Original Article

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The serum/PDGF-dependent "melanogenic" role of the minute level of the oncogenic kinase PAK1 in melanoma cells proven by the highly sensitive kinase assay

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Summary **We previously demonstrated that the oncogenic kinase PAK4, which both melanomas and normal melanocytes express at a very high level, is essential for their melanogenesis. In the present study, using the highly sensitive "Macaroni-Western" (IP-ATP-Glo) kinase assay, we investigated the melanogenic potential of another oncogenic kinase PAK1, which melanoma (B16F10) cells express only at a very minute level. After transfecting melanoma cells with PAK1-shRNA for silencing PAK1 gene, melanin content, tyrosinase activity, and kinase activity of PAK1 were compared between the wild-type and transfectants. We found that (*i*) PAK1 is significantly activated by melanogenic hormones such as IBMX (3-isobutyl-1-methyl xanthine) and α-MSH (melanocyte-stimulating hormone), (*ii*) silencing the endogenous PAK1 gene in melanoma cells through PAK1-specific shRNA reduces both melanin content and tyrosinase activity in the presence of both serum and melanogenic hormones to the basal level, (*iii*) the exogenously added wild-type PAK1 in the melanoma cells boosts the α-MSH-inducible melanin level by several folds without affecting the basal, and (*iv*) α-MSH/IBMX-induced melanogenesis hardly takes place in the absence of either serum or PAK1, clearly indicating that PAK1 is essential mainly for serum- and α-MSH/IBMX-dependent melanogenesis, but not the basal, in melanoma cells. The outcome of this study might provide the first scientific basis for explaining why a wide variety of herbal PAK1-blockers such as CAPE (caffeic acid phenethyl ester), curcumin and shikonin in cosmetics are useful for skin-whitening.**

Keywords: PAK1, melanogenesis, melanomas, tyrosinase, MITF, skin-whitening, serum

1. Introduction

The color of hairs, eye irises, and skin is controlled by a family of pigments called melanins. The intracellular source of melanin is tyrosine, and is converted to melanin through a series of enzymatic oxidation (hydroxylation) which involves tyrosinase, TRP (tyrosinase-related protein) 1 and TRP2. Expression of genes encoding for these melanogenic enzymes requires at least two oncogenic/melanogenic transcription factors (MTFs): beta-catenin and MITF (microphthalmia-associated transcription factor). The majority of herbal compounds in skin-whitening cosmetics either inhibit directly these melanogenic enzymes or down-regulate the MTFs. Tyrosine analogues such as kojic acid belong to the first category (tyrosinase inhibitors), while the majority of remainings such as CAPE (caffeic acid phenethyl ester), curcumin and shikonin belong to the second category (MTF regulators) (*1,2*). Interestingly, many of these MTF regulators including CAPE, curcumin, shikonin and cucurbitacin are known

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to block the oncogenic/ageing kinase PAK1 (RAC/CDC42-activated kinase 1) (*3-5*). Thus, it is most likely that PAK1 is involved in the activation of these melanogenic/oncogenic transcription factors in hair cells, eye irises or skin melanocytes. Indeed, at least beta-catenin is among the direct substrates of PAK1 (*6*). PAK1 phosphorylates beta-catenin at Ser 675 for the activation, leading to malignant transformation. Furthermore, beta-catenin is essential for the activation of MITF, leading to melanogenesis or/and oncogenesis of melanocytes (*7*).

Interestingly, however, it was recently revealed that knocking-out PAK1 gene *per se* in pigmented mice fails to produce any albino mice (Hong He *et al*., unpublished observation), clearly indicating that at least the basal melanogenesis in both hair cells and eye irises is independent of PAK1, although the contribution of PAK1 to skin melanogenesis still remains to be clarified.

We have previously shown that the oncogenic kinase PAK4 (CDC42-dependent kinase 4) is highly expressed in both melanoma and normal melanocyte cell lines, and responsible for their melanogenesis, activating CREB/beta-catenin-MIFT-tyrosinase pathway, while PAK2 (RAC/CDC42-activated kinase 2), another highly expressed member of PAK family, plays no role in their melanogenesis (*8*).

In this study, we provide the first biochemical evidence for a specific role of another oncogenic kinase called PAK1 in skin melanogenesis, despite of the fact that its expression level is minute: shRNA-induced silencing of PAK1 gene in melanocytes (B16F10) derived from mouse melanoma significantly reduced the α-MSH/IBMX-inducible melanogenesis to the basal level, while over-expression of PAK1 gene boosted the α-MSH-inducible melanogenesis without affecting the basal. Furthermore, we found that the α-MSH/IBMX-inducible melanogenesis absolutely requires a serum factor which is most likely PDGF (platelet-derived growth factor). In the serum-free medium, only the basal melanogenesis takes place.

2. Materials and Methods

2.1. *Materials*

B16F10 melanoma cells were purchased from American Type Culture Collection (Rockville, MD, USA). PAK1-SureSilencing plasmids, attractene transfection, endofree® plasmid maxi kit were purchased from Qiagen (Valencia, CA 91355, USA). Primary PAK1-antibodies, biotinylated secondary antibodies, signalfire™ ECL reagent were obtained from Cell Signalling Technology (Danver, MA, USA). Kinase Glo reagent and ATP (ATP_Glo kinase kit) were purchased from Promega (Madison, Wisconsin, USA). Lipofectamin and JM109 competent cells were purchased from Invitrogen and Life Technology. AG1295 and AG1478 were purchased from Calbiochem (San Diego, CA, USA). All other chemicals including human PDGF-bb were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. *Silencing of PAK1 gene in mouse melanocytes (B16F10) by stable transfection with mouse PAK1-specific shRNA*

2.2.1. *Cell culture*

B16F10 melanoma cells were cultured in DMEM supplemented with 10% heat-activated FBS and 1% penicillin/streptomycin (10,000 U/mL and 100 µg/mL) at 37°C in a humidified atmosphere containing 5% CO_2.

2.2.2. *Stable transfection with mouse PAK1-specific shRNA*

ShRNA transfection of B16F10 cell line was carried out basically through the same procedure previously described (*9*), but with mouse PAK1-specific shRNAs (# KM04553, Qiagen). The following four distinct clones of shRNAs were used for the transfection: clone 1 (CCAGAGAAGTTGTCAGCTATT), clone 2 (CATCAAGAGTGACAATATTCT), clone 3 (GTACACACGGTTCGAGAAGAT), and clone 4 (GTACCACCAGTGTCAGAAGAT) as well as shCONTROL nontargeting shRNA as the negative control (WT). However, in this study, clones 1 and 2 turned out to be more effective than clones 3 and 4 for silencing mouse PAK1 gene in this melanocyte line (data not shown). Stable transfectants were selected in the presence of G418 (1 mg/mL) which kills more than 99% of non-transfected cells.

2.2.3. *Western-blot analysis of PAK1 protein level and in vitro assay for the kinase activity of PAK1 in transfectants*

2.2.3.1. *Western blot analysis*

In order to quantify the extremely low level of PAK1 in both control (WT) cell line and PAK1-silenced transfectants (G418-resistant), by minimizing the "non-specific noise" levels, we conducted the western blot analysis using a rabbit polyclonal antibody against PAK1 (#2062, Cell Signaling Technology) as the primary antibody. Briefly, each clone was seeded at the concentration of 2×10^5 cells/well in a 6 well plate, pre-cultured for 48 h, and the cell lysates were boiled in 25 µL of SDS-PAGE buffer for 5 min. Eight µL (containing 15 µg of total protein) of each supernatant per each slot was used for SDS-PAGE. The nitrocellulose was blotted with the anti-PAK1 IgG (1:1,000 dilution as a primary

antibody), and as secondary antibodies the HRP-linked anti-rabbit IgG and anti-biotin IgG (#7074, #7075, Cell Signaling) were used to detect both PAK1 and β-actin bands which were eventually visualized with the ECL system from Amersham. The western blot assays were representative of three independent experiments.

2.2.3.2. *"Macaroni-Western" (IP- ATP_Glo) kinase assay for PAK1 in melanoma cells*

The kinase activity of PAK1 in the WT melanocytes and shRNA transfectants (SHs) was measured by a substantial modification (*5*) of a decade old method (which we coined "Macaroni-Western") developed by an Italian group (*10*). Briefly, B16F10 melanoma cell lines (WT or SHs, 2×10^5 cells/mL) were pre-cultured on 6-well plate for 24 h. Then cells were treated with 100 nM α-MSH or 100 μM IBMX for 48 h. Cells were disrupted by lysis buffer containing 50 mM Tris-HCl pH 7.5 and 150 mM NaCl and 1% Triton-X. For the immunoprecipitation (IP) of PAK1, the cleared cell lysates were incubated with anti-PAK1 IgG (1:50 dilution) and protein A-agarose beads for 1-2 h in cold room with continuous shaking by a rotary mixer (Nissin, Suginami-ku, Tokyo, Japan). The resultant IP (PAK1) from each lysate was incubated with ATP-Glo kinase assay kit (Promega) in the presence of ATP and MBP (myelin basic protein) for 1 h at 37°C, and the remaining ATP level was measured by the ATP-dependent Luciferin-Luciferase reaction which eventually generates a luminescence (*10*). The final suspension was centrifuged, and the supernatant was transferred to 96-well plate for reading. Luminescence was recorded by MTP-880Lab microplate reader (Corona, Hitachinaka-ku, Ibaraki, Japan) with an integration time of 0.5 s per well.

2.3. *Measuring melanin content and tyrosinase activity in transfectants*

2.3.1. *Measurement of melanin content*

Melanin content was determined as previously described (*11*). In brief, B16F10 cells (wild-type or PAK1-specific shRNA transfectants) were plated at a density of 2×10^4 cells/well in a 24-well plate. After 24 h of culture, 100 μM isobutyl-1-methylxanthine (IBMX) or 100 nM α-MSH was added and incubated for an additional 72 h at 37°C. The cells were washed twice with phosphate buffer, then lyzed with 500 μL of a solution containing 1 M NaOH and 10% DMSO and incubated at 80°C for 1 h, to solubilize the melanin, and the melanin content was measured at 490 nm. To compare the melanin content in the wild-type and transfected cells, the total amount of melanin produced by the wild-type melanocytes was considered as the control (100%), and those by the transfectants were calculated accordingly.

2.3.2. *Assay for intracellular tyrosinase activity*

Tyrosinase activity was determined as previously described (*12*), with a slight modifications. B16F10 cells (wild-type or the transfectants) were plated at a density of 2×10^4 cells/well, and after 24 h of culture, 100 μM IBMX or 100 nM α-MSH was added and incubated for an additional 72 h at 37°C. The cells were then washed with ice cold phosphate buffer and lysed with phosphate buffer (pH 6.8) containing 1% Triton-X (500 μL/well). The plates were frozen at −80°C for 30 min. After thawing, 100 μL of 1% L-DOPA was added to each well. Following incubation at 37°C for 2 h, the absorbance was measured at 490 nm.

2.4. *Measuring the melanin content in the wild-type and PAK1-overexpressing melanocytes after α-MSH treatment*

Using Myc vector (2 μg), melanocytes (B16F10) were transfected transiently with either wild-type (WT) PAK1 or so-called "constitutively activated" (CA) PAK1 carrying T423E mutation. After 3 days of culture, each transfected clone was harvested for the further experiment. The effect of PAK1-overexpression on the melanin content was measured through the basically same procedures described previously (8). Briefly, melanocytes, either the wild-type or PAK1-overexpressers which express either the wild-type PAK1 or its CA mutant, were incubated with 100 nM α-MSH for 3 days. Cells were lyzed with a solution containing 1 M NaOH and 20% DMSO to dissolve the melanin, and its content was determined at 405 nm.

2.5. *Melanogenesis in a serum-free medium*

B16F10 cells (wild-type or PAK1-specific shRNA transfectants) were plated at a density of 2×10^4 cells/well in a 24-well plate in the presence of 10% FBS. After 24 h of incubation, the culture medium is replaced with a serum-free medium, and cultured for an additional 72 h with or without 100 μM IBMX or 100 nM α-MSH to measure the melanin content.

2.6. *Effect of PDGF/EGF receptor inhibitors on serum-dependent melanogenesis*

2×10^4 B16F10 cells were seeded in each well for 24 h in 10% FBS-containing medium, and then cultured for additional 72 h in the fresh medium containing 100 nM α-MSH and followings: (1) no serum, (2) 10% FBS alone, (3) 10% FBS plus 2 μM AG1295 (PDGF receptor inhibitor), and (4) 10% FBS plus 400 nM AG1478 (EGF receptor inhibitor), to measure the melanin content.

2.7. *Statistical analysis*

Data are expressed as mean values with their standard errors. Statistical comparisons were performed by one-way ANOVA followed by Duncan's multiple-range test. Statistical analysis was conducted using SAS (release 9.2; SAS Institute, Cary, NC, USA) and $p \leq 0.05$ was considered significant.

3. Results

3.1. *Activation of PAK1 in melanoma cell line (B16F10) by melanogenic hormones*

Two melanogenic hormones, α-MSH (melanocyte-stimulating hormone) and IBMX (3-isobutyl-1-methyl xanthine), are often used to stimulate melanogenesis in melanocytes such as melanoma cell line B16F10. Thus, we first examined if these hormones can activate PAK1 in this cell line, although the protein level of PAK1 there is extremely low, compared with those of PAK2 and PAK4 (*8*). To monitor the changes in kinase activity of PAK1 in cells, we recently developed a highly sensitive/specific kinase assay called "Macaroni-Western" (IP-ATP-Glo) kinase assay protocol by combining the immuno-precipitation (IP) of PAK1 from cell lysates and Promega's ATP_Glo kinase kit (*5*). Using this kinase assay, we found that both α-MSH (100 nM) and IBMX (100 μM) activate PAK1 in melanoma cells, with α-MSH being more potent than IBMX (see in Figure 1). However, we should remind readers that the fold activation of PAK1 shown here is only apparent, because during this time-consuming test tube IP and kinase assay (over 3 h in total) taking place without these melanogenic hormones, PAK1 would be gradually normalized over time. In other words we cannot freeze the exact kinase status of PAK1 at the end of cell culture treated with these stimulators, and the fold activation is only an under-estimated reflection of PAK1 activation taken place during cell culture by these stimulators.

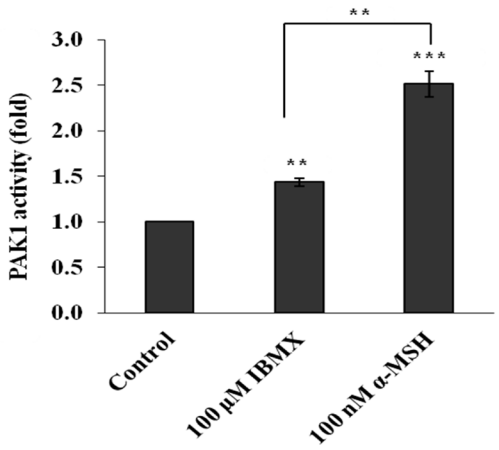

Figure 1. Activation of PAK1 by IBMX and α-MSH in melanocytes. B16F10 cells were treated with IBMX (100 μM) and α-MSH (100 nM) for 72 h. PAK1 was immuno-precipitated from cells lysates, and PAK1 activity was measured by "Macaroni-Western" kinase assay. The results are mean ± SE of three independent experiments. Data have significant difference by ANOVA analysis at $p \leq 0.05$. Statistically significant differences relative to control are indicated by asterisks. * $0.01 \leq p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

3.2. *Silencing of PAK1 gene by shRNAs in mouse melanoma cell line*

In order to investigate further biochemically if PAK1 contributes to the melanogenesis in skin cells (melanocytes), we stably transfected the melanoma cell line B16F10 with mouse PAK1-specific shRNA to silence PAK1 gene selectively.

3.2.1. *Silencing PAK1 gene in melanocytes*

Our preliminary western blot analysis suggested that in two distinct PAK1-silenced clones (SH1 and 2) the PAK1 protein levels are significantly (by around 75%) reduced, but the growth rate of this melanoma cell line *per se* was not significantly affected by the PAK1 silencing (data not shown). However, the accurate quantification of PAK1 protein levels by scanning this western blot was rather difficult, mainly because of the high background noise around the PAK1 band in the effort to visualize its extremely low expression levels. Instead, using the "Macaroni-Western" kinase assay (*5,10*), we verified that the kinase activity of PAK1 in both SH1 and 2 clones are only around 25-30% of that in the control (WT) cells (see Figure 2A).

3.2.2. *Reduction in the melanogenesis by silencing PAK1 gene*

Our next critical question was if such a reduction in the kinase activity of PAK1 affects the melanogenesis. Thus, the melanin content in these clones (SH1 and 2) was compared with the wild-type (WT) melanoma cells, after treating all these cells with α-MSH, a melanogenic inducer, for 72 h. As shown in Figure 2B, the melanin content in these PAK1-deficient melanocytes (SH1 and 2) is reduced by around 50% (51-55%), reaching the basal level in the WT without melanogenic hormone. To see if this reduction in melanin content is associated with the suppression of tyrosinase activity, we measured the tyrosinase activity in PAK1-deficient cells (clones SH1 and 2), compared with the WT. As shown in Figure 2C, the tyrosinase activity in PAK1-deficient cells is only around 45% (33-58%) of that in the WT, again reaching the basal (non-stimulated) level, clearly indicating that PAK1 is essential for α-MSH-induced melanin production by tyrosinase in melanocytes. Furthermore, in a very similar manner the IBMX-induced melanogenesis was also reduced by silencing PAK1 gene in this melanoma cell line (data not shown). However, taking the following two factors into account, it is most likely

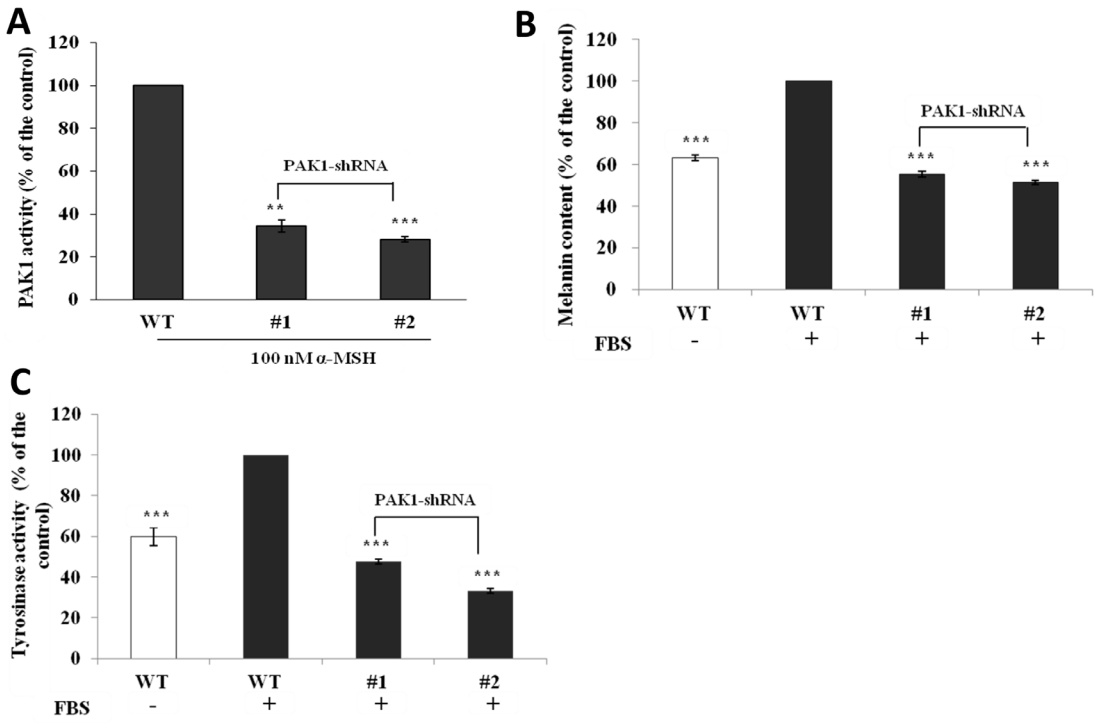

Figure 2. Reduction of melanogenesis by down-regulation of PAK1. (A) Down-regulation of PAK1 by shRNAs. Kinase activity of PAK1 in the transfectant (SH1 and SH2) with PAK1-specific shRNA, compared with the WT melanocytes. Cells were treated with α-MSH (100 nM) for 48 h. PAK1 was immuno-precipitated from cells lysates, and PAK1 activity was measured by "Macaroni-Western" kinase assay. **(B)** Reduction in melanin content by silencing PAK1 gene. The total α-MSH-induced melanin content is reduced around 53% in SH transfectants (1-2), respectively, reaching the basal level in the WT (compare with lane 1). **(C)** Reduction in tyrosinase activity by silencing PAK1 gene. The total tyrosinase activity in SH transfectants (SH1-2) is around 45% of that in the WT, reaching the basal (non-stimulated) level. Values indicate the mean ± SE from three independent experiments. Data have significant difference by ANOVA analysis at $p \leq 0.05$. Statistically significant difference from the control (WT treated with α-MSH) are indicated by asterisks. * $0.01 \leq p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

that only a half of melanogenesis in melanocytes is PAK1-dependent, and the remaining (largely "basic") is PAK4-dependent: (*i*) PAK4 is also essential for the α-MSH-induced melanogenesis in melanocytes (*8*), and (*ii*) around 25% of PAK1 gene appears to be still expressed in these transfectants (SH1 and 2). However, it still remains to be clarified vigorously whether PAK1 is responsible for the hormone-inducible or the basal melanogenesis.

Furthermore, we have confirmed that the endogenous PAK1 is significantly activated by melanogenic inducers such as IBMX and α-MSH in this melanoma cell line (see Figure 1), but without any significant change in its auto-phosphorylation at Thr 423 as judged by the anti-pPAK1 antibody (data not shown).

3.3. *Increase in α-MSH-inducible melanogenesis by over-expressed PAK1*

By over-expression of the wild-type (WT) PAK1 gene in melanocytes (B16F10 cell line) by transfection, we revealed that the exogenously added PAK1 gene boosts the α-MSH-inducible melanin level by several folds (see the left of Figure 3, compare lanes 2 and 4), while it hardly affects the independent "basal" melanin level *per se* (compare lanes 1 and 3 in the left panel of Figure 3), suggesting that PAK1 contributes mainly to α-MSH/IBMX-dependent melanogenesis, and not the basal melanogenesis without exogenous stimulator(s).

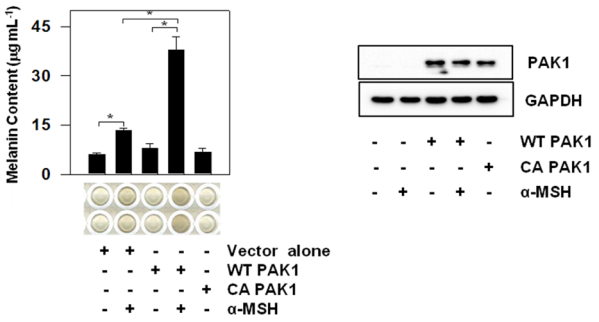

Figure 3. Increase in melanin content by over-expressing PAK1 gene. Left panel: Quantification of melanin content. Lanes 1 and 2 with the control (MYC vector alone) transfection, lanes 3 and 4 with WT PAK1 transfection, and lane 5 with CA PAK1 transfection. The transfection of WT PAK1 gene increased the melanin content more than twice in the presence ofα-MSH (compare lanes 2 and 4). Right panel: western blot analysis. Lanes 3-5 show the expression of transfected PAK1 genes (WT or CA). Data were subjected to ANOVA analysis and statistical significance is indicated by asterisks. ** $p < 0.01$.

3.4. *Serum effect on α-MSH/IBMX-inducible melanogenesis*

More interestingly, we found that the induction of

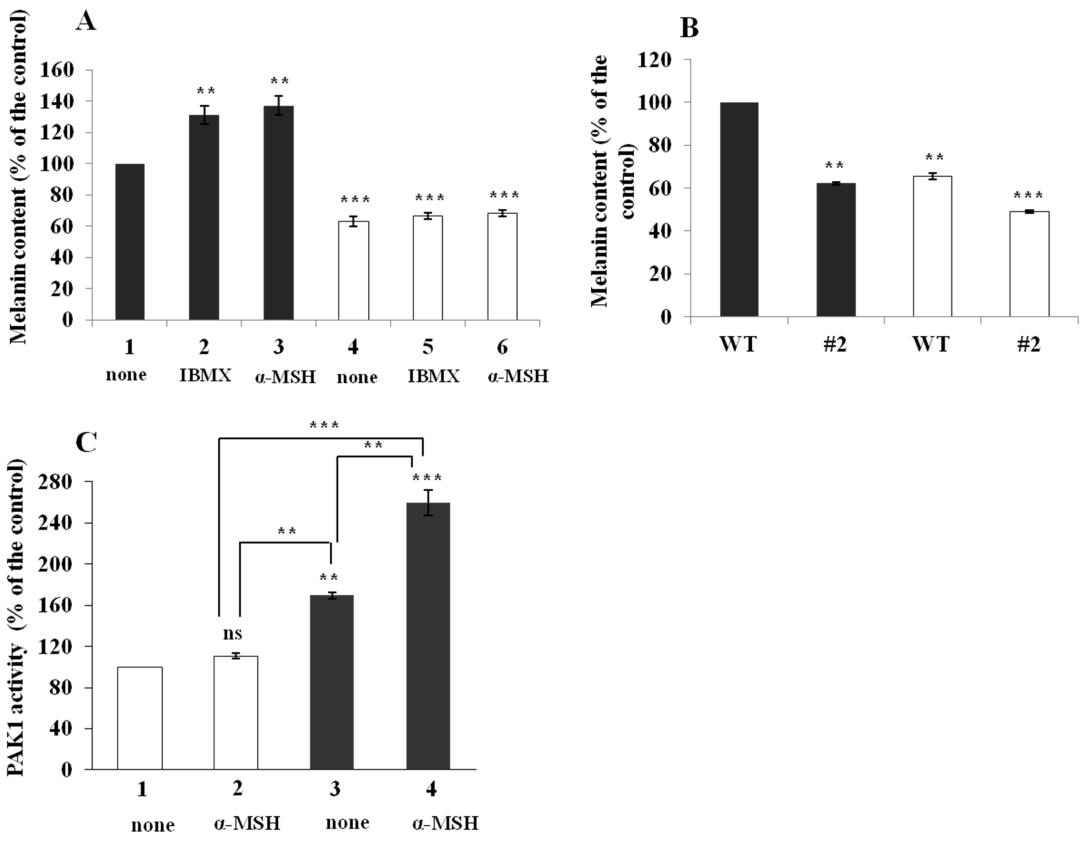

Figure 4. Serum/PAK1-dependency of melanogenesis. Open bars, the control (no serum); closed bars, 10% FBS. **(A)** α-MSH/IBMX requires FBS for their induction of melanogenesis. **(B)** PAK1 is essential for the serum- dependent melanogenesis. WT, the control wild type; #2, PAK1-deficient transfectant. **(C)** α-MSH requires FBS for its robust activation of PAK1. The results are mean ± SE. Data have significance by ANOVA analysis at $p \leq 0.05$. Statistically significant difference in relative to control is indicated by asterisks. ** $p \leq 0.01$, *** $p \leq 0.001$, ns: no significance.

melanogenesis by α-MSH/IBMX absolutely requires 10% FBS (fetal bovine serum) in addition to PAK1. As shown in Figure 4A, in a serum-free medium, either α-MSH or IBMX hardly induced the melanogenesis in the WT cells, although the 10% FBS alone (without α-MSH/IBMX) induced the melanogenesis significantly (by around 60%). Interestingly the melanogenesis in SH transfectants (PAK1-deficient cells) in the presence or absence of serum was basically the same level as that in WT cells in the absence of serum (see Figure 4B), suggesting that the serum-dependent melanogenesis also requires PAK1. In supporting this notion, serum alone significantly activates the kinase activity of PAK1 in WT cells, but the α-MSH-dependent activation of PAK1 in WT cells absolutely requires serum (see Figure 4C).

Regarding the chemical nature of melanogenic serum factor that alone activates PAK1, we speculate that it is PDGF (platelet-derived growth factor) from the following reasons: (*i*) more than a decade ago we have shown that PDGF is the sole growth factor in serum that activates PAK1 through the transactivation of EGF (epidermal growth factor) receptor by PDGF receptor (*13,14*), and very recently others have proven that either PDGF or EGF alone indeed stimulates the melanogenesis of melanocytes (*15,16*).

3.5. *The serum/PDGF-dependent melanogenesis requires EGF receptor*

In an attempt to identify the specific chemical nature of this melanogenic serum factor, we have tested the effect of either AG1295 (inhibitor specific for PDGF receptor) or AG1478 (inhibitor specific for EGF receptor) on the serum-dependent melanogenesis in melanocytes. As shown in Figure 5, either 2 μM AG1295 or 400 nM AG1478 strongly reduced the serum-dependent melanogenesis to the level equivalent to the basic (serum-free) melanogenesis. Since PDGF is abundantly present in serum, but not EGF, and AG1295 does not inhibit EGF receptor, while AG1478 does not inhibit PDGF receptor (*13*), it is most likely that PDGF in serum activates its receptor that in turn trans-activates EGF receptor that eventually activates PAK1 that is essential for serum-dependent melanogenesis (for detail, see Figure 6). Based on this assumption, we shall discuss later the most likely mechanism underlying the apparent synergy between α-MSH and serum/PDGF for the activation of PAK1, leading to the robust melanogenesis.

Lastly, it should be worth pointing out that the "basal" melanin content without α-MSH was not significantly changed by over-expressed CA (constitutively active)

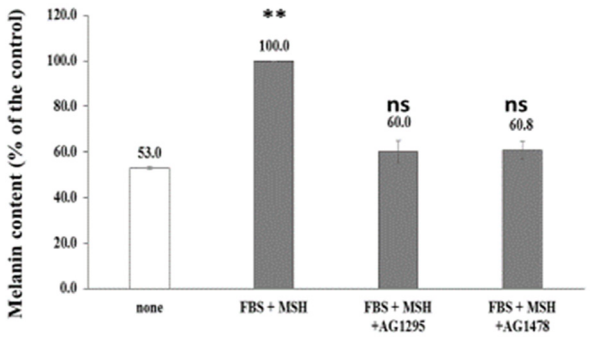

Figure 5. PAK1-dependent serum-induced melanogenesis requires both PDGF and EGF receptors. Open bar, the control (no serum); closed bars, 10% FBS plus α-MSH. The last two bars, plus either AG1295 or AG1478. Both inhibitors equally reduced the serum-induced melanogenesis to the basal (serum-free) level. The results are mean ± SE. Data have significance by ANOVA analysis at $p \leq 0.05$. Statistically significant difference in relative to control is indicated by asterisks. ** $p \leq 0.01$, ns: no significance.

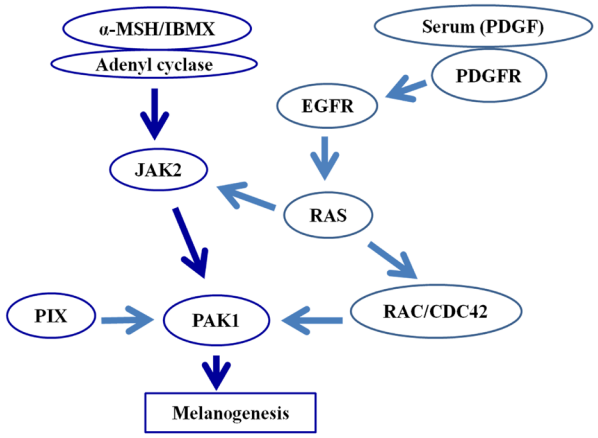

Figure 6. Most likely mechanism underlying the Serun/PAK1-dependent melanogenesis. Either α-MSH or IBMX activates PAK1 through adenyl cyclase that produces cAMP, which in turn activates PKA, and JAK2 that secures the PIX-PAK1 interaction. However, the PIX-PAK1 interaction alone is not sufficient for the full activation of PAK1. It needs RAC/CDC42 which is activated by the oncogenic RAS-PI3 kinase signalling cascade. To activate RAS, cells need either PDGFR or EGFR. Serum is the major source of PDGF, and its receptor (PDGFR) trans-activates EGF receptor. Once RAS is activated, two complementary pathways are activated: one leading to RAC/CDC42 activation, and the other heading for JAK2 activation that secures the PIX-PAK1 interaction. Thus, serum alone can induced melanogenesis through RAC/CDC42- and PIX-dependent PAK1 pathways, but α-MSH or IBMX can further boost the melanogenesis by enhancing the "rate-limiting" PIX-dependent pathway.

mutant of PAK1 carrying T423E mutation (see the left panel of Figure 3, lane 5), as if it were either a DN (dominant negative) or inactive mutant. In fact, α-MSH did not induce the phosphorylation of WT PAK1 at Thr 423 at all (data not shown). Thus, it could be concluded that the auto-phosphorylation of PAK1 at Thr 423 has nothing to do with α-MSH-induced activation of PAK1, which leads to the robust melanogenesis in melanoma cells. Since T423E mutant of PAK1 is well known to be highly oncogenic (*6*), perhaps the auto-phosphorylation at Thr 423 might serve a switch from "melanogenic" to "oncogenic" signalling.

4. Discussion

From our observation on both PAK1-dependent and PAK4-dependent melanogensis in melanocyte/melanoma cells, there rise two potentially interesting issues to be pointed out: (*i*) The "specific" melanogenic activity of PAK1 (only minutely expressed) must be far higher than that of PAK4 (highly expressed) in melanoma cells. (*ii*) It appears that PAK1 is mainly responsible for serum-induced melanogenesis, while PAK4 is mainly responsible for the intrinsic (basal) melanogenesis. In other words, although PAK4-deficiency is embryonically lethal in mice, while PAK1-deficiency alone fails to produce "albino" mice, the apparent difference in the original skin color (basic melanogenesis) between black and white people for instance could be at least partly a reflection of the difference in the expression level of PAK4, as well as difference in the level of melanogenic tyrosinases.

In vivo, using HRM-2 (pigmented but hairless) mice, we have recently shown that a cream containing 10 μM PF3758309 (PAK1/PAK4-inhibitor) reduces the UV-induced melanogenesis in their skin to the basal level, although it still remains to be clarified whether PAK4 or PAK1 is responsible for UV-induction of melanogenesis (*8*). Since PAK1 is responsible for the inducible, but not the basal, melanogenesis, it would be worth testing if PAK1 significantly contributes to the UV-inducible melanogenesis (sun tanning) as well, using the rare PAK1 KO (knock out) mutant derived from C57B16 strain of mice carrying dark hairs and eyes (Hong He *et al*., unpublished observation), in an attempt to understand if a variety of herbal PAK1 blockers in cosmetics are useful for sun-screening agents or not. Interestingly PAK1 was reported to be activated by UV irradiation and other DNA-damaging agents (*17*).

It has been shown that α-MSH activates the Tyr-kinase JAK2 (*18*), which in turn activates PAK1 by the phosphorylation at Tyr 285, (instead of Thr 423), leading to the PIX-PAK1 interaction (*19*). This could explain why neither α-MSH–dependent activation of PAK1 nor melanogenesis involves the autophosphorylation of PAK1 at Thr 423. Thus, we recently investigated if the PAK1-dependent melanogenesis is blocked by a potent herbal JAK2-inhibitor called cucurbitacin I (CBI) from bitter melon (Goya) that inhibits directly JAK2 (*20*), and found that CBI inhibits the melanogenesis in the presence of melanogenic hormones by more than 70%, suggesting the possibility that CBI blocks not only PAK1 but also PAK4 (*5*).

In this context, it would be of great interest to note that the herbal PAK1-blockers such as CAPE, curcumin, shikonin, and FTY 720 could inhibit the α-MSH-

induced melanogenesis in mouse or human skin cells only by around 50% even at their concentrations where PAK1 is almost completely blocked (*1,2*), whereas the synthetic pan-PAK-blocker PF3758309, inhibiting both PAK1 and PAK4, abolishes the melanogenesis in skin cells by around 90% at 300 nM (*8*). In other words, the α-MSH-induced melanogenic system in melanocytes could distinguish the PAK1-specific blockers from pan-PAK-blockers. So far no herbal PAK4-specific blockers (other than PAK4-specific siRNAs) have been identified. However, a very potent quassinoid called glaucarubinone derived from bitter tree grown in Amazon jungles has recently been shown to block both PAK1 and PAK4, inhibiting the growth of pancreatic cancer cells both *in vitro* and *in vivo* (*21*). Thus, it would be of great interest to test if this herbal PAK-blocker suppresses the melanogenesis of skin cells almost completely as does PF3758309.

The major aim of this study was to focus on the specific melanogenic role of PAK1, and not to investigate in detail how PAK1 activates the melanogenic signalling pathway including melanogenic enzymes and MTFs. However, it is most likely that PAK1 activates directly beta-catenin by phosphorylating at Ser 675, leading to the activation of MITF which is essential for expression of genes encoding for melanogenic enzymes such as tyrosinase (Tyr).

Recently we found that PAK4 (CDC42-dependent kinase 4) is also involved in melanogenesis of the same melanoma cell line by activating two transcription factors, CREB and beta-catenin, both of which are essential for MIFT activation (*8*). Since CREB is known to be activated by LIM kinase (*22,23*) which like beta-catenin, is among the common direct substrates of both PAK1 and PAK4 (*6,18*), it is most likely that PAK1 and PAK4 share the same CREB/beta-catenin-MITF signalling pathways to activate the melanogenic enzyme genes.

However, the detailed signal pathways leading to the α-MSH/IBMX-dependent activation of PAK1 could differ significantly from those leading to the activation of PAK4, mainly because the former clearly involve the serum factor (PDGF). The serum alone is capable of activating PAK1 (*13*), and boosts the α-MSH/IBMX-dependent activation of PAK1 as well. In other words, there is a clear synergy between the serum and these melanogenic hormones in both PAK1 activation and melanogenesis.

The following is our working hypothesis as to how this synergy could take place. The major signalling pathway leading to the PAK1 activation is the oncogenic EGFR (epidermal growth factor receptor)-RAS-PI 3 kinase-RAC/CDC42-PAK1 cascade. However, this cascade alone is not sufficient for the full-activation. It needs another factor called PIX, an SH3 adaptor protein that binds directly PAK1 through its Pro-rich motif of 18 amino acids called PAK18. The PIX-PAK1 interaction needs a third protein called JAK2, a Tyr-kinase, that phosphorylates PAK1 at Tyr 285. RAS up-regulates JAK2 through prolactin. According to a few previous findings by us and others (*13-16*), PDGF (platelet-derived growth factor) is the major melanogenic serum factor(s) essential for the α-MSH/IBMX-induced melanogenesis, because it activates its receptor (PDGFR) Tyr-kinase that in turn trans-activates EGFR (epidermal growth factor receptor=ErbB1) Tyr-kinase, leading to the activation of PAK1 through the oncogenic RAS-PI3 kinase-RAC/CDC42 pathway (*13*). Thus, PDGF in serum alone can activates PAK1 and induce melanogenesis up to a half way. However, for the full activation of PAK1-dependent melanogensis, α-MSH/IBMX is needed to activate the JAK2 through a cAMP-dependent pathway (which might involve PKA) that eventually secures the PIX-PAK1 interaction (for detail, see Figure 6).

In conclusion, here we present the very first biochemical evidences which might explain how a variety of herbal PAK1-blockers such as CAPE, curcumin and shikonin in cosmetic creams could contribute to the "skin-whitening" effects. A series of analogous studies with human melanocytes are awaited for the further proof or confirmation.

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