ISSN 0142 5463

Volume 37 Number 3 June 2015

International Journal of Cosmetic Science

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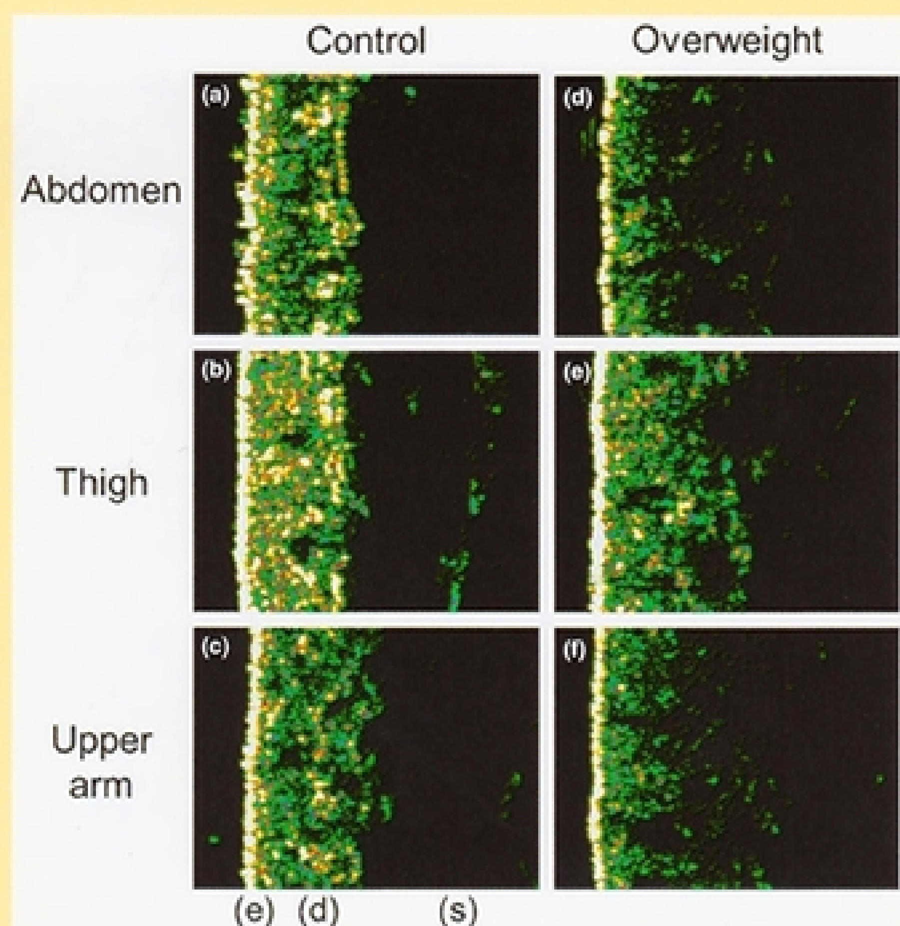

Typical structure of dermis in ultrasonographic images by the 20 MHz ultrasound scanner of control subject and overweight subject in abdomen, thigh and upper arm. In three parts, echogenicity decreased in the lower dermis in an overweight person. (a, b, c) A control subject; 30s male, BMI of 22.6. (d, e, f) An overweight subject; 30s male, BMI of 29.7. (a) Echogenicity of upper dermis; 20.7%, lower dermis; 18.3%. (b) Echogenicity of upper dermis; 32.8%, lower dermis; 27.9%. (c) Echogenicity of upper dermis; 14.3%, lower dermis; 11.4%. (D) Echogenicity of upper dermis; 8.0%, lower dermis; 2.5%. (e) Echogenicity of upper dermis; 18.8%, lower dermis; 7.4%. (f) Echogenicity of upper dermis; 15.5%, lower dermis; 3.0%. e, Epidermis; c, dermis; s, subcutaneous adipose tissue. Courtesy of M. Matsumoto, A. Ibuki, T. Minematsu, J. Sugama, M. Horii, K. Ogai, T. Nishizawa, M. Dai, A. Sato, Y. Fujimoto, M. Okuwa, G. Nakagami, T. Nakatani and H. Sanada.

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Cosmetic Science

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PDGF-AA-induced filamentous mitochondria benefit dermal papilla cells in cellular migration

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Received 06 October 2014, Accepted 26 November 2014

Keywords: ageing, cell culture, hair growth, mitochondria, platelet-derived growth factor-AA, skin physiology

Synopsis

OBJECTIVES: Human dermal papilla cells (HDPCs) play essential roles in hair follicular morphogenesis and postnatal hair growth cycles. Previous reports demonstrated that platelet-derived growth factor-AA (PDGF-AA) enhanced the formation of dermal condensates in hair follicular development. Additionally, PDGF-AA induces/maintains the anagen phase of the hair cycle. It is likely that mitochondrial morphology and functions are tightly coupled with maintenance of these energy-demanding activities. However, little is known about the mitochondrial regulation in HDPCs. Thus, we investigated the PDGF-involved mitochondrial regulation in HDPCs.

METHODS: The mitochondrial morphologies of HDPCs were examined in the presence or absence of PDGF-AA under a fluorescent microscope. ATP production and cellular motility were investigated. The relationship between mitochondrial morphology and the cellular functions was discussed.

RESULTS: We observed that primary HDPCs contained mitochondria with filamentous and/or rounded morphologies. Both types of mitochondria showed similar membrane potentials. Interestingly, in the presence of PDGF-AA, but not PDGF-BB, the balance between the two morphologies shifted towards the filamentous form. Concomitantly, both mitochondrial enzymatic activity and total cellular ATP level were augmented by PDGF-AA. These two parameters were closely correlated, suggesting the mitochondrial involvement in the PDGF-augmented ATP production. Moreover, PDGF-AA accelerated the migration of HDPCs in a gap-filling assay, but did not change the rate of cellular proliferation. Notably, filamentous mitochondria dominated migrating HDPCs.

CONCLUSION: PDGF-AA benefits HDPCs in the process of migration, by increasing the number of filamentous mitochondria.

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This work was presented at the following scientific meetings
8th World Congress for Hair Research
May 14–17, 2014
International Convention Center Jeju, Jeju Island, Korea.Internal Symposium Mitochondria 2013
November 6–7, 2013
Roppongi Academyhills 49, Roppongi, Japan.

Résumé

OBJECTIF: Les cellules de papilles dermiques humaines (HDPCs) jouent un rôle essentiel dans la morphogenèse folliculaire des cheveux et des cycles postnatales de croissance des cheveux. Des rapports antérieurs ont démontré que le facteur de croissance AA dérivé des plaquettes (PDGF-AA) améliore la formation de condensats dermiques pendant le développement folliculaire des cheveux. En outre, PDGF-AA induit/maintient la phase anagène du cycle pilaire. Il est probable que la morphologie et la fonction mitochondriale sont étroitement couplées avec le maintien de ces activités exigeant de l'énergie. Toutefois, on en sait peu sur la régulation mitochondriale dans les HDPCs. Ainsi, nous avons étudié la régulation mitochondriale impliquant le PDGF dans HDPCs.

MÉTHODES: Les morphologies des HDPCs mitochondriales ont été examinées en présence ou en l'absence de PDGF-AA sous un microscope à fluorescence. Nous avons étudié la production d'ATP cellulaire et la motilité. La relation entre la morphologie mitochondriale et les fonctions cellulaires a été discutée.

RÉSULTATS: Nous avons observé que les HDPCs primaires contenaient des mitochondries aux morphologies filamenteux et/ou arrondies. Les deux types de mitochondries ont montré des potentiels de membrane similaires. Fait intéressant, en présence de PDGF-AA, mais pas du PDGF-BB, l'équilibre entre les deux morphologies est déplacé vers la forme filamenteuse. En même temps, à la fois l'activité enzymatique mitochondriale et le niveau de l'ATP cellulaire total ont été augmentés par PDGF-AA. Ces deux paramètres sont étroitement corrélés, suggérant l'implication du PDGF augmenté dans la production mitochondriale d'ATP. De plus, le PDGF-AA a accéléré la migration des HDPCs dans un test de 'scratch', mais ne modifie pas le taux de prolifération cellulaire. Notamment, les mitochondries filamenteuses dominaient la migration des HDPCs.

Introduction

Mitochondria play an essential role in producing chemical energy ATP to achieve a variety of cellular functions. The organelle undergoes morphological changes to meet cellular needs [1]. Previous studies have shown that fragmentation of mitochondria, due to increased activity of fission proteins, is involved in apoptotic cell death [2]. It was recently reported, using mouse embryonic fibroblasts, that elongated filamentous mitochondria produce more energy than shorter mitochondria during autophagy [3]. Also, Mitra *et al.* [4] showed that hyperfused mitochondria are important for regulating G1-S transition by temporarily boosting cyclin E levels and that the elongated mitochondria have greater ATP output than mitochondria at any other

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cell cycle state. Thus, it seems that fused, longer mitochondria show an increased respiratory capacity compared to fragmented mitochondria. However, an inverse correlation between mitochondrial size and metabolic competence was reported, when using the cerebellar cortex of adult rats [5]. Thus, despite the essential biological roles of mitochondria, the relationship between their morphology and function remains confused, which is possibly due to its dependency on cellular types or involvement of different regulators.

Hair follicles are complicated and consist of different types of cells. The follicle contains a ball-like structure, the dermal papilla, at its base. Dermal papilla cells, fibroblast-like cells, play a crucial role in morphogenesis of mammalian hair follicles and the regulation of successive cycles of postnatal hair growth. In the last few decades, a great deal of attention has been paid to HDPCs as the cells became known to be capable of regenerating hair follicle structure in transplantation [6].

It has been reported that skin and hair abnormalities can be attributed to mitochondrial disorders [7, 8]. In addition, a series of genes that encode energy metabolism-related factors, such as Janus kinase, adenylate kinase and tumour necrosis factor-associated protein 1, were downregulated in senescent alopecia [9]. Thus, an understanding of mitochondrial regulation is necessary towards developing a novel treatment for the skin- and hair-related diseases. To elucidate the fundamental molecular basis of hair follicles, numerous signalling molecules and their pathways have been extensively investigated [10]. For example, mice lacking platelet-derived growth factor-AA (PDGF-AA) show small dermal papillae, dermal sheath abnormalities and thin hair. Thus, it is suggested that PDGF-AA is required for cross-talk between the follicle epithelium and its mesenchyme. In fact, PDGF-AA is produced by epidermal keratinocytes of placodes and it enhances formation of dermal condensate (DPC aggregate) at the beginning of follicular morphogenesis [10, 11]. The other essential role of PDGF-AA is to induce and maintain the anagen phase of the hair cycle [12]. In the anagen phase, HDPCs produce a series of growth factors to control hair growth by interacting with surrounding cells, such as hair matrix cells [12, 13]. These physiological activities consume substantial cellular energy and, thus, mitochondrial functions are likely to be correlated with these energy-demanding activities that are triggered by PDGF-AA.

In this work, we describe that PDGF-AA aids directly in transformation of mitochondrial morphology in HDPCs from the rounded type to the filamentous type. The latter produces ATP more efficiently than the former, and therefore, it is beneficial for the cells when energy-demanding migration processes have to be performed.

Materials and methods

Cell culture

The primary HDPCs, derived from a 70-year-old Caucasian female, was purchased from PromoCell GmbH (Heidelberg, Germany). The cells were grown in the provided Follicle Dermal Papilla Cell Basal Medium supplemented with 0.1% hbFGF, 0.1% insulin, 0.4% BPE, 4% foetal calf serum and 50 U mL^{-1} penicillin and 50 μg mL^{-1} streptomycin (Invitrogen, Carlsbad, CA, USA). The cells were kept in a 5% CO_2 atmosphere at 37°C.

Mitochondrial imaging

Coverslips with ~50% confluent HDPCs (passage 6) were prepared. Mitochondria were stained with 100 nM Mito Tracker Red CMXRos (Invitrogen), according to the manufacturer's instruction, followed by formaldehyde fixation. The fluorescent images of mitochondria were visualized under a Eclipse E800 microscope (Nikon, Tokyo, Japan), and a confocal microscope LSM710 (Carl Zeiss, Jena, Germany) was used to verify the observations.

Functional assays

A series of assays were performed, according to the manufacturer's instruction, after 24 h of incubation of HDPC cultures (~50% confluency, passage 6) in the presence of 0–100 ng mL^{-1} PDGF-AA (Wako, Tokyo, Japan) or PDGF-BB (Wako, Tokyo, Japan). To estimate mitochondrial activity, 1.2 mM 3-(4,5-Dimethylthiazol-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (DOJINDO, Tokyo, Japan) was used, and the formation of insoluble formazan, after 4 h of incubation, was determined by measuring optical density (570–650 nm) with a Multiskan FC Microplate photometer (Thermo scientific, Waltham, MA, USA). Total levels of cellular ATP were estimated with a Cell Titer-Glo Luminescent Cell Viability Assay (Promega, Wisconsin, USA), and luminescent counts were determined with a GloMax 20/20 luminometer (Promega). Mitochondrial membrane potential was measured using MitoPT™ JC-1 (Immunochemistry Technologies, Bloomington, MN, USA) and a fluorescence microplate reader, Infinite 200 PRO (TECAN, Männedorf, Switzerland). 1 μM staurosporine (Wako, Tokyo, Japan) and 25 μM carbonyl cyanide m-chlorophenylhydrazone (CCCP) were used as controls.

To estimate cellular proliferation, DNA (Hoechst 33342, DOJINDO, Tokyo, Japan) levels of HDPC cultures were measured, using the fluorescence microplate reader (Ex360 nm, Em450 nm). Turbidity tests (optical density 600 nm) of HDPC cultures were performed additionally after trypsinization. Data are represented as the mean $\pm$ SD of three to six independent experiments. Statistical analysis was performed using Student's t-test.

Migration assay

In gap-filling assays, HDPCs were multilayered (typically three layers) in which a scratch was made. Live-cell imaging was performed in chambers (Lab-Tek II; Nunc, Roskilde, Denmark) at 37°C on the confocal microscope. Images were acquired every 30 min for 8 h.

To perform spheroid assays, microspheres of HDPCs were prepared by transferring trypsinized cells (passage 6) into a low-attachment plate, Ultra Low Cluster Plate (Corning Inc, New York, NY, USA) and incubating the cell cultures for 3 days. To examine migrating cells, the microspheres were then transferred into collagen-coated dishes and were left for 24 h ahead of mitochondrial visualization.

Results

A primary HDPC shows filamentous and/or rounded mitochondria

Mitochondria of primary HDPCs (70-year-old female Caucasian) were visualized with Mito Tracker Red CMXRos. Filamentous and/or rounded types of mitochondria were observed in the fixed primary cells (Fig. 1). About 30% of the HDPCs population contained only filamentous or rounded mitochondria and the rest of the cell showed mixture of the two types (Fig. 1d and Table I). The two types of mitochondria were also observed without the fixation (data not shown). Therefore, we have concluded that there are two

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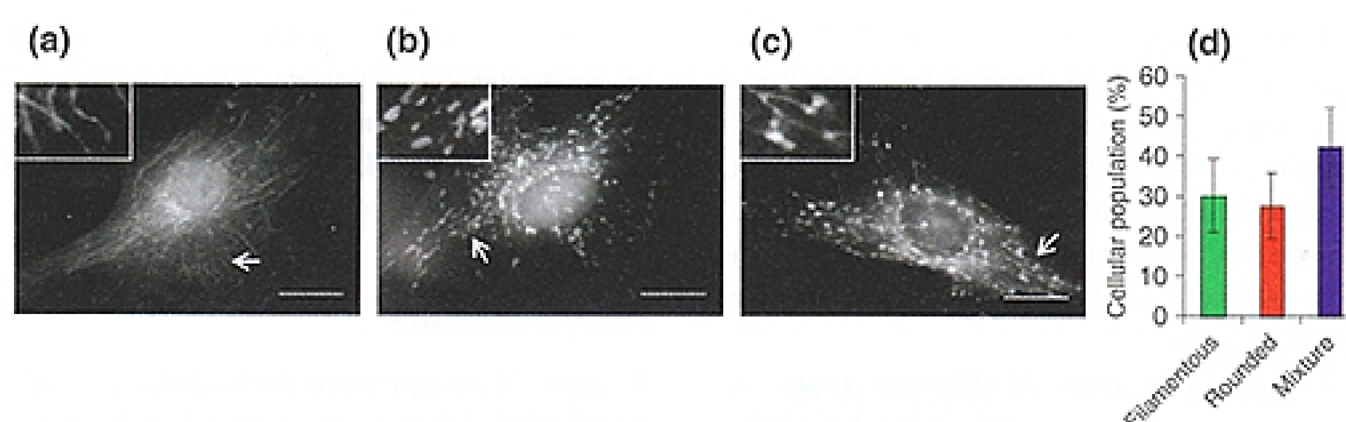

Figure 1 Filamentous and rounded mitochondria in primary HDPCs. Mitochondria of primary HDPCs were visualized with Mito Tracker Red CMXRos. 30% of the cell population showed filamentous mitochondria (a) and 28% of the population showed rounded mitochondria (b). The rest of the cells showed the mixture of the two types (c). Enlarged views are shown in the white boxes. Scale bars = 20 μm. (d) The cellular population with each type of mitochondria. Data are represented as the mean ± SD of six independent experiments.

Table 1 Filamentous mitochondria dominate migrating HDPCs

	Cellular population(%)		
	Filamentous mitochondria	Rounded mitochondria	Mixture
Monolayer[1] Control (n = 291)	30.1 ± 9.1	27.6 ± 8.2	42.3 ± 9.8
Monolayer + PDGF-AA (n = 223)	53.9 ± 9.3	16.8 ± 6.9	29.3 ± 7.5
Monolayer + PDGF-BB (n = 95)	30.0 ± 9.6	23.4 ± 5.7	46.7 ± 12.5
Gap-filling[2] Control (n = 426)	74.4 ± 3.2	0	25.6 ± 3.2
Gap-filling + PDGF-AA (n = 630)	90.4 ± 3.0	0.3 ± 0.5	9.3 ± 3.2
Spheroid[3] (n = 129)	76.4 ± 3.8	4.2 ± 3.6	19.4 ± 3.1

[1]HDPCs were grown as monolayer and mitochondria were observed at ~50% confluency.
[2]In a gap-filling assay, HDPCs were assessed when they were in the process of migration in the gap area.
[3]Migrating cells from spheroids were investigated.
Data are represented as the mean ± SD of three to six independent experiments. Total number of tested HDPCs is shown in parentheses.

different types of mitochondria in HDPCs, and it is possible that HDPCs use them differently as required.

PDGF-AA increases the population of filamentous mitochondria

We examined mitochondrial morphology in the presence of PDGF-AA and found that PDGF-AA shifted the balance between the two morphologies towards the filamentous form. The population of HDPCs with filamentous mitochondria markedly increased in the presence of PDGF-AA, which was not the case with PDGF-BB (Fig. 2 and Table I). The effect of PDGF-AA was dose-dependent. Similar results were obtained from the same source of HDPCs at different passages (passage 7 and 8): PDGF-AA increased the population of filamentous mitochondria from 26.9 ± 3.4% to 55.7 ± 0.4% and from 28.1 ± 3.7% to 57.3 ± 1.2% for passage 7 and 8, respectively. In addition, similar results were observed in a different source of primary HDPCs (53-year-old female Caucasian): PDGF-AA increased the population of filamentous mitochondria from 18.5 ± 0.9% to 40.6 ± 3.4%. Thus, it is likely that PDGF-AA specifically increases filamentous mitochondria in HDPCs.

PDGF-AA augments mitochondrial activity without affecting membrane potential

To understand the functional significance of the PDGF-AA-induced morphological change of mitochondria, we first examined the formation of insoluble formazan. The formation of formazan is dependent on mitochondrial metabolic rate, more specifically, on the enzymatic activities of dehydrogenases in complex I and II. PDGF-AA enhanced the formazan formation in a dose-dependent manner (Fig. 3a). This result can be attributed to either accelerated cellular proliferation activity or increased enzymatic activity of mitochondria. To distinguish these possibilities, we determined the proliferation rate of PDGF-treated HDPCs. Both Hoechst levels and optical densities remained unchanged in the presence of PDGF-AA, at any tested concentration and time points, as shown in Fig. 3(b, c), respectively. These results indicate that PDGF-AA does not accelerate the proliferation rate of HDPCs by itself, which is in good agreement with the previous reports [14]. Thus, we have concluded that PDGF-AA activates mitochondrial function without affecting the proliferation activity of HDPCs.

To further examine the effect of PDGF-AA, mitochondrial membrane potential was examined using 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzamidazolo carbocyanine iodide, commonly known as JC-1. HDPCs were treated with JC-1 and fluorescent intensity was measured. As shown in Fig. 3(d), PDGF-AA as well as PDGF-BB did not change the intensity, in contrast to the marked reduction by an apoptosis inducer (staurosporine) or a mitochondrial uncoupler, carbonyl cyanide m-chlorophenylhydrazone (CCCP).

Collectively, we concluded that PDGF-AA augments mitochondrial enzymatic activity without affecting the cellular proliferation and the mitochondrial membrane potential in HDPCs.

PDGF-AA- induced filamentous mitochondria produce more ATP

Compared to the control, PDGF-treated HDPCs showed elevated levels of ATP in a dose-dependent manner (Fig. 4a). Notably, a linear relationship between insoluble formazan formation and the gross ATP lev-

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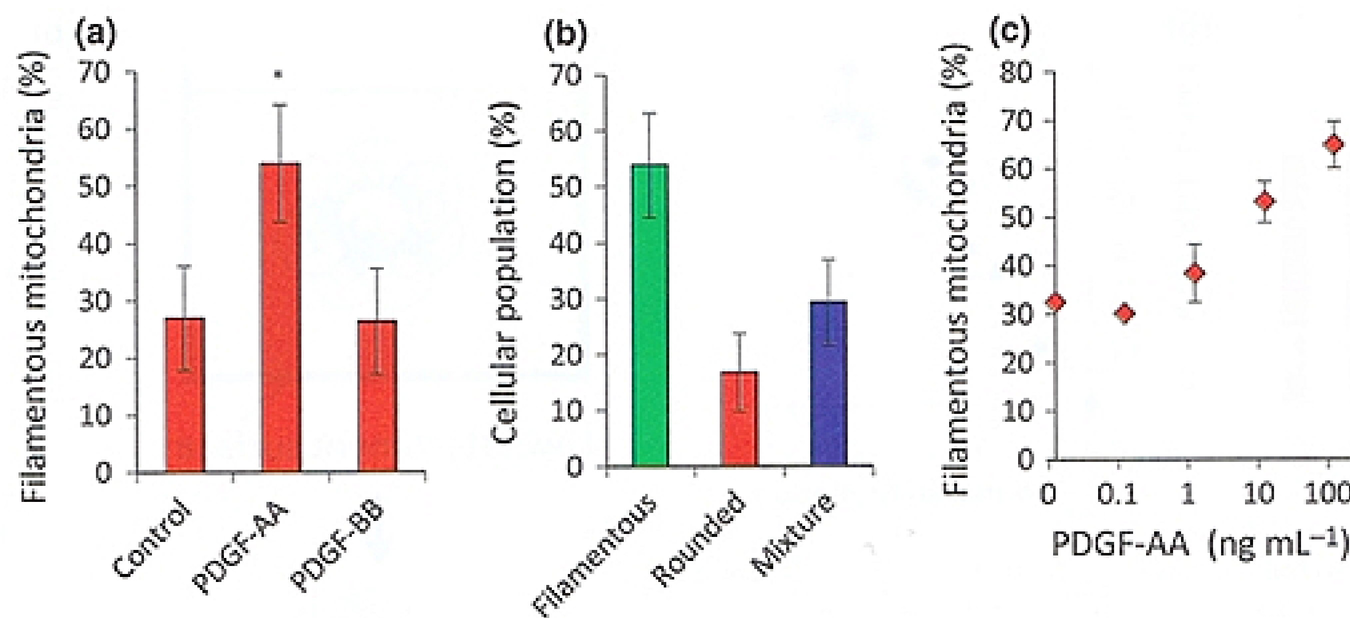

Figure 2 PDGF-AA increases filamentous mitochondria. (a) Population of HDPC with filamentous mitochondria was measured after 24 h of incubation with 100 ng mL^{-1} PDGF-AA or 100 ng mL^{-1} PDGF-BB. Data are represented as the mean ± SD of five independent experiments. (b) The cellular population with each type of mitochondria in the presence of 100 ng mL^{-1} PDGF-AA. Data are represented as the mean ± SD of six independent experiments. (c) Dose-dependent effect of PDGF-AA on the formation of filamentous mitochondria. Data are represented as the mean ± SD of three independent experiments. * indicates P-value <0.05 compared with control.

els was observed (Fig. 4b), suggesting that the elevated ATP production is tightly coupled with the increased mitochondrial activity.

Filamentous mitochondria dominate migrating HDPCs

Finally, we observed migrating mitochondria in a gap-filling assay. Surprisingly, 74% of the HDPC population showed the filamentous mitochondria in the process of migration (Fig. 5a). The increased population of filamentous mitochondria is remarkable compared to the normal monolayer culture (Fig. 1 and Table I). In addition, PDGF-AA further increased the cell population of filamentous mitochondria up to 90%, during cell migration (Fig. 5a). HDPCs migrated faster in the presence of PDGF-AA than its absence (Fig. 5b), which is in good agreement with the previous reports [15]. A different source of primary HDPCs (53-year-old female Caucasian) also showed higher population of filamentous mitochondria during the process of migration (59.2 ± 0.5%) compared with the control normal culture (18.5 ± 0.9%). PDGF-AA further increased the cell population of filamentous mitochondria in this source of HDPCs up to 80.2 ± 3.9%, during cell migration.

A similar result was obtained in another experimental set-up. We first prepared spheroids of HDPCs in a low-attachment plate. When the spheroids are transferred into a collagen-coated plate, the cells attach to the surface and begin to migrate away from the microspheres (Fig. 6a). 76% of the migrating cells showed only filamentous mitochondria (Fig. 6b and Table I).

Discussion

Morphological changes of mitochondria have been widely reported in different cell types, many of which are closely related to physiological cellular activities and human diseases [16]. Therefore, it is possible that an understanding of the molecular basis of the relationship between mitochondrial morphology and energy production will shed light on future therapeutic schemes.

In the current work, we have demonstrated that HDPCs show two different morphologies of mitochondria, filamentous and rounded forms, and that PDGF-AA increases filamentous mitochondria. Our results imply that filamentous mitochondria produce more ATP, which is in good agreement with previous works [3, 4]. The relationship among population of filamentous mitochondria, insoluble formazan formation and the cellular ATP levels supported

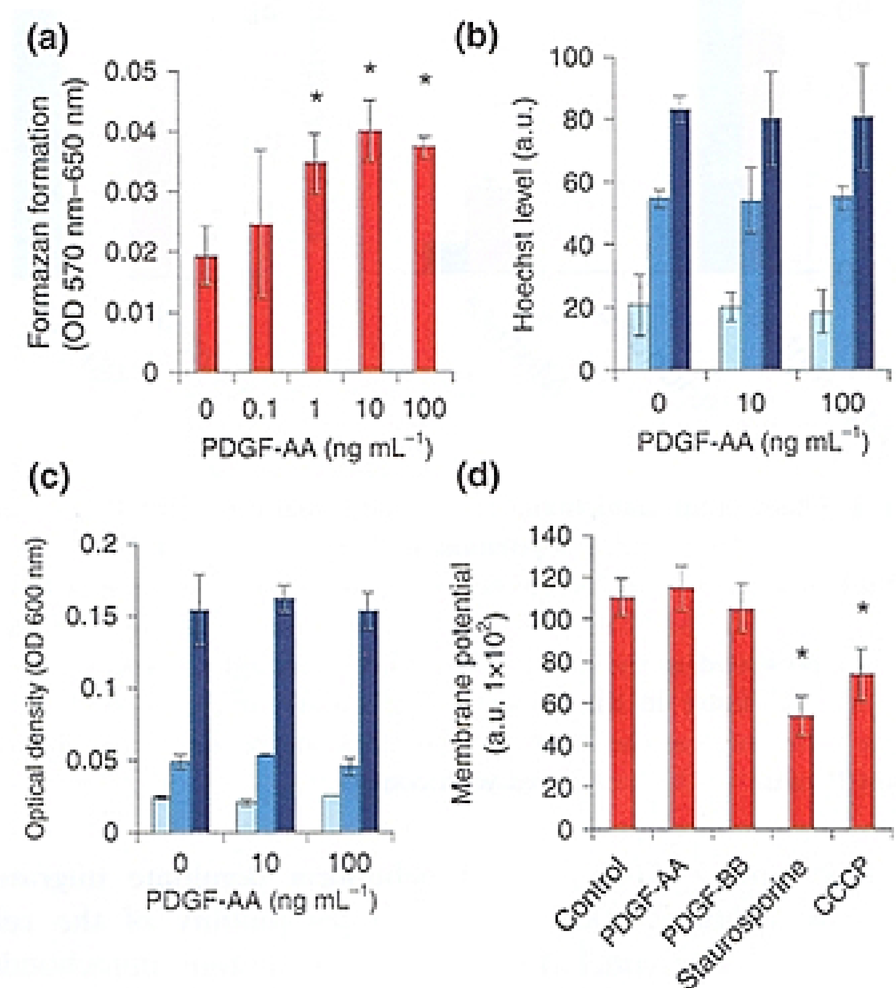

Figure 3 PDGF-AA increases mitochondrial enzymatic activity without changing cellular proliferation rate and membrane potential. (a) Formazan formation was estimated in the presence of 0–100 ng mL^{-1} PDGF-AA. (b, c) HDPCs were incubated with PDGF-AA for 1 (light blue), 4 (blue) and 7 days (dark blue), and the cellular proliferation was estimated by quantifying either DNA level or cellular turbidity. a.u., arbitrary units. (d) PDGF-AA does not change the membrane potential of mitochondria, in contrast to dramatic reduction by staurosporine and CCCP. The cells were incubated in the presence of PDGF-AA (100 ng mL^{-1}, 24 h), PDGF-BB (100 ng mL^{-1}, 24 h), staurosporine (1 μM, 3 h) or CCCP (25 μM, 1 h), followed by analysis with the fluorescent microplate reader (Ex 488 nm, Em 600 nm). a.u., arbitrary units. Data are represented as the mean ± SD of three independent experiments. * indicates P-values <0.05 compared with control.

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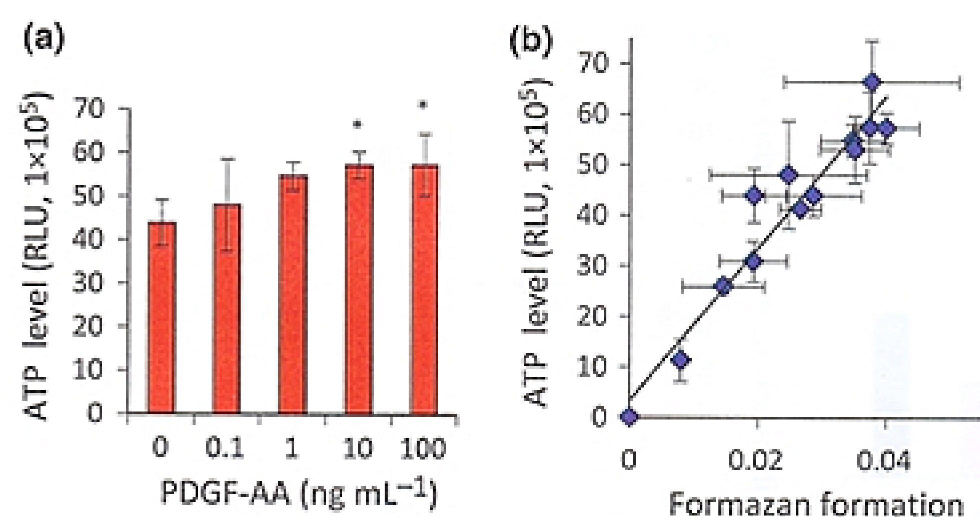

Figure 4 PDGF-AA elevates cellular ATP level. (a) HDPCs were incubated at different concentrations of PDGF-AA for 24 h. Cellular ATP level was measured by a luciferase-based assay. Data are represented as the mean ± SD of three independent experiments.* indicates P-values <0.05 compared with control. (b) The determined ATP levels were replotted against the corresponding activity of formazan formation. Linear least-squares fitting was performed. Data are represented as the mean ± SD. RLU, relative light unit.

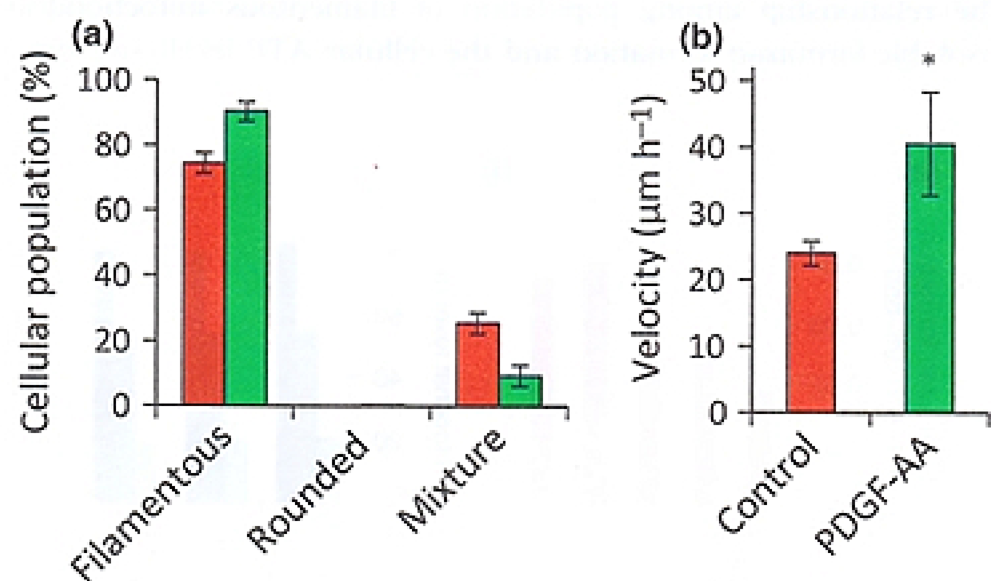

Figure 5 Filamentous mitochondria dominate migrating HDPCs in a gap-filling assay. (a) The cellular population with each type of mitochondria in the migrating cells in the presence (green) and absence (red) of 100 ng mL^{-1} PDGF-AA. The cells were fixed 8 h after scratches were made. Data are represented as the mean ± SD of five independent experiments. (b) HDPCs migrate faster in the presence of PDGF-AA in a gap-filling assay. Data are represented as the mean ± SD of three independent experiments. * indicates P-value <0.001 compared with control.

this. Importantly, filamentous mitochondria dominate migrating HDPCs. In addition, PDGF-AA accelerates motility of the cells. Therefore, we have concluded that the filamentous mitochondria benefit HDPCs in the process of migration by producing more chemical energy.

It was previously shown that the surface area of cristae was increased upon PDGF-AB treatment in rat kidney cells [17]. In addition, an increase of the cristae surface, due to elevated energy requirement, was observed in thyroxine-treated rat hepatocytes and human hepatocellular carcinoma [18, 19]. Thus, it is possible that PDGF-AA triggers mitochondrial structural change at the level of cristae to produce more chemical energy in HDPCs. The filamentous mitochondria could also serve other cellular functions such as efficient homogenization/complementation of mitochondrial DNA and cell protection against apoptosis.

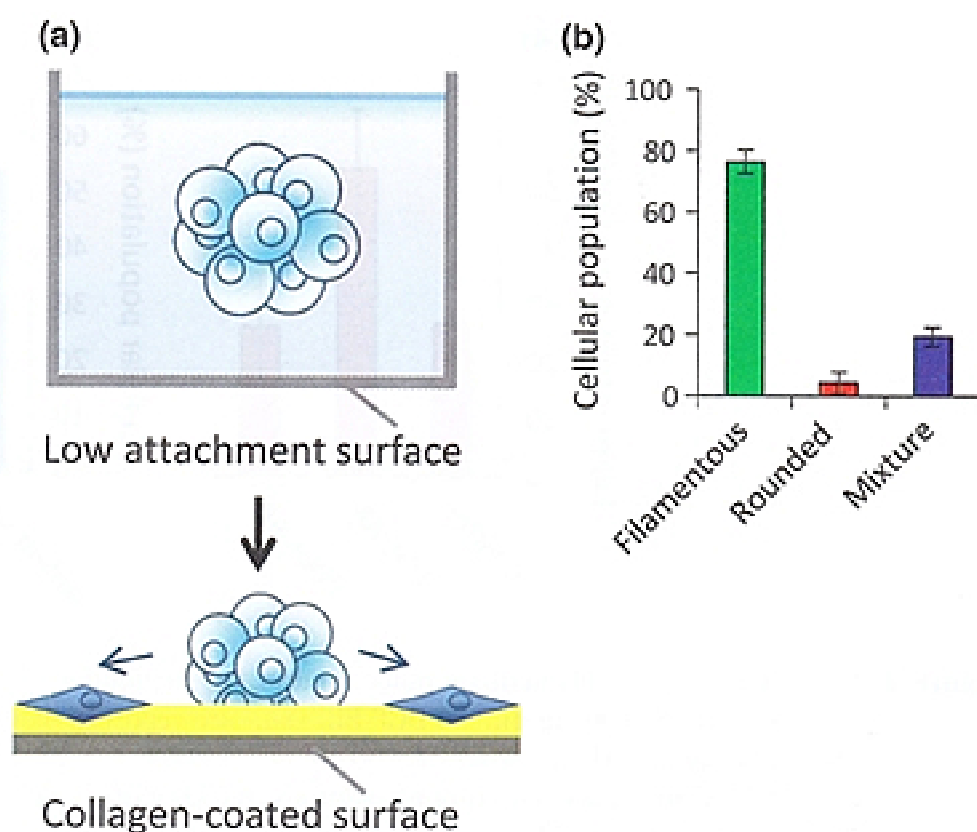

Figure 6 Filamentous mitochondria dominate migrating HDPCs away from spheroids. (a) Schematic drawing of a spheroid assay. Spheroids of HDPCs were formed using a low-attachment dish (top). The microspheres were transferred into collagen-coated dishes and left for a day. The cells partially migrate away from the spheroids during this period (bottom). (b) The cellular population with each type of mitochondria in the migrating cells. Data are represented as the mean ± SD of six independent experiments.

The relatively large rounded mitochondria we observed in the current work seem to be different from small fragmented mitochondria that are described in previous works [2]. These large rounded mitochondria were originally described in the electron microscopic observation of HDPCs in thin sections of human skins [20]. Moreover, this type of mitochondria was observed in other primary cells [21]. The functional roles of the large rounded mitochondria and the physiological significance of the balance of the two types of the organelle have yet to be elucidated. Our current work indicates that HDPCs maintain the balance between the filamentous and rounded mitochondria. It is likely that HDPCs slow down ATP production, in the usual circumstances, using more rounded mitochondria. The rounded mitochondria might be beneficial for quiescent HDPCs in dermal papillae in hair follicles, so that chemical energy is not wasted [22]. On the other hand, the hypothalamic-pituitary –thyroid axis hormones stimulate mitochondrial gene expression and mitochondrial biogenesis especially in outer root sheaths [23], which shows spatial control of mitochondria by different signalling molecules.

In the process of hair follicular formation, the thick overlaid epithelium (placode) produces PDGF-AA, and its receptor is highly expressed in the dermal condensate [10, 11]. PDGF-AA, signalling from the epithelium, triggers the clustering of mesenchymal cells to form a dermal condensate, HDPC aggregate. We assume that filamentous mitochondria are used in the process of the dermal condensate formation when HDPCs migrate. A cooperative effect between PDGF-AA and other signalling molecules, such as Shh, on the mitochondrial morphology and functions, will give us further view.

In anagen, the energy-demanding hair growth phase, adipocyte precursor cells produce PDGF-AA, and the receptors of the signalling molecule in HDPCs are activated. In fact, PDGF-AA-coated beads induce the morphological characteristic of anagen follicles [13].

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Therefore, it is of interest to investigate whether or not the filamentous mitochondria also dominate HDPCs in anagen.

A balance between mitochondrial fission and fusion is regulated by a series of mitochondrial membrane proteins, such as dynamin-related protein 1, mitofusin-1, mitofusin-2 and optic atrophy-1 protein. These molecular mechanisms, by which PDGF-AA affects mitochondrial morphology, are to be investigated. Nevertheless, future research based on the current results will lead us to a complete understanding of the molecular mechanism of hair growth with mitochondrial regulation, and also to the development of novel therapies of mitochondrial diseases based on PDGF-AA regulation [24, 25].

Acknowledgements

We would like to thank Dr. H. Yoshioka, Ms. S. Adachi, H., Mr. Kawazato, Dr. A. Yasuda (Faculty of Medicine, Oita University) and Dr. M. Otsubo (Faculty of Food Science and Nutrition, Beppu University) for providing facilities and assistance. We are grateful to Mr. Hiroyuki Watanabe and Ms. Elyse Mcpherson for their assistance in English language editing.

Conflict of interest

The authors report no conflict of interests.

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