Treatment with cyclohexyl salicylate, an olfactory receptor 2A4/7 agonist, promotes human hair follicle growth and bulge stem cell progeny expansion

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Abstract (Maximum of 250 words)

Background: Hair loss and thinning can cause severe psychological stress in affected individuals. Therefore, topically applicable strategies increasing hair growth and density are highly demanded as alternative or adjuvant to drugs. In this study, we explored, whether cosmetic ligands of the olfactory receptor 2A4/7 (OR2A4/7) improves hair follicle (HF) function and hair growth.

Methods: We evaluated *OR2A4/7* mRNA and protein expression in HFs of freshly frozen human scalp skin samples. Additionally, we organ cultured microdissected HFs in the presence of the OR2A4/7 agonist, cyclohexyl salicylate (CHS), to study its effects on hair cycle as well as hair follicle stem cells and progeny by applying quantitative (immune-) histomorphometry.

Results: We found *OR2A4/7* mRNA expression in the outer root sheath (ORS) and hair matrix (HM) of HFs in human scalp skin, whereas protein expression was mainly restricted to the infundibulum. During organ culture, OR2A4/7 protein expression increased in the HF epithelium. CHS treatment delayed catagen and tendentially increased HM keratinocyte proliferation. While CHS did not impact on K15+ bulge stem cells, the percentage of their immediate progeny (CD34+ cells) was significantly increased in the suprabulbar ORS, as was the percentage of CD71+ transit amplifying cells in the HM and suprabulbar ORS.

Conclusion: Stimulating OR2A4/7 via CHS promotes hair growth and expands the progeny of K15+ stem cells. Therefore, our data invite the use of CHS as a novel cosmetic to inhibit hair loss and thinning

Keywords: Olfactory receptors; Hair loss; Hair Follicle; OR2A4/7

Introduction. The management of hair loss disorders would profit greatly from the development of topically applicable strategies that increase hair density, such as non-drug agents or cosmeceutical adjuvants supporting and enhancing the efficacy of currently available FDA-licensed hair drugs. To prevent or restore hair loss, it is important to effectively suppress hair follicles (HFs) from the premature entering of catagen, and/or to promote epithelial HF stem cell progeny activity, which is reduced in, for instance, androgenetic alopecia [1].

We have recently shown that the synthetic sandalwood-like odorant, Sandalore®, activates olfactory receptor family 2 subfamily AT member 4 (OR2AT4) receptors expressed on human HFs, and that OR2AT4 stimulation with Sandalore® prolongs anagen, promotes hair growth, and reduces telogen effluvium [2, 3]. In light of this, we were interested whether other OR ligands can unfold similar beneficial properties for hair loss management.

We focused our attention on OR2A4 and OR2A7, two proteins with a high degree (~99%) of homology, which are regarded as a single entity (OR2A4/7). OR2A4/7 is known to stimulate keratinocytes proliferation and melanocyte differentiation [4, 5]. Therefore, the aim of this study was to examine the role of OR2A4/7 in the context of HF biology.

Materials and Methods. Human scalp skin and HF specimens were obtained from healthy human donors. We used follicular unit extraction to obtain full-length, microdissected anagen VI HFs. HFs were cultured over a period of six days and 50 µM cyclohexyl salicylate (CHS) or 0.1% DMSO as vehicle control were applied every other day. For hair cycle staging analysis, we evaluated HFs using Masson–Fontana histochemistry and Ki-67/TUNEL immunostaining. *In situ* hybridization and immunohistostainings were performed on freshly frozen HFs to examine *OR2A4/7* mRNA and protein expression, respectively. Additionally, expression of CD34+, K15+ and CD71+ were assessed on freshly frozen samples and staining intensities were evaluated by quantitative (immune-) histomorphometry. Last, we assessed proliferation by quantifying Ki-67+ cells. All data are expressed as mean ± SEM.

We evaluated normality of the datasets with d'Agostino and Pearson omnibus normality test. Data sets considered to follow a normal distribution were compared with the student's t-test; the remaining were compared with the Mann–Whitney test, as indicated. $P < 0.05$ was regarded as statistically significant.

Results. We detected broad *OR2A4/7* mRNA expression in the entire epidermis and the HF epithelium whereas OR2A4/7 protein was found in scalp skin epidermis. In HFs of the scalp, we mainly detected OR2A4/7 protein expression in the infundibulum and the bulge. In *ex vivo* cultured HFs, OR2A4/7 protein expression was increased in the HF bulb in comparison to the *in vivo* situation. These findings suggest that the HF has the potential to express OR2A4/7 protein in various HF epithelial compartments. Next, we aimed at dissecting the functional role of OR2A4/7 in human HFs. Therefore, we applied either CHS or vehicle to organ cultured HFs *ex vivo*. CHS is a synthetic fragrant, belonging to the class of hexyl salicylates, which are widely used in cosmeceutical products, and it is a known, selective activator of OR2A4/7 [6, 7]. In a first step, we investigated the effects of CHS on OR2A4/7 protein expression, and found that treatment with CHS significantly decreased OR2A4/7 expression in compartments of the bulb, but not the bulge. Next, we assessed the functional consequences of OR2A4/7 modulation on HF function. Therefore, we evaluated Keratin 15 (K15+) expression, in cultured HFs *ex vivo*. However, CHS treatment did not affect K15 expression, proportion of K15+ cells, or K15+ cell proliferation. Afterwards, we assessed the effect of CHS on CD34+ and CD71+ expression in the HF and found an increased percentage of CD34+ and CD71+ cells. In a next step, we examined whether CHS treatment would also affect the hair cycle. Application of CHS increased numbers of HFs in anagen, and led to a tendential increase in hair matrix keratinocyte proliferation (Ki-67+ cells). These findings suggest that OR2A4/7 activation by CHS stimulates stem cell progeny, ultimately leading to increased hair growth.

Discussion. In this study, we demonstrate expression of OR2A4/7 in different HF epithelial compartments *in vivo*. Additionally we show that, under organ culture conditions *ex vivo*, OR2A4/7 protein expression is also induced in the bulb, including the proximal ORS, HM and DP, pointing at a "stress-induced" up-regulation of OR2A4/7. Additionally, we

demonstrated that application of CHS primarily increases CD34+ and CD71+ cell numbers, representing putative stem cell progenitor and/or transit-amplifying cell populations. We suggest, that proliferation of these cells ultimately influence the cell cycle and hair matrix keratinocyte proliferation.

Conclusion. Taken together, our results solidify ORs as important regulators of HF function and reinforce the notion that ORs can be pharmacologically or cosmeceutically targeted to improve hair growth. However, further studies will be necessary to understand the interplay between individual HF stem cell populations and hair growth, in dependence of OR2A4/7 activation, and to establish if CHS can be used as a cosmetic adjuvant treatment for hair loss disorders, such as androgenetic alopecia.

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Conflict of Interest Statement. R.P., J.E., and M.B. are employees of Monasterium Laboratory GmbH, a contract research organization specializing in dermatology. D.P. and F.R. are employees of Giuliani S.p.a., R.P. also consults for Giuliani S.p.A.

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