line was tested with nine clones. Black boxes represent methylated sites; white boxes represent no methylation. WM793B = stage 1; WM278 = stage 2; WM1552C = stage 3; A375 = stage 4.

2.4. CpG Island methylation detection by the sequencing of bisulfite converted PCR products from genomic DNA

See Supplementary material.

2.5. Treatment of WM1552C cells with 5-aza-2′-deoxycytidine (5-Aza-dC) or 4-phenylbutyric acid (PBA)

See Supplementary material.

2.6. Northern blot analysis

See Supplementary material.

2.7. DNA pyrosequencing

See Supplementary material.

2.8. Construction of stably-expressing miR-375 melanoma cell line

See Supplementary material.

2.9. Next generation DNA sequencing/RNA-seq

See Supplementary material.

2.10. RNA-Seq data analysis

See Supplementary material.

2.11. Cell proliferation assay

See Supplementary material.

2.12. In vitro wound healing assay

See Supplementary material.

2.13. Invasion assays

See Supplementary material.

3. Results

3.1. Identification of epigenetically modified miRNA-375 in melanoma cells

To identify transcriptional changes in melanoma cells induced by DNA methylation and/or histone modification, we treated the stage 3 melanoma cell line WM1552C with the DNA methyltransferase inhibitor 5-aza-2-deoxycytidine (5AzadC), the histone deacetylase inhibitor 4-phenylbutyrate (4-PBA), or with both. Following exposure, cellular miRNA expression was compared between treated and untreated cells using two miRNA expression profiling platforms (TILDA; ABI and NCode; Lifetech). Both platforms identified the same miRNAs as being up-regulated in WM1552C cells after treatment with 5AzadC and/or 4-PBA. Fig. 1 shows that miRNAs are highly modulated by treatment with 5AzadC and PBA. Several of the up-regulated miRNAs contain

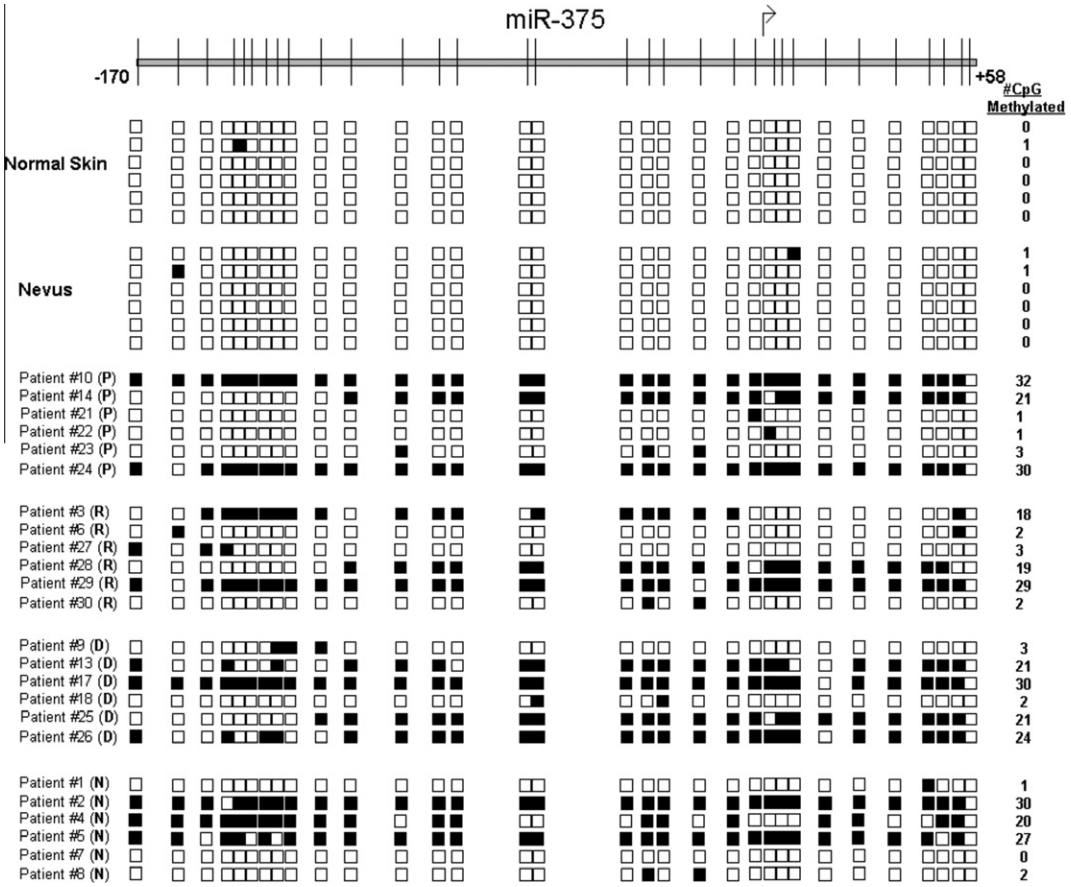

Fig. 3. miR-375 CpG island methylation in patient samples, melanocytic nevus, and normal skin. CpG island methylation frequency in the *mir-375* loci as measured by bisulfite genomic sequencing (annotation conventions are as in Fig. 2). Each patient sample was tested with six clones. Patient samples are: (P) = primary melanoma; (R) = regional metastasis melanoma; (D) = distant metastasis melanoma; (N) = nodal metastasis melanoma.

Table 1
Pyrosequencing of upstream CpG islands of miR-375 in patient samples, normal skin, nevus, and melanoma cell lines. Pyrosequencing measurements are given as an average % of methylation of a given site, using normal skin, nevus, WM278 (stage 1) and A375 (stage 4) melanoma cell lines. The positive methylation cut-off was set at 20%. Patient samples are grouped as primary, regional metastases, distant metastases, and nodal metastases melanoma.

Sample #	miR-375% methylation																					Avg% methylation	
	Primer FS2					Primer FS3										Primer FS4							
	CpG Site 1	CpG Site 2	CpG Site 3	CpG Site 4	CpG Site 5	CpG Site 6	CpG Site 7	CpG Site 8	CpG Site 9	CpG Site 10	CpG Site 11	CpG Site 12	CpG Site 13	CpG Site 14	CpG Site 15	CpG Site 16	CpG Site 17	CpG Site 18	CpG Site 19	CpG Site 20	CpG Site 21		
Normal skin	3	2	2	3	2	2	2	3	3	4	3	3	2	3	2	2/2	3/2	4/3	4/4	2/2	2/1	2.60	Normal skin
Nevus	5	2	2	3	4	2	3	3	2	8	5	9	2	4	4	3/5	3/1	3/3	3/4	3/7	3/4	3.87	Nevus
WH7938	3	3	3	3	3	3	3	3	3	3	2	3	3	4	3	3/4	4/4	3/4	2/2	3/3	2/2	3.00	WH7938
A375	78	75	90	85	85	86	92	96	96	93	86	95	75	96	74	87/96	92/96	91/93	82/82	96/98	63/77	**86.80**	A375
Primary melanomas																							
10	51	48	48	50	47	50	49	51	51	52	12	54	43	58	53	49/54	54/53	52/53	53/53	55/54	43/49	**47.88**	**10**
14	29	28	28	28	27	27	29	30	28	30	28	31	25	34	29	27/30	29/28	33/32	30/31	29/29	23/26	**28.73**	14
21	6	4	5	5	5	2	5	5	4	9	5	7	4	4	5	5/5	6/6	6/3	5/5	5/4	3/4	5.00	21
22	6	6	6	5	6	4	3	5	4	9	4	7	4	5	3	3/3	5/3	5/8,	5/3	5/5	3/3	5.13	22
23	12	10	8	10	11	6	8	7	6	12	9	8	5	10	7	6/10	9/9	8/11	7/9	8/9	5/7	8.60	23
24	47	42	28	91	23	45	42	45	46	43	42	46	38	50	45	43/49	47/46	48/50	44/45	48/47	33/40	**44.87**	24
Regional metastases																							
3	22	20	20	21	17	19	22	20	21	21	17	9	17	24	20	19/22	21/22	29/32	23/25	22/25	24/29	19.33	3
6	10	0	1	2	1	1	9	2	9	2	3	2	1	2	1	1/2	2/1	17/19	1/1	2/1	17/20	3.07	6
27	18	13	11	28	15	19	18	19	25	22	9	18	15	21	20	18/25	21/23	26/26	22/24	20/23	24/28	18.07	27
28	53	49	51	50	40	44	57	51	33	52	45	42	42	42	35	27/29	38/38	40/41	43/42	40/44	38/45	**45.73**	28
29	31	32	32	44	36	32	44	34	32	29	22	28	19	32	15	16/18	25/26	28/29	28/27	25/25	28/31	**30.80**	29
30	4	2	2	2	1	3	5	2	6	6	8	2	1	1	1	2/2	2/1	6/6	1/1	2/1	4/5	3.07	30
Distant metastases																							
9	15	6	5	7	5	5	13	5	16	7	5	2	4	5	5	4/5	5/5	12/14	5/5	6/6	23/27	7.00	9
13	35	36	45	29	32	25	56	37	36	53	35	41	31	36	29	25/20	40/35	45/51	46/46	49/71	39/8	**37.07**	13
17	46	45	46	45	38	43	42	45	37	40	39	44	35	50	43	40/45	44/45	44/48	44/45	46/48	40/48	**42.53**	17
18	8	7	7	9	7	7	9	7	9	10	6	7	7	9	7	7/9	8/7	11/13	8/9	10/11	15/18	7.73	18
25	31	30	29	33	27	31	32	29	31	30	26	15	25	34	31	29/32	32/31	47/50	33/33	31/32	31/35	**28.93**	25
26	36	13	20	25	23	22	34	36	23	35	33	25	23	35	28	26/30	25/22	24/24	34/35	31/32	27/29	**27.40**	26
Nodal metastases																							
1	19	16	17	18	16	14	17	17	17	19	15	17	14	19	16	12/14	17/17	19/20	18/19	17/18	14/17	16.73	1
2	36	36	36	41	35	32	34	38	34	36	33	34	29	41	27	31/35	36/39	38/39	36/37	38/39	50/56	**34.80**	2
4	21	21	18	20	17	19	47	19	47	21	19	21	17	21	17	12/14	21/21	21/22	22/21	21/22	18/20	**23.00**	4
5	34	36	35	34	35	28	31	33	28	35	32	30	27	35	28	16/17	31/33	34/35	33/32	33/34	27/31	**32.07**	5
7	4	3	2	5	4	1	5	3	5	4	3	2	1	2	3	2/2	3/2	5/13	7/7	5/5	5/13	3.13	7
8	11	15	13	11	9	9	11	9	11	13	11	8	9	11	8	8/9	10/10	12/12	10/10	11/11	11/12	10.60	8

Primers FS3 and FS4 overlap at CpG sites 16–21; columns show % methylation for both primers.
Those bolded appear to be 20% methylated or more.

CpG islands in their putative regulatory regions and some have previously been shown by bioinformatic analysis to contain CpG islands [34], suggesting that reactivation of miRNA expression in WM1552C cells may be epigenetically controlled. miR-375 was highly up-regulated after the treatment of WM1552C cells with 5AzadC and 4-PBA, and therefore, we focused our investigation on miR-375.

3.2. miR-375 CpG island methylation in melanocytes, keratinocytes, and melanoma cells

Epigenetically regulated miRNAs often carry modifications such as cytosine methylation in CpG islands located in their upstream regulatory regions. To determine if this was the case for miR-375, we examined methylation patterns within the CpG islands located 1 Kb upstream of miRNA-375 in melanoma cells, and as controls, in normal melanocytes and keratinocytes.

Several CpG islands were identified in the upstream region of miR-375, one of which is located from −170 to +58 bp upstream of miRNA-375 and contains 32 CpG dinucleotides (Fig. 2). For this study, we analyzed CpG methylation solely within this region. To do this, genomic DNA from the cell were bisulfite treated, and the miR-375 upstream CpG island was cloned, sequenced, and the data was compared to the untreated genomic DNA. As shown in Fig. 2, the CpG island located −170 to +58 bp upstream of miRNA-375 was entirely unmethylated in keratinocytes (0.0%), and only poorly methylated in the stage 1 melanoma cell line WM793B (0.5%) and in melanocytes (6.2%). In contrast, extensive methylation was detected in DNA from the stage 2 melanoma line WM278 (53.7%), the stage 3 line WM1552C (68.2%), and the stage

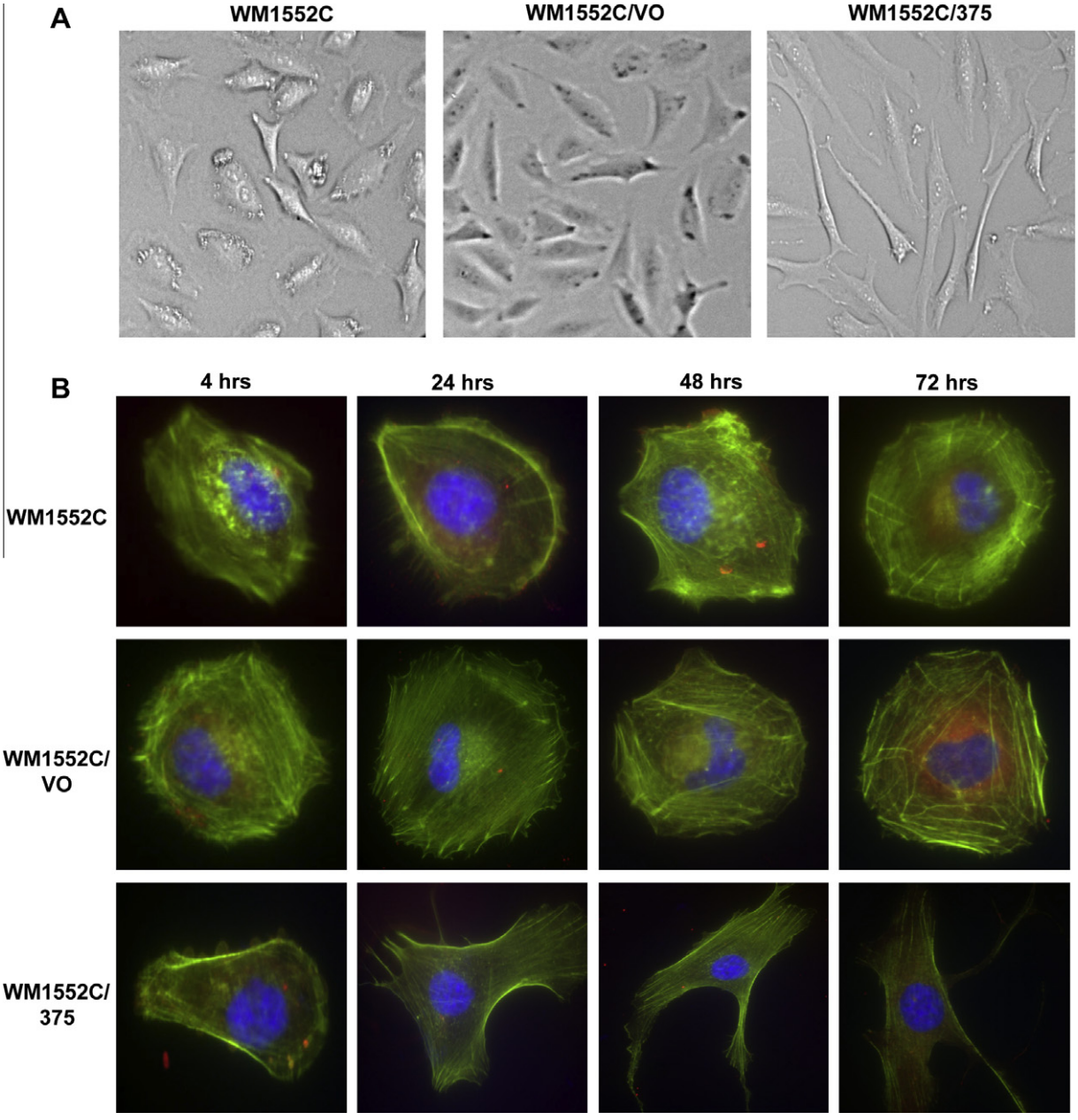

Fig. 4. Effect of miR-375 on melanoma cell morphology. (A) Bright-field images of melanoma cell lines showing growth characteristics (WM1552C, WM1552C/VO, and WM1552C/375). (B) Melanoma cells were seeded and examined by fluorescence microscopy over a period of 72 h. Cells were labeled with DAPI for nuclear staining (blue) and with FITC-labeled phalloidin for staining (green). Melanoma cells that ectopically express miR-375 showed significant changes in cell morphology.

4 line A375 (92.7%). These results show that the proportion of methylated CpG dinucleotides in the miR-375 island increased with advancing melanoma stage, suggesting that epigenetic changes in this CpG island may be stage specific.

To determine if methylation of CpG islands may be responsible for down-regulation of miR-375 expression in melanoma, we first confirmed the effect of 5AzadC treatment on the methylation state of the region. Genomic DNA from 5AzadC-treated WM1552C cells was treated with bisulfite and sequenced. Supplementary Fig. 1A shows that 5AzadC treatment greatly reduced methylation levels from 68.2% to 16.7% at this location. Next, WM1552C cells were treated with 5AzadC, 4-PBA, or both, to determine their effect on miR-375 expression as measured by Northern blotting (Supplementary Fig. 1B). The results show that miR-375 expression was induced by 5AzadC but not by 4-PBA treatment. However, a synergistic effect of 5AzadC and 4-PBA was observed, resulting in greater induction of miR-375 than after treatment with 5AzadC alone. These data show that demethylation of miR-375 induces its expression, confirming that miR-375 is epigenetically regulated.

3.3. CpG island methylation of miR-375 in normal skin and melanoma patient tissues

To confirm that our findings with melanoma cell lines are relevant to human pathology, we next measured CpG island methylation for miR-375 in ex vivo tissue samples from melanoma patients. In samples of normal skin and nevi the miR-375 CpG island was almost entirely unmethylated (Fig. 3), consistent with the lack of methylation seen previously in keratinocytes, the dominant cell type in normal skin (Fig. 2).

Next, we examined miR-375 CpG island methylation in four groups of tissue from 24 melanoma patients: (1) primary melanoma, (2) regional metastases, (3) distant metastases, and (4) nodal metastases (Fig. 3). The results indicate that 3 of the 6 primary melanoma tissues (P), 3 of the 6 regional metastases (R), 4 of the 6 distant metastases (D), and 3 of the 6 nodal metastases (N) were hypermethylated, with average hypermethylation of 77.4%. CpG methylation in these samples was confirmed by DNA pyrosequencing (Table 1). In addition to patient samples, we also

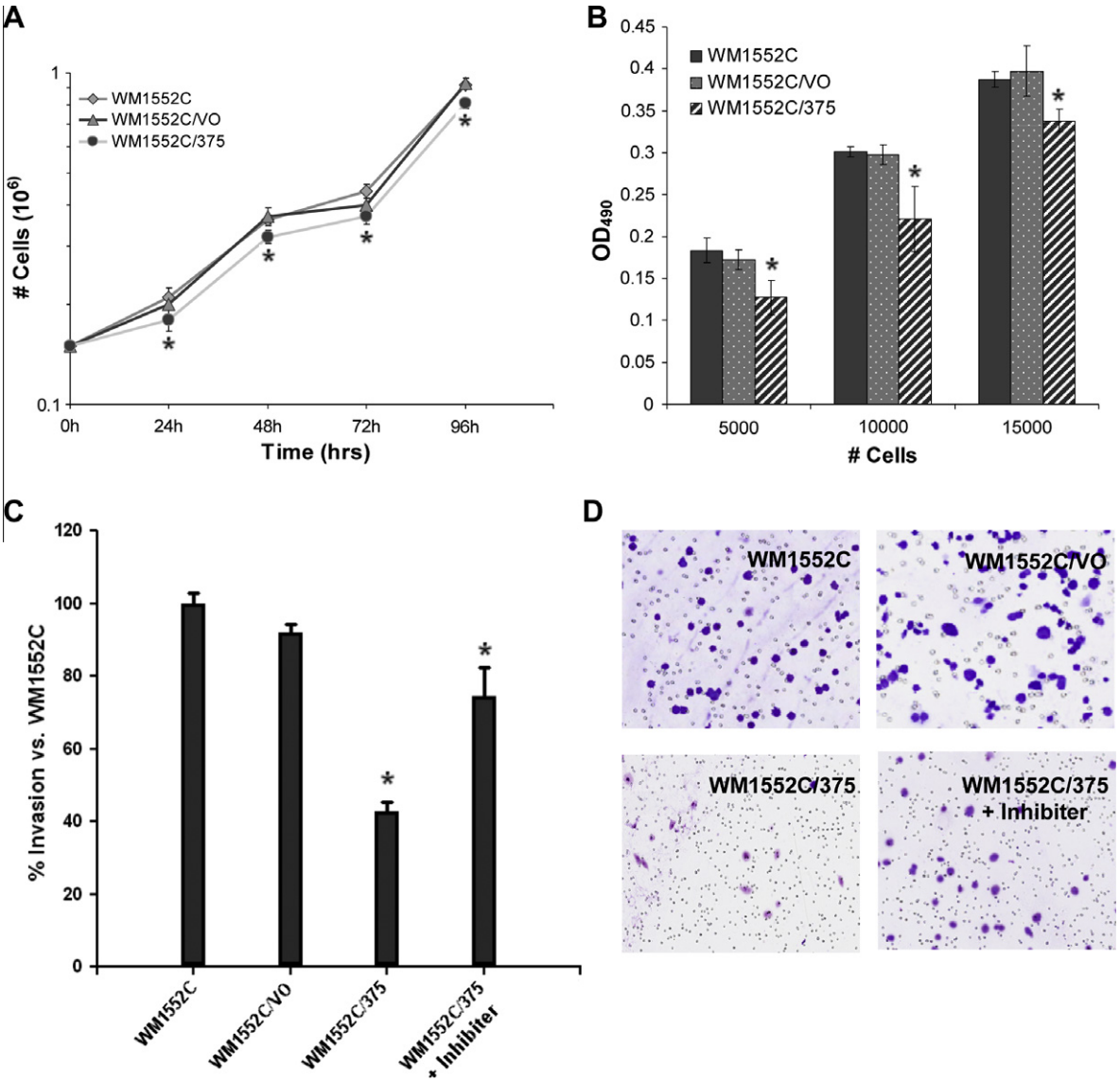

Fig. 5. Effect of miR-375 on melanoma cell growth, viability, and invasion. (A) Proliferation of WM1552C (wild type), WM1552C/VO, and WM1552C/375 cells over 96 h by direct microscopic cell counting. (B) Cell viability of WM1552C, WM1552C/VO, and WM1552C/375 as measured by the MTS assay at cell densities of 5×10^3, 10^4, or 1.5×10^4 cells/well. (C and D) Cell invasion assay of WM1552C, WM1552C/VO, and WM1552C/375 cells. All assays were performed in triplicate. Statistical significance is indicated by an asterisk (Kruskal–Wallis non-parametric test, $P < 0.005$).

pyrosequenced DNA from normal skin, nevus, and the melanoma cell lines WM793B (stage 1) and A375 (stage 3) cell lines as controls. Table 1 shows that the results correlated well with previous bisulfite sequencing results, and revealed low methylation levels in normal skin and nevus, with average methylation of 2.6%, and 3.9%, respectively. As expected, high methylation levels were detected in stage 3 A375 cells (86.8%). Of the patient samples, 12 of the 13 samples positively identified by bisulfite sequencing as having hypermethylated CpG were confirmed by pyrosequencing (with a positive match cut-off set at 20%, only regional metastatic patient sample #3 fell below, with average methylation at 19.33%). qRT-PCR results show that miR-375 expression is low compared to normal skin or nevi (Supplementary Fig. 2). Collectively, these results show that the methylation state of the miR-375 CpG island increases consistently with the transition from normal skin to melanoma, and further indicates the diagnostic potential of this marker for pathological staging of melanoma.

3.4. The effect of miR-375 ectopic expression on cellular morphology, cell growth, migration, and invasion in melanoma cells

To investigate the physiological role of miR-375 in melanoma cells, miR-375 was ectopically expressed in the melanoma cell line WM1552C and assayed for phenotypic changes. Changes in cellular morphology of stable miR-375-expressing melanoma cells were compared with "vector-only" and untransfected cells (Fig. 4). Viewed by light microscopy, the miR-375-expressing cells exhibited a more slender and elongated shape compared to the wild type and vector control cells, which were similar in appearance (Fig. 4A). These cells were also examined by fluorescence microscopy after staining with FITC-labeled phalloidin to visualize filamentous actin. Fig. 4B shows that the morphological changes in miR-375-expressing cells were time-dependant, with changes detectable within 4 h after trypsinization and reseeding, and elongated filapodia-like extensions visible by 24 h. These changes appeared complete by 72 h. By comparison, both untransfected WM1552C and WM1552C/VO cells acquired their characteristic rounded shape by 4 h, and little change in morphology was seen thereafter (Fig. 4B). A background of phospho-cortactin staining can be seen in some cells (red color).

We next examined the effect of miR-375 expression on the cellular properties of these melanoma cells. Fig. 5A shows that proliferation of WM1552C/375 cells was reduced approximately 10–15% compared to that of WM1552C or WM1552C/VO cells, as measured by direct cell counting, and the decrease was sustained over the 96 h assay. Cell viability was examined by the MTS assay, with the results demonstrating that WM1552C/375 cells produced ~15–20% less formazan product than either WM1552C or WM1552C/VO cells (Fig. 5B), indicating a slight reduction in mitochondrial metabolism that may reflect reduced cell viability. Apoptosis was examined by caspase 3/7 assays (Supplementary Fig. 3); however, in this assay no significant differences were noted between miR-375 expressing cells and either wild type or vector-expressing cells.

The invasive properties of miR-375 expressing melanoma cells were measured in standard Boudin invasion assays. Our results (shown in Fig. 5C and D) indicate that WM1552C/375 cells migrated at less than 50% the frequency of either wild type or vector-expressing cells. The reduction in cell invasion exhibited by miR-375-expressing cells is greater than the observed effect on cell proliferation or viability (Fig. 5A or Fig. 5B), suggesting that these two effects of miR-375 expression occur independently. Transfec-

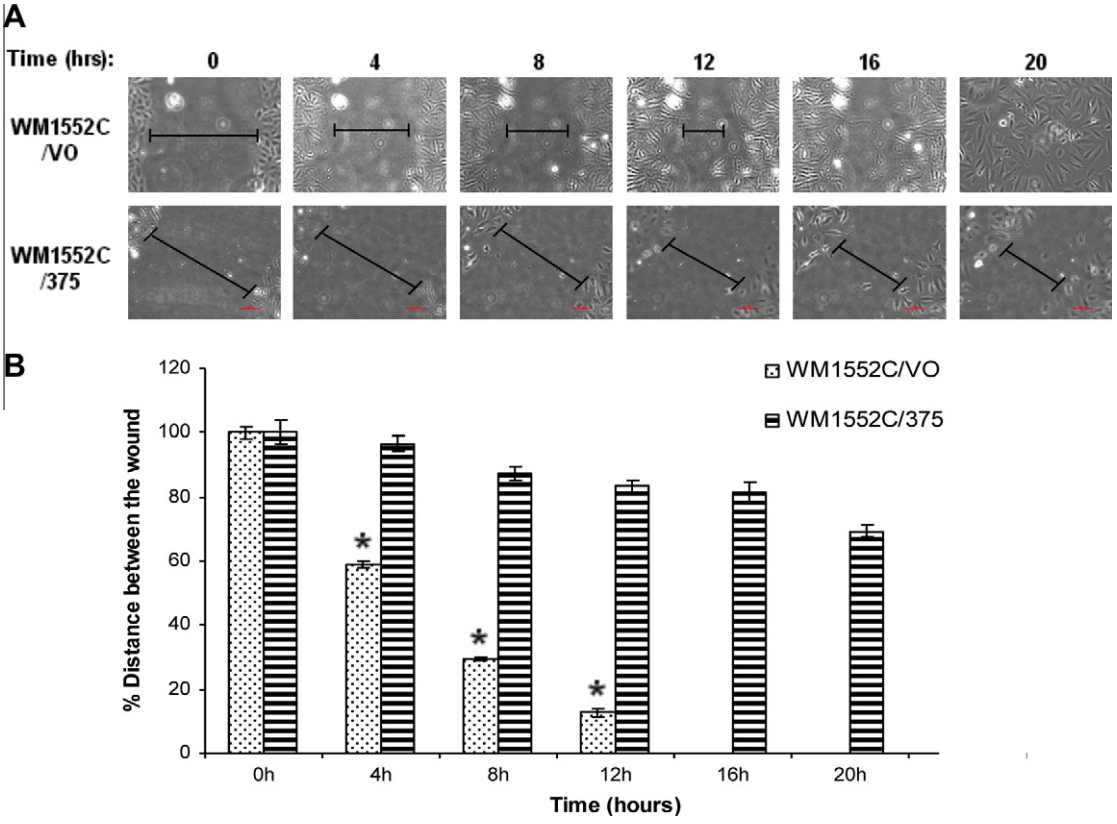

Fig. 6. Effect of miR-375 on melanoma cell motility. (A and B) Wound healing assay of melanoma cells WM1552C/VO and WM1552C/375. (A) Representative images of wound healing assays for WM1552C/375 compared to WM1552C/VO cells over a 20 h period. (B) The bar graph depicts quantitation of the percent surface area between the wounds over a 20 h period, calculated using NIS elements software. Assays were performed in triplicate. Statistical significance is indicated by an asterisk (Kruskal–Wallis non-parametric test, $P < 0.005$).

tion of an miR-375 inhibitor into the WM1552C/375 cells appeared to partially reverse the effect on migration (to ~75% of wild type), confirming that the loss of function is likely due to the up-regulation of miR-375.

The in vitro motility of transfectants was measured in a wound-healing assay, and a striking difference was noted in the rates of motility of cells expressing miR-375 as early as 4 h after initiation of the assay (Fig. 6A and B). By 12 h of incubation, wound healing was nearly complete in cultures of WM1552C/VO cells, whereas migration of WM1552C/375 cells was dramatically reduced with less than 30% wound closure by 20 h.

3.5. The global gene expression profiling of stable miR-375-expressing melanoma cell lines by next-generation sequencing reveals putative target genes

The identification of regulatory targets for miRNAs becomes more significant as their importance in normal cell function and disease becomes apparent. To further probe the pathological relevance of miR-375 on malignant melanoma, total RNA was isolated from WM1552C cells stably expressing miR-375 or transfected with empty vector only (WM1552C/375 or WM1552C/VO). Total RNA was reverse transcribed into cDNA, and analyzed by next-generation sequencing. The sequences were mapped to the human genome and mined for differences in RNA species expression. Twenty candidate target genes were identified as highly differentially expressed (Supplementary Fig. 4). Systems level pathway mapping (Ingenuity pathway maps) revealed that 13 of the 20 candidate genes mapped to a single network regulating cellular and connective tissue development and function (Fig. 7), supporting the notion that miR-375 regulates genes associated with cellular morphology and tissue networking.

4. Discussion

Epigenetic modification of DNA, such as methylation and/or histone modification, is thought to play a key role in cancer progression. In melanoma, epigenetic modifications may silence or reduce the expression of miRNAs but their expression can be reactivated upon treatment with DNA methyltransferase inhibitors such as 5-aza-2-deoxycytidine (5AzadC), or histone deacetylating agents such as 4-phenylbutyrate (4-PBA). To identify miRNAs that may be important in the development of melanoma, we treated a stage 3 melanoma cell line WM1552C, with 5AzadC and/or 4-PBA. Several hypermethylated miRNAs were detected, one of which, miR-375, was highly methylated and was studied further.

Recently, miR-375 hypermethylation was reported in breast cancer and gastric cancer [35,36]. In breast cancer, higher expression of miR-375 in ERα-positive breast cell lines was shown to influence cell proliferation, and miR-375 overexpression caused

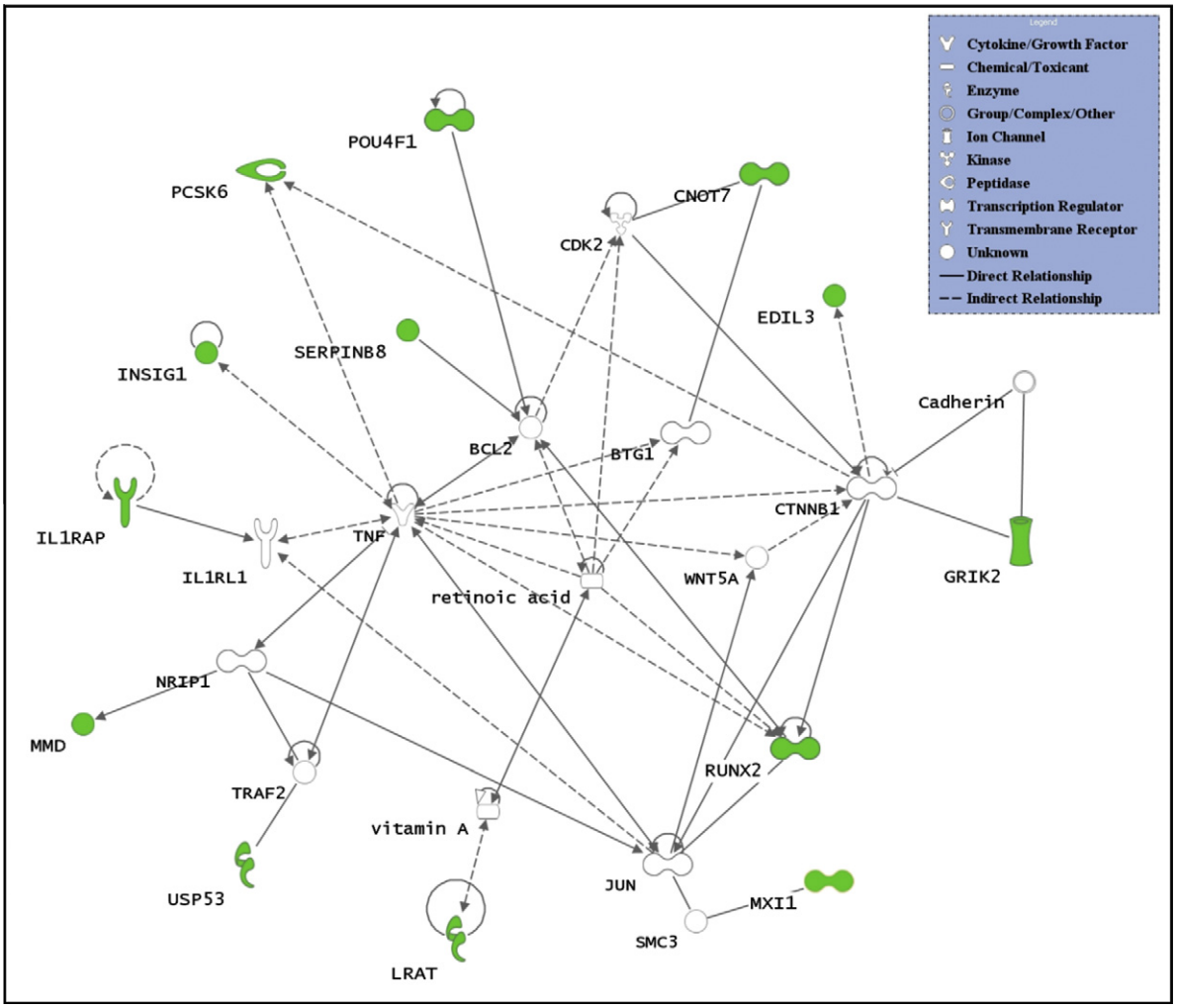

Fig. 7. Pathway analysis of putative miR-375 target genes. Pathway mapping of the 20 most down-regulated miR-375 target genes (TargetScan 5.1) in melanoma cells ectopically expressing miR-375. Thirteen of these putative targets are associated in a network involving the regulation of cellular and connective tissue development and function (Ingenuity Pathway Analysis).

loss of epigenetic marks including H3K9me2 and local DNA hypomethylation. Inhibition of miR-375 in ERα-positive MCF-7 cells reduced both ERα activation and cell proliferation [35]. In gastric cancer, miRNA-375 was found to be the most down-regulated miRNA, and ectopic expression of miRNA-375 in gastric carcinoma cells markedly reduced cell viability by a mechanism involving the caspase-mediated apoptosis pathway [37]. Interestingly, the authors reported that expression of miR-375 inhibited expression of PDK1, a direct target of miR-375, and resulted in the suppression of Akt phosphorylation [36].

Our present results show for the first time that methylation of the miR-375 upstream CpG island is melanoma stage-specific. Minimal CpG island methylation was observed in melanocytes, keratinocytes, and stage 1 melanoma cell lines, but hypermethylation was observed in cell lines from more advanced melanoma. Consistent with this finding, miR-375 CpG island methylation was observed in patient tissue samples from primary, regional, distant, and nodular metastatic melanoma, but not in normal skin and nevus. miR-375 expression was low in patient samples compared to melanocytic nevi and normal skin. Ectopic expression of miR-375 inhibited melanoma cell proliferation, invasion, and wound healing, and induced changes in cell morphology, strongly suggesting a physiological role for miR-375 in cytoskeletal architecture, cell motility, and migration. Collectively, our data show that miR-375 is epigenetically regulated in melanoma, but not in melanocytes, keratinocytes, or melanocytic nevi, and plays an important role in melanoma cell invasion and proliferation. Together, these data indicate that miR-375 may have an important function in the development and progression of human melanoma.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2011.06.025.

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