BMB
reports

Regulation of melanocyte apoptosis by Stathmin 1 expression

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Undesirable hyperpigmentation that can arise from increased melanocyte activity may be alleviated by targeting active melanocytes for apoptosis. The role of Stathmin 1 as an important regulator of microtubule dynamics is well documented. The current study examined the potential of Stathmin 1-targeting strategies in eliminating active melanocytes. A vector to overexpress Stathmin 1 and vectors to express three distinct small hairpin RNAs to knockdown Stathmin 1 expression in normal melanocytes were produced and in cell cultures acted accordingly. Both overexpression and knockdown of Stathmin 1 led to a marked increase in melanocyte apoptosis, as indicated by the accumulation of apoptotic cells and increased levels of cleaved caspase-3. Both up- and down-regulation of Stathmin 1 expression inhibited the activity of differentiated melanocytes, as indicated by decreases in both melanin production and tyrosinase activity. Taken together, these results indicate that hyperactive melanocytes can be inhibited by altering Stathmin 1 expression. [BMB reports 2008; 41(11): 765-770]

INTRODUCTION

Skin hyperpigmentation may be caused by abnormal melanin production from cutaneous melanocytes and is commonly regarded as a result of combinations of local injury, inflammation, scarring, and aging. Skin biopsies from the abdomen have confirmed that tissue darkening is associated with an increase in the number of active melanocytes (1). The increased activity and/or the number of melanocytes has been linked to insufficient serum 25(OH)D_3 (2).

Stathmin 1 [also called oncoprotein 18 (Op18)] is the first identified member of the stathmin family and a very conserved vertebrate protein that is highly expressed during embryogenesis. During postnatal development, Stathmin 1 is progressively downregulated in the brain and spinal cord (3). In contrast, a high level of Stathmin 1 expression has been found in rapid self-renewal cells and tissues, such as hematopoietic cells, and tissues with pathological hyperproliferation, such as cancer (4), indicating that Stathmin 1 regulates cell proliferation in general. In addition, Stathmin 1 plays an important role in cell mitosis (5) and transformation (6), suggesting that it may have a role in carcinogenesis. Stathmin 1 can bind to tubulin with high affinity, promoting the disassembly of tubulin-based microtubules (4). The activity of Stathmin 1 in mitosis is regulated by the phosphorylation status of four serine residues of Stathmin 1 (Ser^{16}, Ser^{25}, Ser^{38}, and Ser^{63}) by various distinct protein kinases, such as MAPK (Mitogen-activated protein kinase) (7, 8), CDK (cyclin-dependent kinase) (7, 9) and PKA (Protein Kinase A) (10). Increased Stathmin 1 phosphorylation correlates with a decreased binding affinity for tubulin, leading to reductions in both Stathmin 1-dependent microtubule disassembly (11) and tubulin sequestration (12). Additionally, phosphorylation of Stathmin 1 at multiple serine sites is required for cell cycle progression (5).

Stathmin 1 has been implicated in signal transduction through direct interaction with KIST (kinase interacting stathmin) (13, 14) and may also interact with other proteins of known or unknown functions, such as p27, Pin2-interacting protein X1, Von Hippel-Lindau tumor suppressor (VHL), HLA class I, B-42 alpha chain precursor (HLA b), and ubiquilin-4. These results indicate that the roles of Stathmin 1 are more complicated than previously thought and that regulating its expression and activity may be a promising approach for disease prevention and treatment.

In this study, the function of Stathmin 1 in melanocytes was examined using various gene manipulation methods, including gene overexpression and gene knockdown. For the knockdown of Stathmin 1, we constructed three Stathmin 1-targeting small-hairpin RNA (shRNA) expression vectors to test their effects through RNA interference on the Stathmin 1 expression in human epidermal melanocytes.

RNA interference is a highly conserved mechanism for degrading of specific RNAs based on sequence complementarity (15). Naturally occurring small interfering RNAs (siRNAs) are 21- to 23-nucleotides long, generated through the action of the RNase III endonuclease Dicer, and, based on this phenomenon, RNA interference technology has gained a great deal of attention for its highly specific ability to silence gene expression (16). In particular, recently developed DNA vector-based small hairpin RNA (shRNA) technology allows the synthesis of shRNA from a DNA template for the stable suppression of specific mammalian gene expression (17).

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Received 24 April 2008, Accepted 16 June 2008

Keywords: Melanin, Pigmentation, Programmed cell death, RNA interference, Tyrosinase

MATERIALS AND METHODS

Cell lines and cell culture

An immortalized normal human melanocyte cell line [a gift from Dr. Huigang Shi, Da An GENE Co. Ltd, Guangzhou, China] was cultured in DMEM (Gibco, BRL, Guangzhou, P. R. China) supplemented with $NaHCO_3$ and 10% fetal bovine serum (FBS, Jianghai Bio Co., China) at 37°C with 5% CO_2.

Construction of shRNA and pAdTrack vectors

Three oligonucleotide sequences predicted to have the highest probability of knocking down the human Stathmin 1 gene were selected using the online program, http://www2.takara-bio.co.jp/sirna-d/, and were named Stathmin 1-si 1, Stathmin 1-si 2, and Stathmin 1-si 3. Stathmin 1-si 1 was targeted at the 3' untranslated region (UTR) of the gene, Stathmin 1-si 2 at a loop in the coding region, and Stathmin 1-si 3 at a loop-stem-loop sequence in the coding region. To ensure that these shRNAs would not cause off-target inhibition of other known human genes, a BLAST search against the human genome database was also performed. Small hairpin RNA-encoded sequences were generated by two-step PCR. Briefly, the common template used for the first step was a pBlue-U6 plasmid-based cDNA library at an annealing temperature of 52°C. The resulting DNA fragments were diluted 100-fold and used as the templates for the second PCR step at an annealing temperature of 45°C. The PCR primers were synthesized by BioAsia, Co. (Shanghai, P. R. China). The resultant double-stranded DNAs were ligated into the EcoRI/XhoI-linearized pBlueScript-KS(+)-based expression vector, pBlue-U6, where the expression of shRNA was under the control of the constitutively active U6 promoter. To construct the Stathmin 1-overexpression vector, the full-length Stathmin 1 cDNA was subcloned into the Sal I-BglII sites of a pAdTrack shuttle vector (a gift from Dr. Xiaohu Ge, Hong Kong Polytechnic University, China). Purified plasmids were sequence-verified by Invitrogen (Shanghai, P. R. China).

Transient transfection

The melanocytes at 60% confluence were transfected with a pBlue-U6-based shRNA or pAdTrack-based vector or empty vectors, using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Reverse-transcription and quantitative real-time PCR

Twenty-four hours after transfection, the melanocytes were harvested, total RNA was extracted with Trizol reagent, and the amount of total RNA was determined by ultraviolet (UV) spectrophotometry. For the reverse transcription, 4 μl of RNA was incubated for 60 min at 42°C with 2 μl of dNTP (10 mMol/L, Roche, Guangzhou, P. R. China), 0.5 μl of oligo-dT (10 μMol/L, Invitrogen), 1 μl of RNase inhibitor (TOYOBO, Guangzhou, P. R. China), 4 μl of 5X reaction buffer, 1 μl of reverse transcriptase Ace (TOYOBO), and 7.5 μl of sterile RNase-free distilled deionized water (ddH_2O). The reactions were terminated by a 5-min incubation at 99°C.

One microliter of cDNA from each reverse transcription reaction was added to the real-time PCR cocktail, which contained 2 μl of 10X buffer, 1 μl of dNTP (10 mMol/L), 1 μl of each of forward primer (10 μMol/L) and reverse primer (10 μMol/L), 2 μl of SYBR Green, 0.2 μl of Taq polymerase (5.0 units/μl), and sterile ddH_2O in a total volume of 20 μl. The reaction mixtures were incubated at 95°C for 2 min followed by 40 cycles of PCR amplification by an ABI7300 real-time PCR instrument. The real-time PCR program was composed of a denaturation step at 95°C for 15 sec, annealing at 60°C for 30 sec, and extension at 68°C for 30 sec. Data analysis was automatically accomplished by the ABI7300 system software, SDS, which determines the threshold cycle numbers (Ct value). The amount of sample cDNA was estimated by the ratio of the Ct for Stathmin 1 to that of an internal control, β-actin.

Western blotting

Cells were lysed with T-PER tissue protein extraction reagent (Probe Co. Ltd. Guangzhou, P. R. China). Protein concentrations were measured using the BCA protein assay kit (Bio-Rad, Guangzhou, P. R. China). Samples with equal amounts of protein were separated by 15% SDS-PAGE and transferred onto PVDF membranes. The membranes were rinsed with TBST (Tris-buffered saline and Tween, Probe Co. Ltd., Guangzhou, P. R. China) and incubated in blocking buffer (5% dried milk in PBS) for 1 h at 37°C, followed by incubation with primary antibodies at 4°C overnight. A polyclonal antibody against Stathmin 1 was obtained from Sanying Co. Ltd., (Wuhan, P. R. China) and was used at a 500-fold dilution. Monoclonal antibodies against procaspase-3 or cleaved caspase-3 (Sigma, USA) were used at a 1000-fold dilution. Monoclonal antibody against GAPDH (Kangcheng Co. Ltd., Shanghai, P. R. China) was used at a 3000-fold dilution. After three washes with TBST, the membranes were incubated with their corresponding secondary antibodies for 1 h. The bound secondary antibodies were detected by chemiluminescence and autoradiography.

Cell proliferation assay

The melanocytes were seeded into a 96-well plate (1×10^4 cells/well) and cultured in DMEM containing 10% FBS. When the cells reached 80% confluence, they were transiently transfected as described above. Following the transfection, viable cells were quantified at the indicated time points, using the MTT assay ($A_{570\ nm}$) according to the manufacturer's instructions (Sigma, USA)

Flow cytometric analysis

Parental or transfected melanocytes were subjected to flow cytometric analysis for chromosomal DNA. DNA labelling was performed using the Cycle TESTTM PLUS DNA Reagent kit (BD Biosciences, Pharmingen, USA). Briefly, cells were washed 3 times with PBS, mixed with 250 μl of solution A, in-

cubated for 10 min at 25°C, and mixed with 200 μl of solution B for 10 min at 25°C. Finally, 200 μl of solution C was added into each reaction, followed by a 10-min incubation on ice in the dark. The samples were then analyzed using a flow cytometer (Beckman Counter, Guangzhou USA).

Quantification of melanin

After treatment, cells (1×10^6) were lysed to measure melanin levels using the method of Hunt (18), and the production of chromogen measured by absorption spectrophotometry at 400 nm.

Tyrosinase activity assay

Cell lysates were analyzed for tyrosinase activity through assay of a chromogenic substrate, as described by Nakajima (19), measured by absorption spectrophotometry at 490 nm.

RESULTS

To determine the effects of Stathmin 1 on melanocytes, we constructed both Stathmin 1-encoding and Stathmin 1 shRNA-encoding vectors. As shown in Fig. 1, transfection with pAdTrack-Stathmin 1 increased the level of Stathmin 1, both at the mRNA level (not shown) and the protein level, significantly above the existing abundant expression of the parental melanocytes. In addition, transfection with Stathmin 1-shRNA constructs (si 1-3) significantly reduced the endogenous expression of Stathmin 1 at the protein level after 48 hours. Based on densitometric analysis of the Western blots using the SynGene GeneTools Analysis Software (Version 3.02.00), the knockdown of Stathmin 1 protein by Stathmin-si 1, Stathmin 1-si 2, and Stathmin 1-si 3 was 94.1%, 83.6%, and 84.6%, respectively. Similar results were obtained at the mRNA level as measured by quantitative real-time PCR and the suppressions of Stathmin 1 were found to be statistically significant (all $P < 0.01$). The expression of Stathmin 1 was not significantly altered by transfection of melanocytes with either the empty pBlue-U6 vector or the empty pAdTrack vector, using the expression of GAPDH as loading controls.

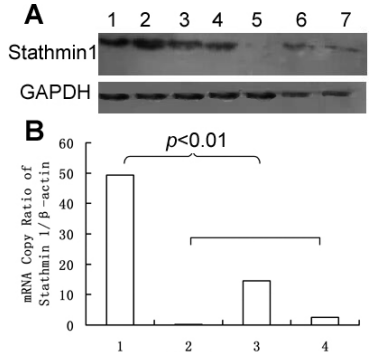

Fig. 1. Detection of Stathmin 1 in normal human melanocytes. A. Western blotting of Stathmin 1 and GAPDH (loading control) proteins in parental melanocytes (lane 1); melanocytes transiently transfected with pAdTrack-stathmin 1 (lane 2); two melanocyte cultures transfected independently with pAdTrack and pBlue-U6 (lane 3 and 4); melanocytes transfected with pBlue-U6-si 1, pBlue-U6-si 2, and pBlue-U6-si 3 (lanes 5, 6, and 7, respectively). B. Quantitative real-time PCR detection of Stathmin 1 mRNA in melanocytes transfected with pBlue-U6 empty vector (lane 1), and pBlue-U6-si 1, pBlue-U6-si 2, and pBlue-U6-si 3 (lanes 2, 3, and 4, respectively). The critical threshold cycle numbers were normalized to the internal control (β-actin) in each reaction and are the average of triplicate experiments. The P values from the paired *t*-tests of control (lane 1) vs. siRNA-transfected cells (lanes 2-4) were all less than 0.01.

To examine the effects of altered Stathmin 1 expression on cell proliferation, transfected cells were analyzed by the MTT assay. As shown in Fig. 2, the growth rates of melanocytes transfected with the empty pBlue-U6 vector or the empty pAdTrack vector and of parental cells were similar. In contrast, Stathmin 1 knockdown or overexpression dramatically reduced cell proliferation, which was associated with increased apoptosis as shown by the FACS analyses of chromosomal DNA (Fig. 3A). The apoptosis rates (the M1 fractions in Fig. 3A) in cells either expressing Stathmin 1 shRNAs or overexpressing Stathmin 1 were significantly higher than that of the control cells ($P < 0.05$). Twenty percent of the parental cells were observed to be apoptotic under the same conditions, typical of a normal cell line. In a parallel analysis using the human nasopharyngeal carcinoma cell line, CNE-2, no significant apoptosis was observed (data not shown), indicating that a basal level of apoptosis in normal cells exceeds that of cancer cells under the same conditions. Consistent with the FACS data, Stathmin 1 knockdown led to significant cell rounding (data not shown), indicating dramatic cytoskeletal collapse as a prelude to apoptosis. Furthermore, Western blot analyses demonstrated that both overexpression and knockdown of Stathmin 1 significantly increased the cleavage of pro-caspase-3 (Fig. 3B),whose proteolytic activation is a critical step in the execution of apoptosis, leading to the specific cleavage of poly(ADP-ribose) polymerase (PARP) and ultimately to DNA fragmentation (20).

Interestingly, cell fractions in G1, S and G2 phases of the cell cycle remained unchanged after melanocyte transfection

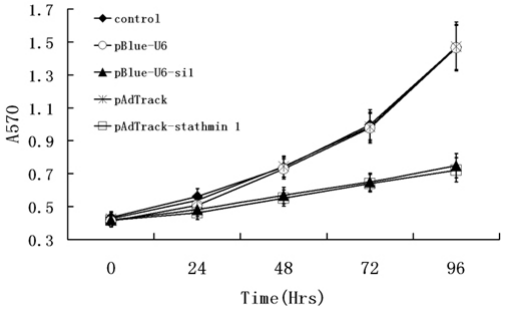

Fig. 2. Cell proliferation analysis. Parental and transfected melanocytes were cultured in triplicate, quantified over a 96-hour period, and the triplicate data averaged.

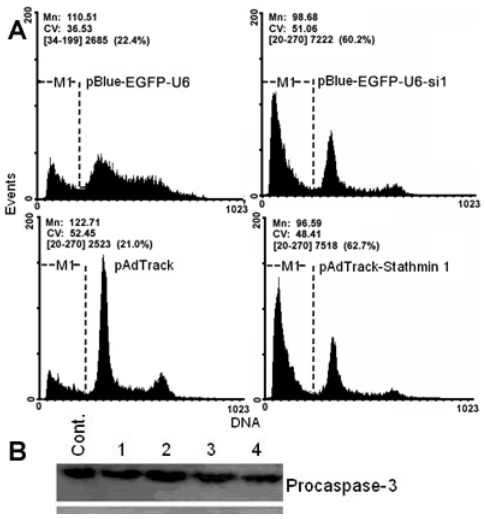

Fig. 3. Analysis of apoptosis. A. Representative results of flow cytometric sorting of chromosomal DNA in melanocytes transfected by pBlue-U6 or pBlue-U6-si 1 and in melanocytes transfected with pAdTrack or pAdTrack-Stathmin 1, with each reaction repeated five times. B. Western blotting of procaspase-3 (p32) and cleaved caspase-3 (p17) in melanocytes transfected by pBlue-U6 (lane 1) or pBlue-U6-si 1 (lane 2), in melanocytes transfected with pAdTrack (lane 3) or pAdTrack-Stathmin 1 (lane 4), and in untreated parental melanocyte controls (Cont.). As loading controls, the same membranes were stripped and re-probed for GAPDH detection.

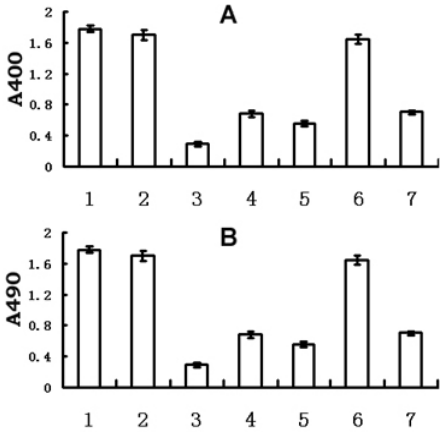

Fig. 4. Analyses of melanocyte-specific activities. Melanin production ($A_{400\ nm}$ in A) and cellular tyrosinase activity ($A_{490\ nm}$ in B) were assayed in triplicate and averaged, using 1×10^6 melanocyte samples that were untreated (bar 1) or transfected with pBlue-U6 (bar 2), pBlue-U6-si 1, pBlue-U6-si 2, pBlue-U6-si 3 (bars 3, 4, and 5, respectively), pAdTrack (bar 6), or pAdTrack-Stathmin 1 (bar 7).

with any of these plasmid vectors ($P > 0.05$, data not shown). Since the cells used for transfection were not pre-synchronized, it is possible that only cells in a specific cell cycle phase would commit to Stathmin 1 alteration-induced apoptosis. Nevertheless, the overall biological activities of differentiated cells in melanocytes transfected with pAdTrack-Stathmin 1 or Stathmin 1 shRNA vectors, as measured by the production of melanin and the activity of tyrosinase, were significantly reduced relative to the control cells (both with $P < 0.05$, Fig. 4). All the melanocytes used in melanin production and tyrosinase activity analyses in each of the transfected groups were living cells sorted with FCM to avoid the confounding effects from cell death after transfection. The similar effects of Stathmin 1 on melanin production and tyrosinase activity were not surprising since tyrosinase plays a key role in melanin production (1). There was no significant difference between the cells transfected with Stathmin 1-overexpressing or Stathmin 1-silencing vectors ($P > 0.05$). Thus, it is possible that Stathmin 1 also plays a role in the regulation of melanocyte function independent of its role in mitosis.

DISCUSSION

Melanocyte proliferation and/or activity may be increased in response to aging, stress, or trauma, leading to an increased melanin deposition. In exploring the biological function of Stathmin 1 in melanocytes, our cell biological and biochemical data suggest that Stathmin 1 plays a vital role in the homeostasis of melanocytes to such a degree that apoptosis can result from either up-regulation or down-regulation of Stathmin 1 expression, a result which can be explained by the role of stathmin in the regulation of microtubule dynamics (11). Our data are consistent with earlier evidence that both silencing and overexpression of Stathmin 1 regulate the cell cycle of myelogenous leukaemia K562 (4).

Our data indicate that Stathmin 1 plays a critical role in the balance between life and death in melanocytes and is consistent with earlier findings that Stathmin 1 acts to sequester tubulin and favour microtubule disassembly. Furthermore, specific to human melanocytes, it appears that Stathmin 1 may be a promising molecular target for therapies designed to block excessive melanin deposition (hyperpigmentation) due to increased melanocyte proliferation and activity. *In vitro* studies of K562 cells suggest that the inhibition and overexpression of Stathmin 1 arrest cell cycle progression at different stages; Stathmin 1 overexpression prevents mitotic spindle formation in early mitosis and inhibition seems to interfere with a later stage of mitosis (4). Present observations suggest that, in cells that have not committed to apoptosis, Stathmin 1 may act as a direct inhibitor of normal differentiated melanocyte functions and support the idea that agents which modulate Stathmin 1 expression have therapeutic potential for the reduction of melanin deposition in a circumscribed area when locally applied.

While the current study needs to be validated by *in vivo* studies, several new and intriguing mechanistic questions need to be addressed by *in vitro* methods. There is a need to confirm Stathmin 1's role in the above discussed differential effects on melanocyte cell functions, to establish possible

Stathmin 1 dose-dependent relationships to these functions, and to elaborate Stathmin 1's role in the regulation of melanocyte functions, particularly as it effects tyrosinase activity, as it is involved in both melanin production and the differentiation of melanocytes (21). It is particularly important to investigate whether Stathmin 1 regulates cell differentiation in a tubulin-dependent manner.

The present study demonstrated that Stathmin 1 shRNAs were highly effective in silencing Stathmin 1, supporting further development of shRNA-based therapies and, in particular, therapies for melanocytes. Interestingly, the most effective shRNA construct, Stathmin 1-si 1, targets the 3'-UTR sequence of Stathmin mRNA. This result was not entirely surprising since 3'-UTR sequences tend to be more specific than open reading frames, which may encode variable, but evolutionarily conserved motifs or domains. In fact, highly efficient silencing effects by RNA interference with 3'-UTR have been reported elsewhere (22), and some of these UTR-targeting siRNAs may also have potential as therapeutic agents for a variety of diseases. For example, Prabhu reported that siRNAs targeting the untranslated sequence of the hepatitis C virus (HCV) internal ribosome entry site effectively induce target mRNA degradation (23). Since Stathmin is required for cell proliferation, the application of Stathmin-targeting shRNAs has been tested *in vitro* for anti-cancer effects and, indeed, Stathmin 1 knockdown results in G2/M phase cell cycle arrest in several tumor cell lines (5, 24, 25).

In summary, we provide here the first evidence that forced alteration of Stathmin 1 expression in normal human melanocytes leads to cell apoptosis, concurrent with significant decreases in melanocyte function, particularly in melanin production. Our results also support the pursuit of future shRNA-based Stathmin 1-targeted drug development.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (Grant 30672358 to Zhenyu Zhu). We are grateful to Dr. Kong at the Department of Inspection of the First Affiliated Hospital of Sun Yat-sen University for his skillful assistance with our flow cytometric analyses.

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