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Lipocalin-type prostaglandin D synthase as a regulator of the retinoic acid signalling in melanocytes

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Lipocalin-type prostaglandin D synthase (L-PGDS) catalyses the formation of prostaglandin D_2 (PGD_2) and also functions as a transporter for lipophilic ligands, including all-*trans* retinoic acid (RA). Here, we show that human epidermal melanocytes produce and secrete L-PGDS and PGD_2 in culture medium, whereas L-PGDS is not expressed in human melanoma cell lines, HMV-II, SK-MEL-28, 624 mel and G361. Treatment with RA (1 or 10 μM) for 4 days decreased the proliferation of melanocytes (30% decrease), but not melanoma cells. We therefore isolated L-PGDS-expressing cell lines from 624 mel cells. Treatment with RA decreased the proliferation of L-PGDS-expressing cells by 20%, but not mock-transfected cell lines lacking L-PGDS expression. RA induced expression of a cyclin-dependent kinase inhibitor $p21^{Cip1}$ in L-PGDS-expressing cells, but not mock-transfected cells. Moreover, RA increased the transient expression of a reporter gene carrying the RA-responsive elements in L-PGDS-expressing cell lines (at least 5-fold activation), compared to the 2-fold activation in mock-transfected cell lines, suggesting that L-PGDS may increase the sensitivity to RA. Lastly, the knockdown of L-PGDS expression by RNA interference was associated with the restoration of the RA-mediated decrease in proliferation of human and mouse melanocytes. In conclusion, L-PGDS may fine-tune the RA signalling in melanocytes.

Keywords: all-*trans* retinoic acid/lipocalin-type prostaglandin D synthase/melanocytes/melanoma/proliferation.

Abbreviations: L-PGDS, lipocalin-type prostaglandin D synthase; MITF, microphthalmia-associated transcription factor; NHEM, normal human epidermal melanocytes; PG, prostaglandin; RA, all-*trans* retinoic acid.

Epidermal melanocytes play an important role in the protection against solar radiation by producing melanin. Development and survival of melanocytes depend on microphthalmia-associated transcription factor (Mitf) (*1–3*). Homozygous Mitf-mutant mice, black-eyed white $Mitf^{mi\text{-}bw}$, lack melanocytes in the skin and inner ear and thus exhibit the complete white coat colour and deafness (*4*). $Mitf^{mi\text{-}bw}$ mice also exhibit augmentation of ventilatory responses to hypoxia and hypercapnia (*5*), which may be related to a poorly characterized neuroendocrine function of melanocytes (*6*). We have identified lipocalin-type prostaglandin D synthase (L-PGDS) as a melanocyte marker by cDNA microarray analysis between the skin of wild-type and $Mitf^{mi\text{-}bw}$ mice (*4*,*7*). L-PGDS catalyses the isomerization of prostaglandin (PG) H_2 to produce PGD_2 (*8*). L-PGDS and PGD_2 may contribute to neuroendocrine function of melanocytes (*6*). L-PGDS is also secreted into various body fluids (*8*), such as plasma and cerebrospinal fluid, and binds lipophilic ligands, including retinaldehyde and retinoic acid, with high affinities ($K_d = 30-80$ nM) (*8*, *9*). In addition, the structural and functional analysis of Zebrafish and chicken L-PGDS homologues has revealed that non-mammalian L-PGDS shows weak or no catalytic activity (*10*). It is therefore conceivable that L-PGDS may primarily function as a transporter for lipophilic ligands.

It has been shown that PGD_2 is a potent inhibitor of the growth of cultured human melanoma cells (*11*, *12*) and also inhibits metastasis of B16 mouse melanoma cells (*13*, *14*). In addition, all-*trans* retinoic acid (RA) inhibits the proliferation of cancer cells (*15*), including melanoma cells (*16*). RA has been also shown to inhibit UVB-induced melanogenesis (*17*) and decrease the degree of pigmentation in melasma and a certain type of lentigo (*18*). RA induces proliferation arrest, differentiation and apoptosis through retinoic acid receptor (RAR) and activates transcription of RAR target genes in a wide variety of cell types (*19*). We have reported that L-PGDS mRNA is expressed in normal human epidermal melanocytes, but is undetectable in human melanoma cell lines (*7*). In this context, human melanoma cells are often associated with RA resistance (*16*, *20*, *21*). The sensitivity or resistance to RA is determined by many factors, including RA-degrading enzymes and cellular retinoic acid-binding proteins (*22*).

In the present study, we explored the hitherto unknown role of L-PGDS in the biological actions of RA and provide the evidence for the link between L-PGDS and the RA signalling.

Materials and Methods

Cell culture

HMV-II human melanoma cells were obtained from RIKEN Cell Bank and cultured in nutrient mixture Ham's F12 medium containing 10% foetal bovine serum (FBS). Human melanoma cell lines, G361 and SK-MEL-28, were obtained from Cell Resource Center for Biomedical Research (Institute of Development, Aging and Cancer, Tohoku University, Miyagi, Japan). Human melanoma cells (624 mel) were provided by Dr Y. Kawakami (Institute for Advanced Medical Research, School of Medicine, Keio University, Tokyo, Japan). G361 and 624 mel cells were cultured in RPMI-1640 medium supplemented with 10% FBS. SK-MEL-28 cells were cultured in minimum essential medium eagle supplemented with 10% FBS. Normal human epidermal melanocytes were obtained from KURABO (Osaka, Japan) and cultured in Medium 154S (KURABO) containing human melanocyte growth supplement (KURABO). Melan-a murine immortalized melanocytes, a gift from Bennet D. (*23*), were grown in minimum essential medium supplemented with 10% FBS and 200 nM phorbol 12-myristate 13-acetate.

RT–PCR

Total RNA was extracted from human epidermal melanocytes and melanoma cell lines, HMVII, 624 mel, SK-MEL 28, and G361 using TRI Reagent (Sigma, St Louis, MI, USA), and subjected to RT–PCR. PGD_2 receptor consists of DP1 (*24*) and DP2, that is identified as chemo-attractant receptor-homologous molecule expressed on Th2 lymphocytes, CRTH2 (*25*). Cyclooxygenase 1 (COX-1) and COX-2 are upstream enzymes essential for PG synthesis, including PGD_2. The primer sets used were 5′-GAC AAG TTC CTG GGG CGC TGG T-3′ and 5′-GCT GTA GAG GGT GGC CAT GC-3′ for L-PGDS; 5′-CCA TGC GCA ACC TCT ATG CGA T-3′ and 5′-AAT TGC TGC ACC GGC TCC TGT A-3′ for DP1; 5′-CCT CTG TGC CCA GAG CCC CAC GAT GTC GGC-3′ and 5′-CAC GGC CAA GAA GTA GGT GAA GAA G-3′ for DP2; 5′-CCC CCA CCT ACA ACT CAG CAC A-3′ and 5′-AGG GCG GGT ACA TTT CTC CAT C-3′ for COX-1; 5′-CTT TGG TCT GGT GCC TGG TCT GAT GAT GTA-3′ and 5′-CAG AAG GGG ATG CCA GTG ATA GAG GGT GTT-3′ for COX-2; and 5′-ACC ACA GTC CAT GCC ATC AC-3′ and 5′-TCC ACC ACC CTG TTG CTG TA-3′ for glyceraldehyde-3-phosphate dehydrogenase (G3PDH). The PCR conditions were 30 cycles of 94°C for 1 min, 68°C for 1 min and 72°C for 1 min for L-PGDS and DP1, 30 cycles of 94°C for 1 min, 68°C for 1 min and 72°C for 2 min for DP2 and G3PDH and 30 cycles of 94°C for 15 s and 60°C for 1 min for COX-1 and COX-2.

Real-time RT–PCR for components of the RA signalling was also performed. The PCR conditions were 95°C for 1 min and 40 cycles of 95°C for 15 s and 60°C for 1 min. The primers used were 5′-CCT AAT CCG CCC ACA GGA A-3′ and 5′-ACC TCC GGG AGA GAG GAA AA-3′ for $p21^{Cip1}$; 5′-CCG GCT AAC TCT GAG GAC AC-3′ and 5′-AGA AGA ATC GTC GGT TGC AG-3′ for $p27^{Kip1}$; 5′-GTA AAT ACA CCA CGA ATT CCA GTG CTG A-3′ and 5′-CAG ACG TTT AGC AAA CTC CAC GAT CTT A-3′ for RARβ; and 5′-GAT ATG CTC ATG TGG TGT TG-3′ and 5′-AAT CTT CTT CAG TCG CTC CA-3′ for 18S rRNA.

Measurement of PGD_2

PGD_2 concentration was measured in the culture media of normal human epidermal melanocytes, 624 mel human melanoma cells and other melanoma-derived transfectants cultivated for 7 days, with Prostaglandin D_2-MOX EIA Kit (Cayman Chemical, MI, USA) according to the manufacturer's instructions.

Cell proliferation assay

Cells were seeded in the 96-well plate (1×10^3 cells/well) and cultivated overnight, and then incubated under various conditions indicated. Cell proliferation was assessed with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay kit (Nakalai, Kyoto, Japan). MTT values, obtained at OD_{570}, reflect the number of viable cells. Cells were treated with the indicated concentration of PGD_2 (Cayman Chemical, Ann Arbor, MI, USA) or RA (Sigma, St. Louis, MI, USA) for 3 or 4 days, and subjected to the MTT assay.

Western blot analysis

Whole cell extracts were prepared from the cells by the method of Schreiber *et al*. (*26*) and then subjected to western blot analysis (20 μg/lane) using anti- $p21^{Cip1}$ (Santa Cruz, California, USA), anti-$p27^{Kip1}$ (Santa Cruz), anti-L-PGDS (Novus Biologicals Inc., Littleton, CO, USA), or anti-β-actin (Sigma), as described previously (*27*).

RNA interference

RNA interference analysis against human L-PGDS was performed with short interfering RNA (siRNA), known as stealth RNAi (Invitrogen, Carlsbad, CA, USA). The three different siRNAs against human L-PGDS were L-PGDSi-1 (UAU UGU UCC GUC AUG CAC UUA UCG G), L-PGDSi-2 (AGG CGG UGA AUU UCU CCU UUA ACU C) and L-PGDSi-3 (AUC CAC AGC GUG UGA UGA GUA GCC A). Negative control RNAi was designed according to the manufacturer's instructions. Normal human epidermal melanocytes were cultured for 16 h after plating in 3.5-cm dishes or 96-well plates (1×10^3 cells/well), and then transfected with each L-PGDSi or negative control RNAi by Lipofectamine™ RNAiMAX protocol (Invitrogen). After incubation with each RNAi for 6 h, RA or vehicle was added to the culture medium. Cells were then incubated for 4 days and harvested for RNA preparation, whereas the conditioned media were subjected to western blot analysis. Cells seeded in a 96-well plate were used for MTT assay. RNA interference analysis against mouse L-PGDS was also performed with stealth RNAi (Invitrogen) as described above. The siRNAs used were mL-PGDSi-1 (UAG CAG AGC GUA CUC GUC AUA GUU G), mL-PGDSi-2 (UGU AGA GGG UGG CCA UGC GGA AGU C) and mL-PGDSi-3 (AAA GGU GGU GAA UUU CUC CUU CAG C).

L-PGDS immunoassay

The cells were untreated or treated with vehicle (ethanol) or RA (1 or 10 μM), and incubated for 4 days, and each culture medium was subjected to L-PGDS immunoassay, with human lipocalin-type Prostaglandin D Synthase EIA kit (Cayman chemical, MI, USA) according to the manufacturer's instructions. The amount of L-PGDS measured was divided with the cell number per dish.

Plasmid construction

Human L-PGDS cDNA was amplified by PCR from human retinal epithelial cell line, ARPE-19 and inserted into the expression vector pUB6/V5-His (Invitrogen, Carlsbad, CA, USA), yielding pUB6/V5-L-PGDS. The predicted L-PGDS protein contains the entire amino acid residues and is tagged with V5 epitope and hexa-histidine at the C-terminus. The sequence of the obtained L-PGDS cDNA was confirmed. L-PGDS protein contains the entire amino acid residues and is tagged with V5 epitope and hexa-histidine at the C-terminus. Further, the DNA fragment, containing L-PGDS, V5 and hexa-histidine, from pUB6/V5-L-PGDS, was ligated with pIRESneo (Clontech, CA, USA), yielding pIRESneo-L-PGDS. pGL3-basic (Promega, Madison, WI, USA) was ligated with enhancer-less SV40 promoter derived from pGL2-Control (Promega), yielding pGL3sv. The two copies of retinoic acid-responsive element (RARE), tagggttcaccgaaagttca, in the gene promoter of the mouse RARβ (*28*), were inserted into the upstream of the SV40 promoter, yielding pGL3sv-2RARE.

Establishment of melanoma cell lines expressing L-PGDS

Cells (624 mel) were transfected with pUB6/V5-L-PGDS or parent vector pUB6/V5-His using FuGENE 6 (Roche Diagnostics, Mannheim, Germany), and selected with blasticidin (4 μg/μl). The vector pUB6/V5-His encodes a resistant gene for blasticidin, which is a nucleoside antibiotic, and a potent translational inhibitor in both prokaryotic and eukaryotic cells. Thus, independent cell lines were established from L-PGDS vector-transfected cells and mock-transfected cells. The secreted L-PGDS protein tagged with

hexa-histidine was collected from the media using Ni-column, and subjected to western blot analysis with anti-V5 antibody (Invitrogen) and anti-α-tubulin antibody (NeoMarkers, Fremont, CA, USA).

Northern blot analysis

Northern blot analysis for L-PGDS mRNA was performed with the DIG Northern Starter Kit (Roche). The human L-PGDS cDNA of positions 76–648 (NM_000954) was amplified by PCR and then cloned into pGEM-T Easy (Promega), named T-PGDS. SP6 RNA polymerase was used for transcription of RNA probe containing the entire coding region of L-PGDS.

Luciferase assay

L-PGDS-expressing cell lines, L-PGDS #1 and #2, and mock-transfected cell lines, Mock #1 and #2, were cultured in 12-well plates 1 day before transfection to reach 50–80% confluence. Cells were transfected with FuGENE 6 for 24 h using 10 ng of Renilla luciferase control plasmid pRL-CMV vector as an internal control (Promega) and 490 ng of pGL3sv-2RARE per well. Following the 12 h incubation, cells were treated for 48 h with 10 μM RA or vehicle. Expression of reporter genes and pRL-CMV was determined with the Dual-Luciferase™ Reporter Assay System (Promega).

Mass culture of melanoma cells expressing L-PGDS

HMVII, 624 mel, SK-MEL 28 and G361 human melanoma cells were transfected with pIRESneo-L-PGDS, and selected with an antibiotic G418 (600 μg/μl). We thus established L-PGDS-expressing transfectants in a mass culture. Because pIRESneo-L-PGDS produces mRNA, encoding L-PGDS and Neo^R G418 resistance gene linked with internal ribosomal entry site (IRES), L-PGDS and Neo^R are translated from the same mRNA. G418-resistant cells therefore simultaneously express L-PGDS.

Statistical analysis

All data are mean ± SD of at least three independent experiments. Two-tailed Student's *t*-test was used for comparison between the two groups. The two groups compared are specified in each figure legend. Differences between mean values were considered significant when $P < 0.05$.

Results

L-PGDS expression is associated with the RA-mediated proliferation inhibition of human melanocytes

RT–PCR analysis revealed that mRNAs for L-PGDS and the PGD_2 receptors, DP1 and DP2, are expressed in normal human epidermal melanocytes, but not in human melanoma cell lines, HMVII, 624 mel, SK-MEL 28 and G361 (Fig. 1A). In contrast, COX-1 and COX-2 mRNAs are expressed in both human melanocytes and human melanoma cell lines (Fig. 1A). We also confirmed the production of PGD_2 in normal human epidermal melanocytes; namely, the PGD_2 concentrations in the conditioned media were 13.6 ± 0.6 pM after cultivation of 7 days. In contrast, PGD_2 was undetectable in the conditioned media of melanoma cells. The distinct expression profiles of L-PGDS mRNA led us to hypothesize that L-PGDS may influence the growth potential of melanocytes and melanoma.

We then examined the effect of PGD_2 on the proliferation of human epidermal melanocytes and 624 mel melanoma cells (Fig. 1B). The proliferation of human melanocytes (left panel) was decreased by the treatment with PGD_2 for 3 days at the concentration of 1 or 10 μM, each concentration of which was much higher than that detected in the media of human melanocytes (Fig. 1B). PGD_2 at 0.1 μM did not show the

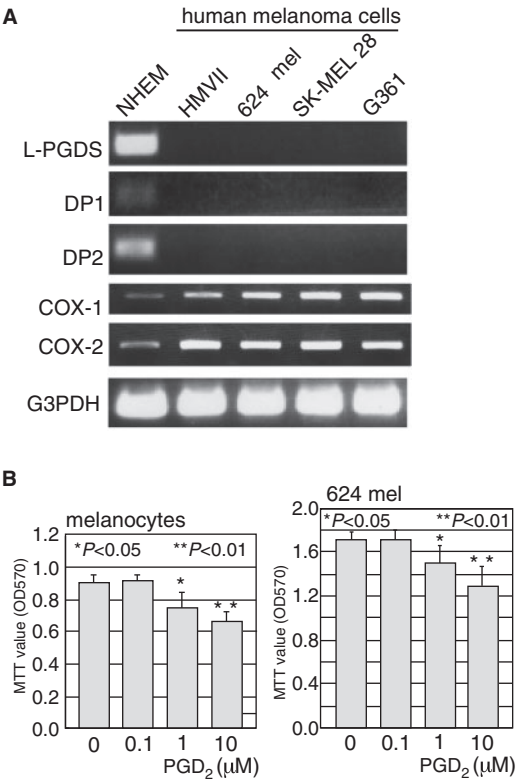

Fig. 1 Expression of L-PGDS and the effect of PGD_2 on proliferation of human melanocytes. (A) RT–PCR analysis showing expression profiles of mRNAs for L-PGDS, PGD_2 receptors DP1 and DP2, COX-1 and -2 in normal human epidermal melanocytes (NHEM) and human melanoma cell lines. G3PDH mRNA was included as a control. (B) Effect of PGD_2 on the proliferation of melanocytes and melanoma cells. Normal human epidermal melanocytes (left panel) or 624 mel human melanoma cells (right panel) were treated with PGD_2 at the indicated concentrations for 3 days. Proliferation was assessed by MTT assay. Shown at the ordinate are MTT values that reflect the number of viable cells. The data are means ± SD of more than three independent experiments (*$P < 0.05$, **$P < 0.01$, compared to vehicle).

noticeable effect on cell proliferation. PGD_2 at 1 or 10 μM also decreased the proliferation of 624 mel melanoma cells (right panel) and other melanoma cell lines, HMVII, SK-MEL 28 and G361 (data not shown), despite that PGD_2 receptors were undetectable in the melanoma cell lines analysed. These results indicate that the PGD_2-mediated decrease in proliferation is independent of the PGD_2 receptors, DP1 and DP2.

Human melanoma cells are often associated with RA resistance (*16*,*20*,*21*). Considering the RA-transporting function of L-PGDS (*8*, *9*), we analysed the effect of RA on the proliferation of human epidermal melanocytes and melanoma cells. Treatment with RA (1 or 10 μM) for 4 days decreased the proliferation of human melanocytes by ~30% (Fig. 2A, left panel). The time-course study revealed that the RA-mediated decrease in proliferation of melanocytes was detected at 4 and 6 days of RA exposure (1 or 10 μM), but not at 2 days (Fig. 2B). In contrast, RA treatment for 4 days did not cause noticeable change in the proliferation of melanoma cells that lack L-PGDS expression (Fig. 2A, right panel). No inhibitory effect was

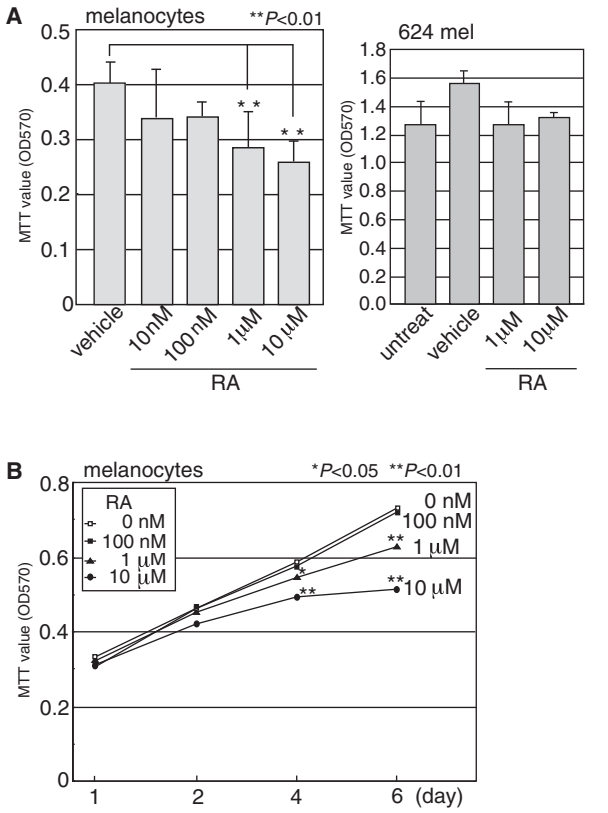

Fig. 2 Different sensitivity to RA in human melanocytes and melanoma cells. (A) Effect of RA on the proliferation of melanocytes and melanoma cells. Normal human epidermal melanocytes (left panel) or 624 mel melanoma cells (right panel) were treated with RA at the indicated concentrations for 4 days. The data are means ± SD of more than four independent experiments (**$P<0.01$, compared to vehicle). Note that RA did not show the noticeable effect on the proliferation of melanoma cells. The lower MTT values, compared to the time-course study shown below, were due to the small number of melanocytes, when the experiments were performed. (B) Time-course study of RA effect on the proliferation of normal human melanocytes. Normal human epidermal melanocytes were treated with RA at the indicated concentrations for 1, 2, 4 and 6 days. The data are means ± SD of more than four independent experiments [*$P<0.05$, **$P<0.01$, compared to 0 μM (vehicle) at a given time-point]. The time-course data with 10 nM RA are not presented, because no noticeable effect was detected.

detected in melanoma cells even after the treatment with 10 μM RA for 6 days (data not shown). These results suggest the link between L-PGDS and the RA-mediated decrease in proliferation of melanocytes.

L-PGDS is involved in the RA signalling in human melanocytes

To study the mechanism for the RA-mediated decrease in proliferation of melanocytes, we measured the expression of cyclin-dependent kinase inhibitors, $p21^{Cip1}$ and $p27^{Kip1}$, each of which is associated with cell cycle arrest and is induced by RA (*20*, *29*, *30*). Treatment with RA (1 or 10 μM) increased the expression levels of $p21^{Cip1}$ mRNA and protein in melanocytes (Fig. 3A), while the expression of $p27^{Kip1}$ remained unchanged (Fig. 3A, right panel). These results suggest that the induction of $p21^{Cip1}$ may be responsible for the RA-mediated decrease in proliferation of human epidermal melanocytes.

We next performed RNA interference against L-PGDS using three different siRNAs (L-PGDSi-1, −2 and −3). Among these siRNAs, only L-PGDSi-1 effectively reduced the expression of L-PGDS mRNA (~50% reduction) (Fig. 3B, left panel); thus, L-PGDSi-1 was used in the subsequent experiments. The expression level of L-PGDS mRNA remained unchanged with negative control RNAi. L-PGDSi-1 also reduced the amount of secreted L-PGDS in culture medium of epidermal melanocytes (Fig. 3B, right panel). These results indicate the validity of RNA interference. It is also noteworthy that human melanocytes produce and secrete L-PGDS protein under basal conditions (14.6 ± 3.6 ng/10^6 cells). We then analysed the effect of RA on the expression of $p21^{Cip1}$ mRNA in melanocytes treated with L-PGDSi-1 or negative control RNAi. Partial knockdown (~50% reduction) of L-PGDS mRNA with L-PGDSi-1 significantly decreased the degree of the $p21^{Cip1}$ induction at 10 μM RA (~17% decrease), compared with negative control RNAi (Fig. 3C). Likewise, the degree of the $p21^{Cip1}$ induction at 1 μM RA was decreased in L-PGDSi-1-treated melanocytes, compared with negative control RNAi, although the decrease was not statistically significant (Fig. 3C). We further analysed the effect of RA on cell proliferation. L-PGDSi-1 treatment significantly decreased the degree of the RA-mediated proliferation restriction (~10% restoration), compared with negative control RNAi (Fig. 3D). Taken together, these results suggest that L-PGDS may be involved in the RA-mediated decrease in proliferation of human melanocytes in part through the induction of $p21^{Cip1}$.

Isolation of L-PGDS-expressing cell lines and their sensitivity to RA

To obtain further evidence for the role of L-PGDS in the RA signalling, we took the advantage that L-PGDS is not expressed in melanoma cells (Fig. 1A) and thus established independent L-PGDS-expressing cell lines from 624 mel melanoma cells. The expression vector, pUB6/V5-L-PGDS, codes for V5-epitope- and hexa-histidine-tagged L-PGDS. The expression of L-PGDS mRNA was detected in L-PGDS vector-transfected cell lines (L-PGDS #1 and #2), but not in 624 mel cells (parent cell line) and a L-PGDS vector-transfected cell line (L-PGDS #3) (Fig. 4A). We also detected V5 epitope-tagged L-PGDS protein in the whole cell extracts and culture media of L-PGDS #1 and #2 cells, but not L-PGDS #3 cells (Fig. 4B). L-PGDS #3 was used as one type of control in the subsequent experiments. In addition, the amount of secreted L-PGDS from L-PGDS #1 cells was 220.9 ± 111.9 ng per 10^6 cells, but L-PGDS was undetectable in culture media from 624 mel cells (parent cell line) and a mock transformed cell line, Mock #1. It is therefore conceivable that L-PGDS protein is actively secreted into the culture medium, which may account for the low levels of L-PGDS protein in the whole cell extracts of L-PGDS #1 and #2 cells (Fig. 4B). Moreover, PGD_2 concentrations in the conditioned media of L-PGDS #1 cells were 9.0 ± 0.2 pM after cultivation of 7 days, which is comparable to the

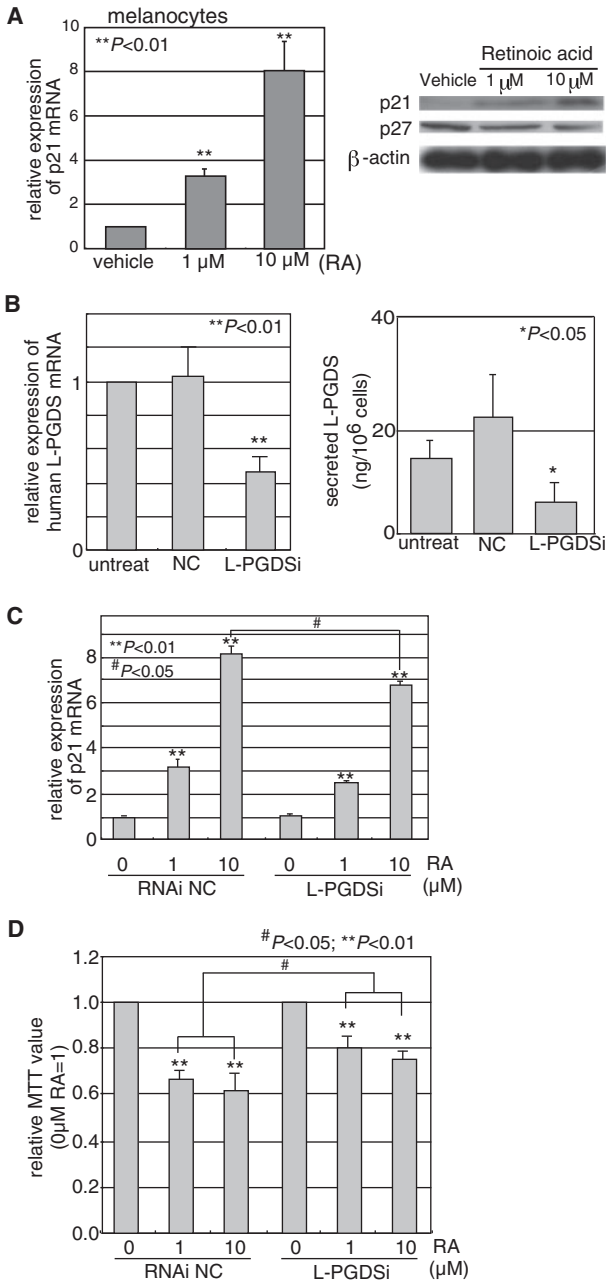

Fig. 3 Role of L-PGDS in the RA-mediated decrease in proliferation of human melanocytes. (A) Induction of $p21^{Cip1}$, a cyclin-dependent kinase inhibitor by RA. Total RNAs or proteins were prepared from epidermal melanocytes, treated with 1 μM RA, 10 μM RA, or vehicle (ethanol) for 4 days, and subjected to real-time PCR (left panel) and western blot analysis (right panel) for assessment of $p21^{Cip1}$ and $p27^{Kip1}$ mRNAs and proteins, respectively. The mRNA level measured was normalized with 18S rRNA level (internal control) and shown as a ratio to the expression level of vehicle treatment. The data are means ± SD of three independent experiments (**$P<0.01$, compared to vehicle treatment). In right panel, expression of β-actin protein is shown as a loading control. (B) RNA interference analysis against human L-PGDS with L-PGDSi-1 (L-PGDSi). Human epidermal melanocytes were left untreated (untreat) or transfected with L-PGDSi-1 or negative control RNAi (NC) for 4 days, and then used for real-time PCR of L-PGDS mRNA expression (left panel) or L-PGDS immunoassay for the amount of secreted L-PGDS (right panel). The amount of secreted L-PGDS was divided with the cell numbers. L-PGDSi reduced the expression of L-PGDS mRNA by ~50% (left panel; **$P<0.01$) as well as the amount of secreted L-PGDS (right panel; *$P<0.05$) compared with negative control RNAi and untreated control. (C) Knockdown of L-PGDS expression attenuates the RA-mediated $p21^{Cip1}$ induction. Melanocytes were treated with each RNAi and RA (1 or 10 μM) or vehicle (ethanol) for 4 days. Thereafter, the expression level of $p21^{Cip1}$ mRNA was measured by real-time PCR. RA induced the expression of $p21^{Cip1}$ mRNA in melanocytes treated with negative control RNAi (RNAi NC) or L-PGDSi (**$P<0.01$, compared with vehicle control). Note that L-PGDSi decreased the magnitude of the RA-mediated $p21^{Cip1}$ induction compared with RNAi NC ($^{\#}P<0.05$). (D) Role of L-PGDS in the RA-mediated proliferation inhibition. Melanocytes were treated with each RNAi and RA (1 or 10 μM) or vehicle (ethanol) for 4 days. The data are shown as the ratio to the MTT value of vehicle treatment (0 μM). RA (1 or 10 μM) repressed the proliferation of melanocytes treated with negative control RNAi (RNAi NC) or L-PGDSi-1 (L-PGDSi) (**$P<0.01$, compared with vehicle control). Note that the magnitude of the RA-mediated proliferation inhibition was smaller in melanocytes treated with L-PGDSi than that with negative control RNAi ($^{\#}P<0.05$).

level (13.6 ± 0.6 pM) seen in the conditioned media of human melanocytes, whereas PGD_2 was undetectable in the culture media of 624 mel cells and mock cell lines. These results indicate that the tagged L-PGDS protein is catalytically active. However, despite the expression of L-PGDS and production of PGD_2, there was no noticeable difference in the proliferation rate of L-PGDS #1 and #2 cells (Fig. 4C, open column). On the other hand, treatment with RA (10 μM) for 4 days decreased the proliferation of L-PGDS #1 and #2 cells by ~20% (Fig. 4C). In contrast, RA exerted no noticeable effect on the proliferation of cell lines, Mock #1, Mock #2 and L-PGDS #3, in which L-PGDS is not expressed.

We next examined the combined effect of PGD_2 and RA on the proliferation of melanocytes and melanoma transfectants. Human melanocytes (Fig. 4D, left panel) and 624 mel transfectants, Mock #1 and L-PGDS #1 (Fig. 4D, right panel), were treated with 1 μM PGD_2 in the presence or absence of RA (1 μM) for 4 days. Treatment with RA or PGD_2 alone decreased the proliferation of melanocytes by 16 or 27%, respectively; namely, there was no significant difference in the proliferation inhibitory effect between RA and PGD_2. However, the treatment with both RA and PGD_2 decreased the proliferation of melanocytes by 43%, indicating the additive effect of RA and PGD_2 (Fig. 4D, left panel). Likewise, PGD_2 decreased the proliferation of Mock #1 cells (20% reduction) and L-PGDS #1 cells (23% reduction). The co-treatment with RA and PGD_2 decreased the proliferation of L-PGDS #1 cells by 40%; the degree of proliferation restriction was greater than that obtained with a single treatment. In contrast, no additive effect of RA and PGD_2 was detected in Mock #1 cells. These results indicate that the RA-mediated proliferation restriction is dependent on L-PGDS expression, but not PGD_2 receptors, whereas the PGD_2-mediated proliferation restriction is independent of L-PGDS expression.

To further confirm the role of L-PGDS in the inhibitory effect of RA on proliferation of L-PGDS #1 cells, we performed RNA interference with L-PGDSi-1 (data not shown). Treatment with L-PGDSi-1 reduced L-PGDS mRNA expression to the undetectable level and abolished the RA-mediated decrease in proliferation of L-PGDS #1 cells. These results also support

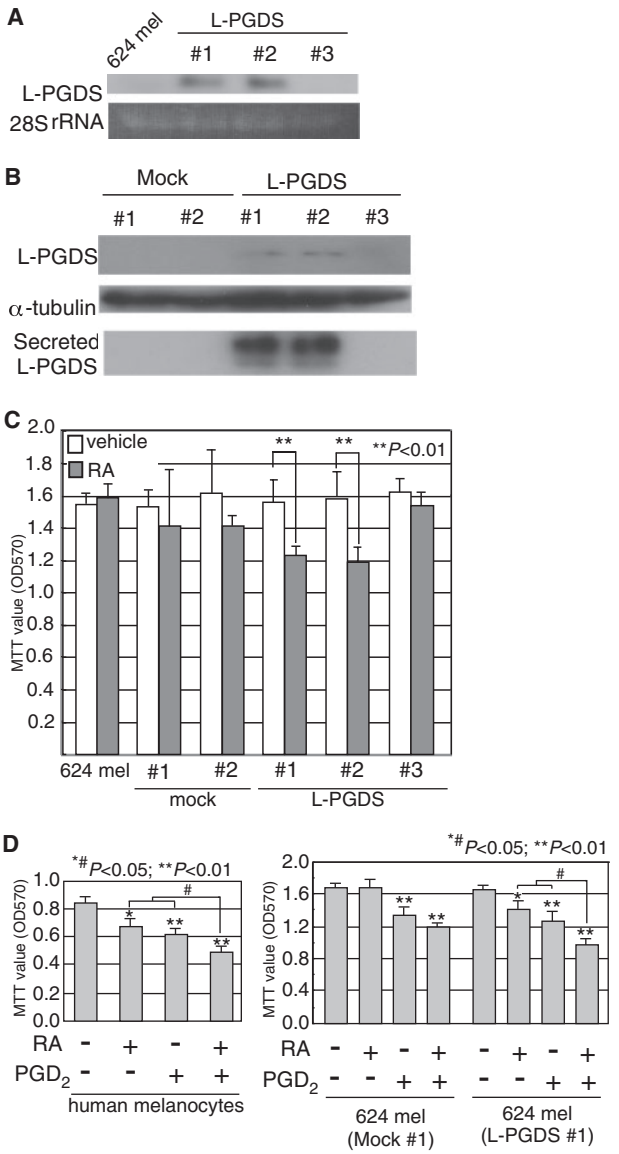

Fig. 4 Isolation and characterization of L-PGDS-expressing cell lines. (A) Northern blot analysis of L-PGDS-expressing cell lines, derived from 624 mel cells. L-PGDS mRNA is expressed in isolated cell lines, L-PGDS #1 and #2, but not in L-PGDS #3 and in Mock #1 and #2 (not shown). (B) Western blot analysis for expression of tagged L-PGDS protein. L-PGDS protein was detectable with anti-V5 epitope antibody in cell extracts (top panel) and culture media (bottom panel) of L-PGDS #1 and #2 cells. The exposure time was 18 h for the detection of L-PGDS protein in the whole cells extracts (top panel), while the exposure time was a few seconds (middle panel) or a few minutes (bottom panel). (C) Effect of RA on cell proliferation. Cells were treated with 10 μM RA for 4 days. Proliferation was assessed by MTT assay. The data are means ± SD of more than four independent experiments (**$P<0.01$, compared to vehicle control). Note that RA inhibited the proliferation of L-PGDS #1 and #2 cells, each cell line of which expresses L-PGDS. (D) Effect of PGD_2 on the RA-mediated proliferation restriction. Human melanocytes (left panel) and 624 mel transfectants, Mock #1 and L-PGDS #1 (right panel), were treated with RA and/or PGD_2 at each concentration of 1 μM for 4 days. Proliferation was assessed by MTT assay. The data are means ± SD of three independent experiments (*$P<0.05$, **$P<0.01$, compared to vehicle control, or $^{\#}P<0.05$, compared to RA or PGD_2 alone).

our proposal that L-PGDS expression may sensitize cells to RA.

L-PGDS ensures the RA signalling in melanoma-derived transformed cell lines

Consequently using the established cell lines, L-PGDS #1 and #2, we explored the effect of RA on the expression of $p21^{Cip1}$ cyclin-dependent kinase inhibitor. RA at 10 μM increased the expression levels of $p21^{Cip1}$ mRNA and protein in L-PGDS #1 and #2 cells, but not in mock-transfected cell lines (Fig. 5A and B). In contrast, the expression level of $p27^{Kip1}$ protein remained unchanged, which is similar to the results observed in melanocytes (Fig. 3A). We also examined the expression of RARβ, which is a direct target gene for RA signal transduction (*28*) and a potential tumour suppressor (*31*). The basal expression levels of RARβ mRNA were similar in L-PGDS and mock transfectants that were treated with vehicle (Fig. 5C). In contrast, treatment with RA for 4 days caused significant increases in the expression levels of RARβ mRNA in both mock and L-PGDS-expressing cell lines (Fig. 5C), with the greater magnitude of the induction in L-PGDS #1 and #2 cells. These results suggest that L-PGDS is involved in the RA-mediated induction of $p21^{Cip1}$ and RARβ, which in turn may lead to the growth inhibition. Furthermore, we examined the effect of RA on the RAR− mediated transactivation in L-PGDS-expressing cell lines, using the luciferase reporter gene under the control of RARE (Fig. 5D). The basal luciferase activity (vehicle treatment) was similar in Mock #1 cells and L-PGDS-expressing cell lines (L-PGDS #1 and #2). Treatment with RA significantly increased luciferase activity in both Mock #1 cells and L-PGDS-expressing cell lines, with the greater degree (>2-fold) of induction in L-PGDS #1 and #2 cells. Thus, L-PGDS may enhance the RAR-mediated transactivation. Taken together, these results indicate that L-PGDS expression is associated with the higher susceptibility to RA.

Forced expression of L-PGDS increases the sensitivity of melanoma transfectants to RA

To confirm the regulatory role of L-PGDS for RA signalling detected in the 624 mel-derived cell lines, we established L-PGDS-expressing and mock transfectants in a mass culture from each of human melanoma cell lines, HMVII, 624 mel, SK-MEL 28 and G361. The L-PGDS expression vector, pIRESneo-L-PGDS, allowed G418-resistant cells to express L-PGDS tagged with V5-epitope and hexa-histidine. L-PGDS transfectants, derived from each melanoma cell line, secreted L-PGDS protein in culture medium (Fig. 6A), whereas L-PGDS protein was not detectable in the culture media of mock transfectants (data not shown). The L-PGDS proteins of different molecular sizes are probably due to the different degree of glycosylation (*8*). We then analysed the effect of RA (10 μM) on the proliferation of the melanoma-derived transfectants. RA treatment significantly decreased the proliferation of L-PGDS transfectants (~30% decrease), irrespective of the parent melanoma cell line (Fig. 6B), whereas RA treatment did not cause noticeable

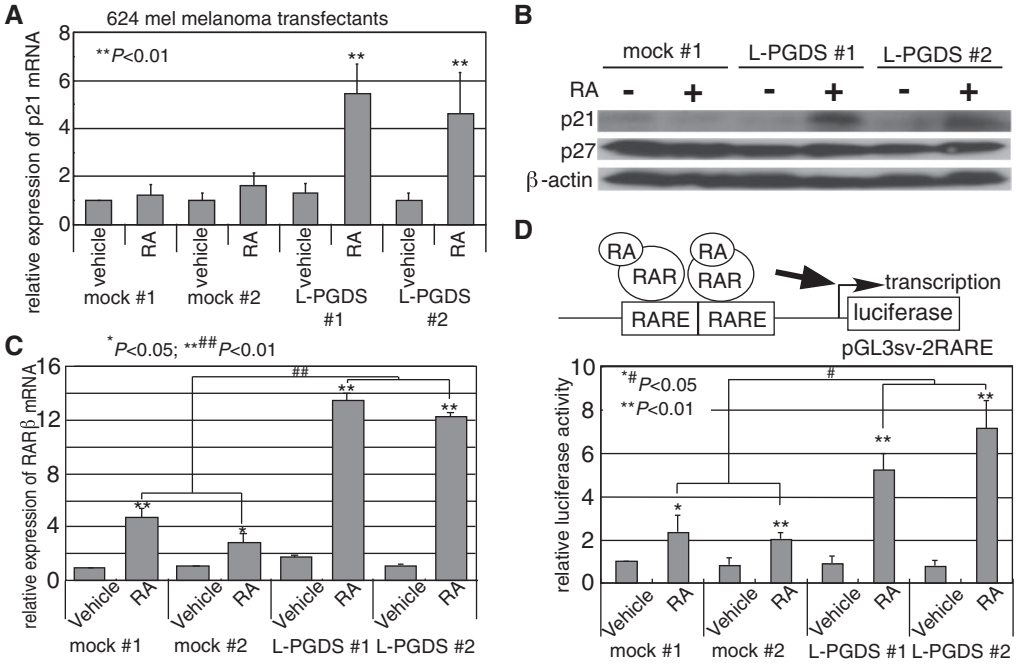

Fig. 5 L-PGDS enhances the effects of RA signalling in L-PGDS-expressing cell lines. RA induced the expression of $p21^{Cip1}$ mRNA (A) and protein (B) in mock and L-PGDS-expressing cell lines. Each cell line was treated with 10 μM RA or vehicle (ethanol) for 4 days. Total RNAs and proteins were prepared from Mock #1 and #2 cells and L-PGDS #1 and #2 cells, and subjected to real-time PCR (A) or western blot analysis (B). In (A), the data are means ± SD of three independent experiments (**$P<0.01$, compared to vehicle). (C) Real-time PCR showing the induction of RARβ mRNA expression by RA. Treatment with RA increased RARβ mRNA levels in Mock #1 and #2 cells and L-PGDS #1 and #2 cells. The data are means ± SD of three independent experiments (*$P<0.05$ and **$P<0.01$, compared to vehicle control). Note that the magnitude of induction of RARβ mRNA was greater in L-PGDS #1 and #2 cells than that in Mock #1 and #2 cells ($^{\#\#}P<0.01$). (D) L-PGDS enhances the RA-mediated transcriptional activation. RA binds RAR, which in turn transactivates target genes through the retinoic acid-responsive element (RARE). Shown at top is a model construct pGL3sv-2RARE. Indicated cell lines were transfected with pGL3sv-2RARE and pRL-CMV (internal control), incubated for 24 h, and treated with 10 μM RA or vehicle (ethanol) for 48 h. RA treatment increased luciferase activity in Mock #1 and #2 cells and L-PGDS #1 and #2 cells. The data are means ± SD of three independent experiments (*$P<0.05$ and **$P<0.01$, compared to vehicle control). In addition, the magnitude of the increase was greater in L-PGDS #1 and #2 cells than that in Mock #1 and #2 cells ($^{\#}P<0.05$).

changes in the proliferation of mock transfectants. It is therefore conceivable that L-PGDS expression may enhance the sensitivity of human melanoma cells to RA, thereby reducing the proliferation of L-PGDS-expressing transfectants.

L-PGDS expression is correlated with the RA-mediated proliferation inhibition of mouse melanocytes

To further obtain the evidence for the physiological significance in the regulatory role of L-PGDS in RA signalling, we examined whether L-PGDS modulates the effect of RA in mouse melanocytes. Taking the advantage that L-PGDS is expressed in melan-a mouse melanocytes (*7*), we treated melan-a cells with RA. As expected, RA (1 or 10 μM) significantly inhibited the proliferation of melan-a cells (~40 or 50% reduction, respectively; Fig. 7A). We then knockdowned the expression of L-PGDS protein in melan-a cells with each of three different siRNAs (mL-PGDSi-1, -2 and -3) (Fig. 7B). In addition, treatment with each mL-PGDSi significantly attenuated the RA-mediated proliferation inhibition compared with negative control RNAi (~25% reduction, Fig. 7C). These results suggest that L-PGDS may be responsible for the RA-mediated decrease in proliferation of melan-a cells. Thus, the function of L-PGDS as a regulator of RA signalling is conserved between human and mouse melanocytes. The marginal reduction of 25%, despite the efficient knockdown of L-PGDS expression, may reflect the involvement of other factors that contribute to RA sensitivity, such as RA-degrading enzymes or cellular RA-binding proteins.

Discussion

In the present study, we have shown that treatment with RA significantly decreases the proliferation of cultured human epidermal melanocytes and melan-a mouse melanocytes, both of which endogenously express L-PGDS. In contrast, RA exerted no noticeable effect on the proliferation of human melanoma cells that lack L-PGDS expression. We have provided several lines of evidence that L-PGDS may sensitize melanocytes to the RA signalling. In addition, we have shown that human epidermal melanocytes produce and secrete L-PGDS and PGD_2 into culture medium. It is therefore conceivable that L-PGDS may negatively regulate the proliferation of melanocytes in the human epidermis.

One important question remains to be answered is the mechanism, by which L-PGDS enhances the growth inhibitory effect of RA on melanocytes. In

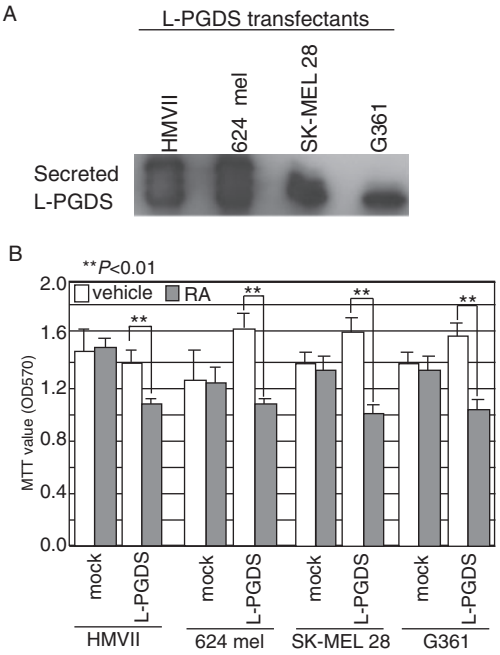

Fig. 6 Forced expression of L-PGDS in melanoma transfectants increases the sensitivity to RA. (A) Secretion of L-PGDS protein in culture media from L-PGDS-expressing transfectants. HMVII, 624 mel, SK-MEL 28 and G361 human melanoma cells were transfected with pIRESneo-L-PGDS, and selected with G418 (600 μg/μl) in a mass culture. Shown is western blot analysis of the condition media with anti-V5 epitope antibody. (B) Effect of RA on cell proliferation, assessed by MTT assay. The indicated transfectants were treated with 10 μM RA or vehicle (ethanol) for 4 days. The data are means ± SD of more than four independent experiments (**$P < 0.01$).

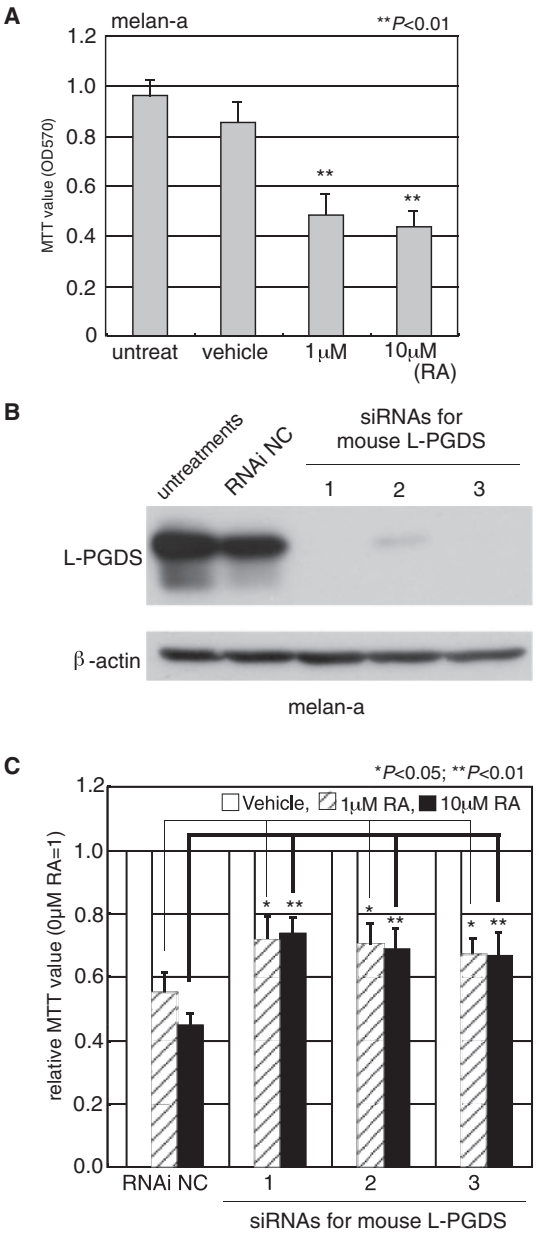

Fig. 7 L-PGDS expression is associated with the RA-mediated decrease in proliferation of mouse melanocytes. (A) Effect of RA on the proliferation of melan-a mouse melanocytes. Melanocytes were left untreated (untreat) or treated with RA at the indicated concentrations or vehicle (ethanol) for 4 days. Proliferation was assessed by MTT assay. The data are means ± SD of three independent experiments (**$P < 0.01$, compared to vehicle and untreat). (B) RNA interference analysis against mouse L-PGDS. Mouse melanocytes were left untreated (untreat) or transfected with each of mL-PGDSi-1, -2 and -3 (siRNAs for mouse L-PGDS 1, 2 and 3) or negative control RNAi (RNAi NC) for 4 days, and harvested. Whole cell extracts were used for western blot analysis of L-PGDS (top panel). Bottom panel shows β-actin protein as loading control. (C) Role of L-PGDS in the RA-mediated proliferation inhibition. Melanocytes were treated with each siRNA and RA (1 or 10 μM) or vehicle (ethanol) for 4 days. The data are shown as the ratio to the MTT value of vehicle treatment (0 μM). Note that each siRNA decreased the magnitude of the RA-mediated proliferation inhibition compared with negative control RNAi (*$P < 0.05$; **$P < 0.01$).

this connection, RA has been reported to inhibit the enzyme activity of L-PGDS (*32*). Furthermore, treatment with PGD_2 decreased the proliferation of human melanocytes at the concentrations of 1 and 10 μM (Fig. 1B), each concentration of which was much higher than that (~10 pM) detected in the conditioned medium of melanocytes. These results suggest that the amount of PGD_2, produced by melanocytes, may not be sufficient to restrict the proliferation of melanocytes. We therefore propose that L-PGDS may confer the RA sensitivity on human melanocytes as a RA-binding protein rather than as an enzyme that produces PGD_2.

Loss of L-PGDS expression was reported in human prostate cancer cells, derived from epithelial cells, while L-PGDS is expressed in normal human prostate cells, such as epithelial, stromal and smooth muscle cells (*33*). Those authors suggested that locally produced PGD_2 inhibited the growth of prostate cancer cells (*33*). Such direct involvement of PGD_2 in the prostate cancer seems to be different from our finding in melanocytes, in which L-PGDS indirectly decreased cell proliferation through RA. In fact, forced expression of L-PGDS did not influence the growth of human melanoma cells (Figs 4C and 6B). Furthermore, a recent study reported that PGD2 induced the expression of a transcription factor SOX9 in A375 human melanoma cells (*21*) and that overexpression of SOX9 enhanced the RA-mediated decrease in the proliferation of human melanoma cells (*21*). Thus, PGD2 may increase the sensitivity to the RA-mediated proliferation inhibition in human melanoma cells. In this context, we showed that

PGD_2 at 1 μM increased the magnitude of the RA-mediated proliferation restriction of human melanocytes and L-PGDS-expressing cells (Fig. 4D). Taken together, these results suggest that L-PGDS may inhibit cell proliferation by producing PGD2 and/or through the RA-mediated mechanism, depending on cell types or cellular microenvironments. The loss of L-PGDS expression in human melanoma and prostate cancer cells may be one of escape mechanisms from tumour surveillance (*34*).

The earlier study of other investigator indicated that the half-life of RA was ~4 days in the serum-containing medium without cells (*35*). Indeed, treatment with RA (10 μM) for 4 days induced the expression of $p21^{Cip1}$ cyclin-dependent kinase inhibitor in human melanocytes (Fig. 3A) and melanoma-derived L-PGDS-expressing cells (Fig. 5B). In addition, treatment with RA for 4 days induced RARβ mRNA expression by >3-folds in mock transfectants that lack L-PGDS expression (Fig. 5C), although the greater magnitude of the induction was detected in L-PGDS-expressing cell lines. These results suggest that RA is effective at least for 4 days in the culture condition used in the present study. Furthermore, L-PGDS expression is associated with the increase in the RAR-mediated transactivation (Fig. 5D). We thus propose that RA induces expression of RARβ and $p21^{Cip1}$, which in turn leads to proliferation inhibition of melanocytes and melanoma-derived L-PGDS-expressing cells.

It is unknown whether intracellular or secreted L-PGDS enhances RA signal transduction, which in turn leads to proliferation inhibition. There is a possibility that intracellular L-PGDS may function as a coactivator of the RAR-mediated transcription. In fact, it was reported that preferentially expressed antigen in melanoma (PRAME) binds RAR and functions as a corepressor of RAR (*20*). Alternatively, L-PGDS may stabilize RA in the culture medium through its binding, because the large amount of L-PGDS was detected in the media (Fig. 4B).

In the present study, we used the pharmacological concentrations of RA (1 and 10 μM) in cell cultures, although the physiological concentration of RA in human serum is ~10 nM (*34*). It is, however, noteworthy that peak plasma level of RA could be 2 μM after ingestion of RA in patients with acute promyelocytic leukaemia (*35*). In addition, RA concentration is ~1 μM in mouse embryonic eye (*36*). It is, therefore, conceivable that the local concentration of RA (1–10 μM) may be achieved under a certain physiological or pathological condition.

Unlike the human melanoma cell lines examined in the present study, B16 mouse melanoma cells express L-PGDS (*7*). In addition, it has been reported that RA inhibits proliferation of B16 melanoma cells (*20*, *37*). We also confirmed that B16 melanoma cells were sensitive to proliferation inhibition caused by RA at 1 μM (~72% reduction after 4 days). These results are consistent in part with our proposal that L-PGDS enhances the susceptibility to RA, which leads to the RA-meditated proliferation restriction of melanocytes. On the other hand, treatment with RA (even at 10 μM) did not cause any noticeable change in proliferation of ARPE-19 human retinal pigment epithelial cells (data not shown), despite the expression of L-PGDS and production of PGD_2 in ARPE-19 cells (*38*). It is therefore conceivable that the newly recognized function of L-PGDS may be important in the maintenance of epidermal homoeostasis.

In summary, L-PGDS may restrict the proliferation of melanocytes by modulating the RA signalling. Considering the evolutionary conserved RA signalling (*39*) and the conserved transporter function of L-PGDS (*10*), we suggest that L-PGDS may fine-tune the action of RA, which contributes to the regulation of epidermal pigmentation.

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Conflict of interest

None declared.

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