Hair greying and stimulation of hair pigmentation

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Abstract (Maximum of 200 words)
Hair greying is a universal process resulting from a decline of hair melanocytes to produce and transfer melanin into the hair shaft. Grey hair affects age perception and therefore impacts self-esteem and psychological wellbeing. Although hair dying is common practice, it may negatively impact scalp, hair, and environment, despite continuous improvements of their chemistry and packaging. We therefore decided to investigate on active ingredients which trigger endogenous hair pigmentation without using exogenous hair dyes.
From screening of 48 peptides for melanin production and tyrosinase activity we selected dipeptide P2. P2 induced melanin production by 223% in melanocytes and by twelve-fold in isolated hair follicles. It increased dendritic length in melanocytes (+54%) and phagocytosis in keratinocytes (+283%) indicating enhanced melanin transfer. Pharmacological inhibition of pathways involved in melanogenesis, showed that P2 acts via PKA/CREB pathway, without modulating P38, PI3K or ERK. P2 induced *CREB*, *MITF* and *TYRP1* gene expressions in melanocytes by 56%, 42% and 54% respectively. MITF protein levels in hair follicles were also increased. Clinical evaluations showed that P2 improved hair pigmentation on >70% of the volunteers. White hair density decreased, while black/white hair ratio increased. P2 effect was remanent as pigment persisted after the last application.

Keywords: Hair greying; Hair pigmentation; Peptide; Melanogenesis

Introduction.

As we age, hair go through multiple changes including hair loss, hair follicle miniaturization, structural changes to the hair shaft and hair greying [1,2].
Hair follicle is a complex structure composed of 3 regions: the infundibulum, the isthmus, and the bulb [3,4]. The infundibulum extends from the surface of the epidermis to the sebaceous duct. The isthmus comprises the area between the sebaceous gland and the insertion site of the arrector pili muscle, also known as the bulge. The bulge corresponds to the niche of hair follicle stem cells including melanocytic and epithelial stem cells of the hair [4] . The bulb comprises all the structures underneath the bulge [3,4].
At the base of the bulb lies the dermal papilla, which sends signals to stimulate hair growth. Surrounding and above the dermal papilla is the germinative matrix, containing hair melanocytes and keratinocytes [3,4]. These cells derive from the bulge and travel to the germinative matrix during hair growth (anagen phase). Melanocytes produce melanin that is stored in vesicles called melanosomes that travel through melanocytes dendrites and reach keratinocytes where they enter by phagocytosis. Keratinocytes that have acquired their pigment, divide, produce keratin and give rise to the pigmented hair shaft [3,4]. At the end of the anagen phase, melanogenesis stops and dendrites retract [2].

Melanin production starts with L-Tyrosine conversion to Dopaquinone via the tyrosinase. Dopaquinone goes through 2 different enzymatic reactions including TYRP1 and TYRP2 (tyrosinase related proteins) that give rise to eumelanin (brown/black pigment) and pheomelanin (red/yellow pigment).
At the molecular level, melanin production is controlled by multiple signaling pathways leading to the activation of the key melanogenesis master-regulator MITF (Microphtalmia Associated Transcription Factor), involved in melanocyte differentiation and survival. There are 3 major pathways involved in MITF-induced melanogenesis:

1-CREB (cAMP-dependent protein kinase response element-binding protein) Upon UV induction, α -MSH (α-melanocyte-stimulating hormone) binds to its receptor, MC1R (melanocortin 1 receptor), and leads to increase cAMP which in turn activates PKA. PKA phosphorylates CREB that induces MITF transcription by directly binding to its promoter [5,6].

2- MAPKs (Mitogen-activated protein kinases): MAPKs are involved in multiple processes. Phosphorylation of MITF by ERK is a tag for proteasomal degradation. On the other hand, phosphorylation of MITF by p38 stabilizes it and triggers melanogenesis [5,6].

3- GSK3β- pathway (Glycogen synthase kinase-3β): in a similar way to CREB, GSK-3β also binds MITF gene to induce its transcription. PI3K (phosphatidylinositol 3 kinase) phosphorylates and inhibits GSK-3β therefore downregulating MITF transcription [5,6].

Even though the mechanism of hair greying is not completely identified, hair is linked to a strong decrease in active melanocytes because of ageing and oxidative stress. There is therefore a decrease in tyrosinase activity causing reduced melanin production but also a reduction in melanin transfers due to a decrease of dendrite size[7] [7].
Because the importance of hair-greying has in people's appearance, well-being and self-esteem, we therefore focused on identification of a peptide able to re-activate melanin production and pigment hair.

Materials and Methods.

Melanin quantification and tyrosinase activity

Primary human melanocytes (Invitrogen) with moderate melanin production were seeded in 24 well plates (Falcon) and cultivated at 37°C, 5% CO2 in M-254 (Invitrogen) growth media. When cells were confluent, media was replaced by DMEM-F12 (Gibco), supplemented with P2 (5 ppm, 10ppm and 12.5ppm) or its control (0.1% DMSO) for 10 days. Media was renewed every 2-3 days. Melanin was extracted using NaOH (Sigma-Aldrich) and its quantity was assessed with a spectrophotometer at 490 nm.
In parallel, tyrosinase activity was assessed through conversion of L-DOPA to dopaquinone, at 37°C. Dopaquinone production was measured at 490 nm.
To homogenize and compare the data obtained, melanin quantity and tyrosinase activity were reported to protein amount assessed by BCA (Bicinchoninic acid) (Invitrogen).

Pigmentation of hair follicle *ex-vivo*

Scalp was isolated from lifting surgery on a 51 years-old woman. Hair follicles, including their lightly pigmented bulb, were isolated and placed in separated wells of a 48-well cell culture plate

(Falcon) in presence of 10ppm P2 or its vehicle (0.1% DMSO) for 8 days. Media was renewed every 2 days. After the incubation time, hair follicles were fixed in formol for 24 hours and included in paraffin. 7µm sections were performed for either quantitative assessment of their pigmentation after carmine staining (n=6) or for immunostaining (n=6).

Dendrite length assessment

Primary human melanocytes were cultivated in DMEM-F12 (Gibco). Cells were then treated with 10 ppm P2 for 48h. Cells were then fixed and stained with NKiBeteb antibody in order to have the whole melanocyte surface. Nuclei were stained with Hoechst 33258. For dendrite length, pictures were taken and analyzed using MetaXpress (Molecular Device). Size of dendrites length were reported to cell number and shown as dendrite length per 100 cells.

Phagocytosis assay in keratinocytes

Primary human keratinocytes (Cellntec) were cultivated in their growth media (CnT57c, Cellntec) according to manufacturer's instructions. They were then seeded in collagen-coated well-plates (Falcon) in KSFM media (Invitrogen). Keratinocytes were treated with 10ppm P2. Fluorescent microspheres™ (Molecular Probes) were applied for 3h. Cells were rinsed with a PBS/ 0,5% BSA solution and phagocyted beads were quantified. Cell nuclei were stained with Hoechst 33258 and data were normalized to cell number.

Gene expression

Primary human melanocytes were seeded in 12-well plates (Falcon) in M-254 (Invitrogen). Media was replaced by DMEM-F12 (Gibco), supplemented with 10ppm P2 or its vehicle (DMSO). 3 days later, RNA extraction was performed using RNA extraction kit (Qiagen). RNA quantity was assessed using Nanodrop and 1µg RNA was reverse transcribed using iScript cDNA Synthesis kit (Biorad, USA). Gene expression was assessed using SYBRGreen (Biorad, USA) and CFX technology (Biorad, USA). Primers targeting *MITF, TYRP1 and CREB* were purchased from Biorad's library of validated primers. Fold-change of gene expression was calculated using $\Delta\Delta$Ct method.

Pharmacological inhibition

Mouse-derived B16 melanoma cells (ECACC) were seeded in 48-well plates in DMEM media (Gibco). The next day, cells were treated with an inhibitor of CREB pathway (H89, Cell Signaling), an inhibitor of P38 (SB203580, Cell Signaling), an inhibitor of PI3K/GSK3β pathway (LY294002, Cell Signaling) or an ERK inhibitor (PD98059, Cell Signaling)) for 1 or 4h. Cells were then rinsed and treated with P2 for 48h. Melanin and tyrosinase were extracted. Quantity of melanin and tyrosinase activity were assessed as described previously.

Clinical evaluation

Four independent studies were conducted at four different European sites involving a total of 84 volunteers with grey hair. The average age of the entire panel of volunteers chosen was 42 years old. Tests were performed at various times, in different seasons and on different application sites, using various phototypes and methods. Lotions containing 90 ppm of P2 were applied daily for at least 3 months. Photos were taken and assessed parameters included: white+grey hair surface and ratio of black/white hair number.

Statistical analysis

For *in-vitro* assays, one-way analysis of variance (ANOVA) was used to determine whether there was any significant difference between the means of two or more independent groups. Difference between two means with similar variances was performed with Student's t-test. A p-value $p<0.05$ or $p<0.01$ were considered statistically significant.
For clinical studies, statistical analysis were performed using Student- or Wilcoxon-t test; two tailed, paired series. A p-value $p<0.05$ or $p<0.01$ were considered statistically significant.

Results.

Identification of P2 as the best candidate for hair pigmentation

48 peptides were tested on their ability to induce melanin production and tyrosinase activity in both B16 cell line and human melanocytes. P2 increased melanin production in a dose-dependent manner by up to 223 % (Figure 1A) and tyrosinase activity by 27% in moderately pigmented human melanocytes, making P2 the best candidate. We assessed expression of genes involved in melanogenesis: the master regulator of melanogenesis: MITF; its direct transcriptional activator CREB and the enzyme involved in the conversion of dopaquinone in eumelanin TYRP1. P2 induces these targets (Figure 1B). We then tested the capacity of P2 to induce melanin in hair. Hair follicles with low or no melanin were isolated and treated with 10ppm P2. P2 significantly increased pigment in hair follicles and expression of MITF and M1CR *ex vivo* (Figure 1C, 1D). Altogether these data showed that the identified candidate increases melanin production in both 2D cell culture and ex-vivo hair follicles. We then focused on understanding the mechanism of action by which P2 activates melanin production and melanin transfer.

P2 activates melanin transfer from melanocytes to keratinocytes.

To understand the pigmentation mechanism observed in hair follicle, we focused on two parameters involved in melanin transfer: 1) melanin dendrite length and 2) keratinocyte phagocytosis.
When treated with P2, melanocytes dendrite length was increased by 57% (Figure 2A). In keratinocytes, P2 activated phagocytoses by 283% (Figure 2B).
Even though, we do not have direct proof, data on melanin dendrite length, phagocytosis in keratinocytes and capability of P2 to pigment hair follicle, strongly suggest that melanin transfer is activated.

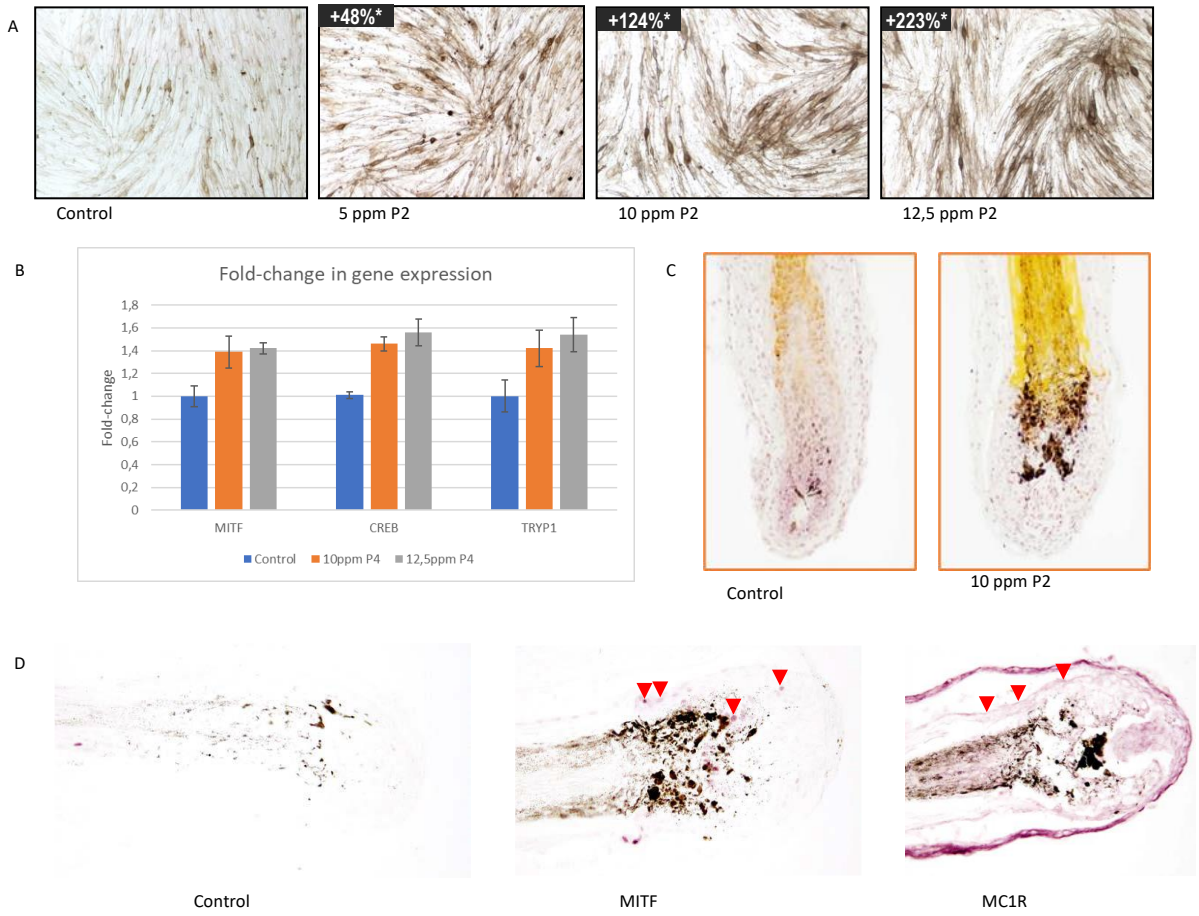

Figure1: Assessment of melanin-inductive properties of P2. A. Induction of melanin production in human primary melanocytes. B. Expression of genes involved in melanogenesis in human primary melanocytes treated with P2. C. Effect of P2 in pigmentation of hair follicles *ex vivo* and D. Expression of MITF and MC1R in hair follicles *ex vivo*

P2 acts via PKA/CREB pathway.

Melanin production is a complex process regulated by multiple signaling pathways. In order to identify which pathways are targeted by P2, we went through pharmacological inhibition of PKA/CREB, P38, PI3K and ERK. Melanin quantity was determined in each case.

Only inhibition of PKA/CREB by H89 blocked melanin induction by P2. H89 alone inhibited melanin production by 31,9% ($p<0,01$). P2 was not able to induce melanin levels when CREB was inhibited. This means P2 induction of melanin production goes through the activation of PKA/CREB pathway. SB203580, LY294002 and PD98059 inhibiting respectively: P38 MAPK, PI3K and ERK did not alter effect of P2, meaning that P2 did not act on these pathways.

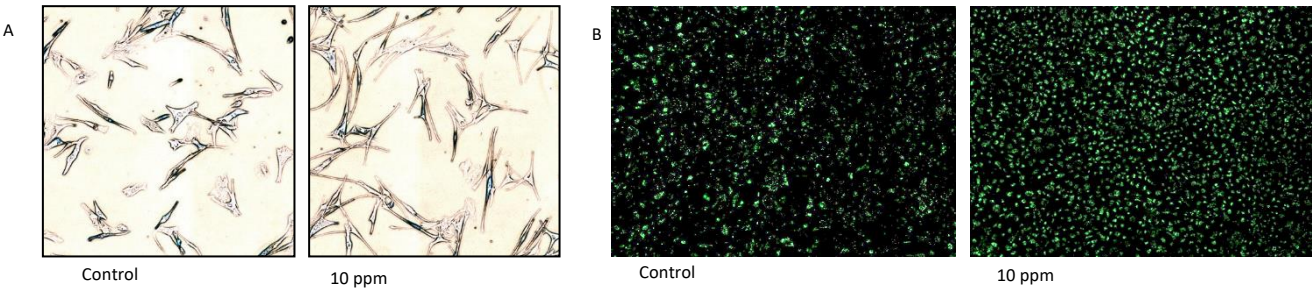

Figure 2: Melanin-transfer mechanism. A. Size of melanocytes dendrites treated with P2; B. Phagocytosis in keratinocytes after P2 treatment.

Clinical trials

Given the promising data obtained both *in-vitro* and *ex vivo*, we decided to test P2 efficacy *in vivo*. A lotion containing 90 ppm P2 was applied daily for 3-4 months. Scalp was massaged during product application. Placebo was tested in an identical protocol, on a separate trial and showed no effect. We therefore focused on evaluating grey hair density and black/ white hair ratio during time. Hair pigmentation increased in >70% of volunteers. For 3 studies, grey hair density decreased by 32% ($p<0.01$) on average after 3 months of treatment. In one study, black/ white hair ratio increased by 27% ($p<0.05$) after 4 months of treatment (representative pictures are shown in figure 3). On two studies, volunteers were asked to stop applying the product for up to 4 months and grey hair density was measured to determine the remnant effect of P2. Grey hair density continued decreasing after last application. Indeed, on both studies, there is still a significant reduction in grey hair density. There is even a slight decrease of -7% and -3% compared to last time of application. Altogether, clinical trials showed that P2 induced hair pigmentation *in vivo* and that its effect is remnant.

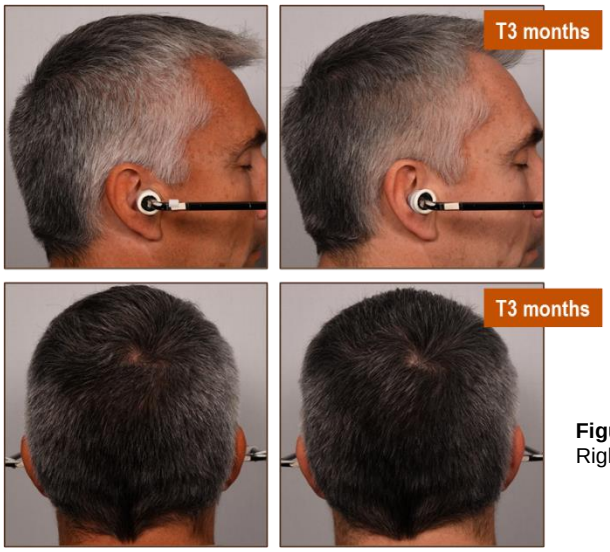

Figure 3: Example of P2 effect *in vivo*. Left: Before treatment. Right: After treatment

Discussion.

In this study, we showed that P2, a palmitoylated di-peptide, stimulated melanocytes pigmentation in hair follicles through activation of CREB pathway resulting in upregulation of MITF, TYRP1 and tyrosinase activity. Effect of P2 on dendrite lengths in melanocytes and

phagocytosis in keratinocytes, suggests that this peptide improves melanin transfer from melanocytes to keratinocytes. Further studies on melanocytes / keratinocyte co-culture would help us understand melanin transfer mechanism. *In-vitro* and *ex vivo* data suggested that P2 would be the best candidate to induce hair pigmentation. We therefore performed *in vivo* evaluations. Four clinical evaluations, performed in four independent laboratories, confirmed the preclinical results showing an improvement of hair pigmentation observed in majority of volunteers. Interestingly, an unexpected remanent effect was also observed 4 months after the last application. In conclusion, peptide P2 is a new strategy for recovering natural hair colour and for reducing hair greying.

Conclusion.
In the attempt to find a product able to induce hair pigmentation, we developed a screening platform to identify molecules triggering melanin production. This strategy helped us identify an efficient and suitable candidate for hair pigmentation.

Acknowledgments.
The authors wish to thank BIO-EC/ Eurofins for their study on isolated hair follicles.

Conflict of Interest Statement.
NONE.

References.
All the references must be quoted numerically in the order in which they appear in the manuscript. This must be done in between square brackets [1].

Examples:

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