Thymic Peptides Differentially Modulate Human Hair Follicle Growth

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TO THE EDITOR

Topical preparations containing thymus-derived protein extracts have long been claimed to stimulate human hair growth (Renner *et al.*, 1986; Sawaya and Shapiro, 2000), but there is still no convincing evidence available that they really modulate human hair growth. Despite their historical name, most thymic peptides (TPs), such as thymosin β4 (TB4), prothymosin alpha (PTMA), and thymulin (TYL), are much more widely distributed than in the thymic epithelium where they were first identified (Kato *et al.*, 1981; Moll *et al.*, 1996; Folch *et al.*, 2010; Supplementary Information S1 online). The *PTMA* gene is prominently expressed in many rodent tissues (Moll *et al.*, 1996), including fetal murine hair follicles (HFs). PTMA is involved in many cellular processes such as apoptosis, chromatin remodeling, and transcriptional regulation (reviewed in Hannappel and Huff (2003)); Supplementary Information S1 online). TYL has been claimed to be thymus specific, although TYL immunoreactivity has also been reported in murine epidermis and skin appendages (Kato *et al.*, 1981) and in murine spleen (Folch *et al.*, 2010). Interactions between TYL and the neuroendocrine system have been reported to lead to a release of prolactin, thyrotropin, and ACTH (Hadley *et al.*, 1997; Brown *et al.*, 1998). As these neurohormones are well appreciated as regulators of human HF growth, cycling, and pigmentation (Paus, 2011), it is particularly interesting to investigate TYL in a hair research context.

Topically applied TB4 reportedly enhances hair growth in rats and mice and stimulates early differentiation of rat vibrissae epithelial progenitor cells (Philp *et al.*, 2004), whereas it is unknown whether TB4 impacts on human hair growth. Therefore, we wished to clarify in the current study whether human scalp HFs express any TPs, and whether selected TPs exert measurable effects on human scalp HFs in organ culture (Kloepper *et al.*, 2010).

TYL, TB4, and PTMA are predominantly expressed in differentiating epithelial compartments of the human HF

First, we asked whether human HF epithelium expresses any defined TPs on the gene and/or protein level. As no gene coding for TYL has been identified (see Supplementary Information S1 online), only TB4 and PTMA gene transcription could be examined. As shown in Figure 1a, strong and specific mRNA signals for both genes were identified, thus confirming that human anagen HF epithelium expresses both *TB4* and *PTMA*.

By immunohistochemistry or immunofluorescence (Supplementary Information S2 online), strong PTMA immunoreactivity was detected in the hair matrix, the cortex, and inner and outer root sheaths (IRS and ORS, respectively) of the lower HF epithelium and, more weakly, in the dermal papilla (Figure 1b). Interestingly, TB4-like immunoreactivity was only found in the nuclei of the IRS, ORS, and cuticle. Strong TYL immunoreactivity was detected in Henle's layer of the IRS, in the ORS, and in the hair shaft cortex (Figure 1b).

Expression of these TPs on the gene and/or protein level suggests the hypothesis that endogenous, intrafollicularly produced TPs are used by human HFs to regulate some of their functions.

TYL, thymosin alpha 1, and TB4 exert subtle, but distinct, effects on human hair shaft production *in vitro*

This hypothesis was tested in serum-free human HF organ culture (Supplementary Information S2 online). We first studied whether thymosin alpha 1, a peptide comprising amino acids 2–29 of PTMA, (Supplementary Information S1 online), TYL, and TB4 modulate hair shaft production (i.e., the hair shaft elongation rate) *in vitro*. Cultivation of HFs with 10 pg ml^{-1} TYL for 7 days in three independent experiments from three different human donors resulted in an increased hair shaft growth rate compared with vehicle-treated HFs (Figure 2a). In contrast, hair shaft elongation rates of HFs treated with 100 or 1,000 ng ml^{-1} thymosin alpha 1 for 7 days were slightly lower than those of control HFs (Figure 2a). In TB4-treated HFs, hair shaft production was 10–20% lower than in the vehicle control (Figure 2a and Supplementary Figure S1 online).

TYL prolongs anagen, whereas TB4 shortens it

Next, we studied by quantitative hair cycle histomorphometry (Kloepper *et al.*, 2010) whether the tested TPs had an effect on the transformation of anagen VI HFs into the regression stage of the hair cycle (catagen). This transformation is the clinically most relevant parameter one can study in HF organ culture, as any prolongation effect on anagen would be expected to correlate with a reduction of telogen effluvium *in vivo* (Cotsarelis and Millar, 2001; Paus and Foitzik, 2004; Kloepper *et al.*, 2010). Moreover, the effect of each peptide on the hair cycle was further assessed

Abbreviations: HF, hair follicle; IRS, inner root sheath; ORS, outer root sheath; PTMA, prothymosin alpha; TB4, thymosin β4; TP, thymic peptide; TYL, thymulin

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by determining the hair cycle score in each treatment condition (Figure 2b and c; Supplementary Information S2 and Supplementary Figure S1b online).

These analyses showed that HFs treated with 10 pg ml^{-1} TYL for 7 and 9 days stayed longer in anagen VI than vehicle-treated controls (Figure 2b, Supplementary Figure S1b online). By two-tailed Student's *t*-test, these differences did not come up as significant in the 7-day treatment group where the effect of TYL 10 on the distribution of anagen vs. catagen had a *P*-value of 0.06. Hair cycle score analysis further indicated that treatment with TYL for 7 and 9 days inhibited the progression of HFs from anagen to catagen (Figure 2c, Supplementary Figure S1b online). This anagen-prolonging effect of TYL (which reached significance in the 9 day cultures) was independently corroborated by the demonstration that, compared with vehicle controls, treatment of HFs with 10 pg ml^{-1} TYL for 7 days increased the number of Ki67 + cells in the hair matrix of anagen VI HFs, whereas the number of TUNEL + (i.e., apoptotic) cells was reduced (Figure 2d).

Despite its slight growth-inhibiting effect, treatment with 1,000 ng ml^{-1} thymosin alpha 1 for 7 days did not markedly change HF cycling *in vitro* in three independent experiments with HFs from three different patients (Figure 2d). Interestingly, there even was a stimulatory effect on hair matrix keratinocyte proliferation of anagen HFs (Supplementary Figure S2 online). Instead, treatment with 1,000 ng ml^{-1} TB4 for 7 days shortened the duration of anagen and prematurely induced

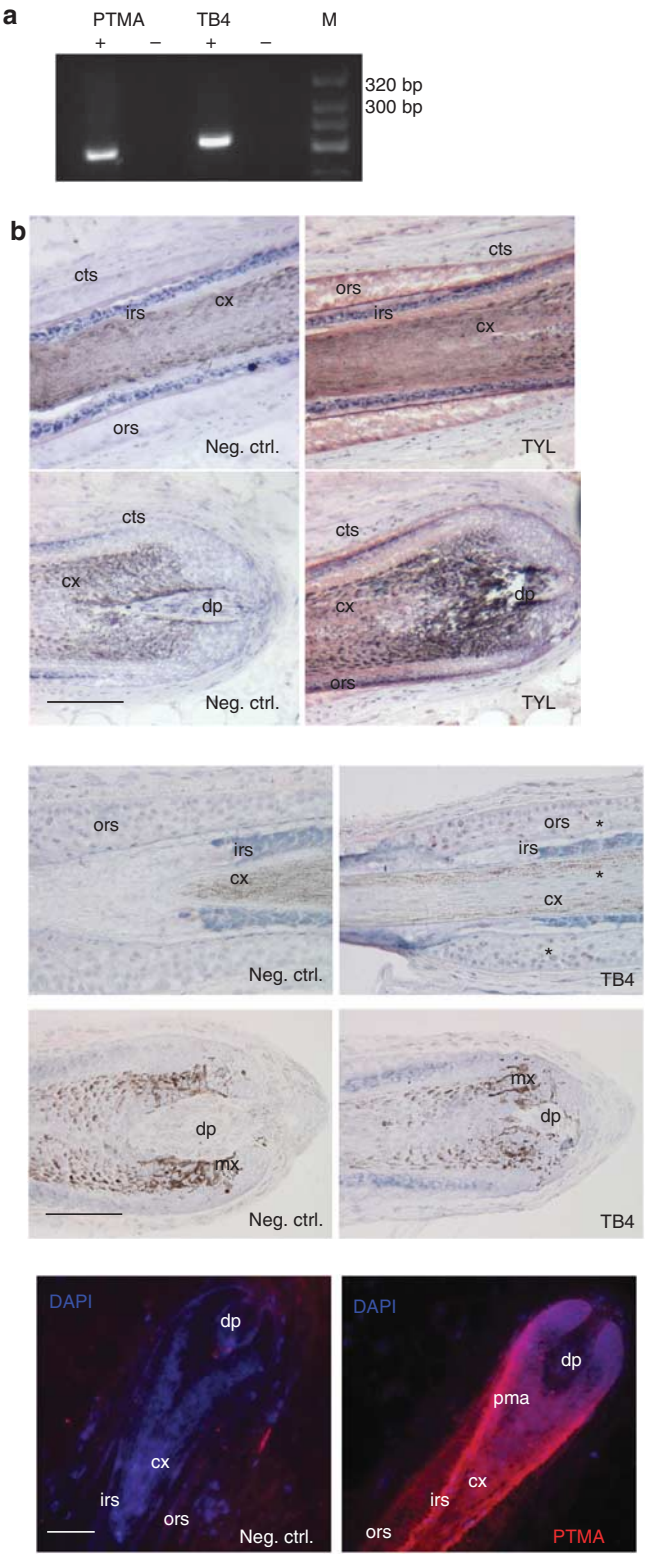

Figure 1. Localization of thymulin (TYL), thymosin β4 (TB4), and prothymosin alpha (PTMA) in the different compartments of the human hair follicle (HF). (**a**) Reverse transcription-PCRs to detect PTMA1 and TB4 transcripts in complementary (cDNA) derived from RNA from the epithelial part of female anagen human HFs follicles. The samples were run on 2% agarose/TAE gel; PTMA amplicon: 292 bp; TB4 amplicon: 315 bp (+ lanes). The lanes labeled with a (–) indicate the negative control (Neg. ctrl.) reactions that were run without cDNA. M, DNA size marker. (**b**) Exemplary immunohistochemistry and immunofluorescence results with TYL-, TB4-, or PTMA-specific antibodies. All three proteins were detected in the cortex (cx), inner and outer root sheath (irs and ors, respectively). TYL immunoreactivity was PTMA immunoreactivity was also found in the precortical matrix (pma). TB4 signals were strictly confined to the nuclei (*) of the mid and upper regions of the hair follicle not in the matrix (mx) and the dermal papilla (dp). Negative controls: experiments conducted without primary antibody. DAPI, 4′-6-diamidino-2-phenylindole; TAE, Tris-acetate-EDTA. Bars = 50 μm.

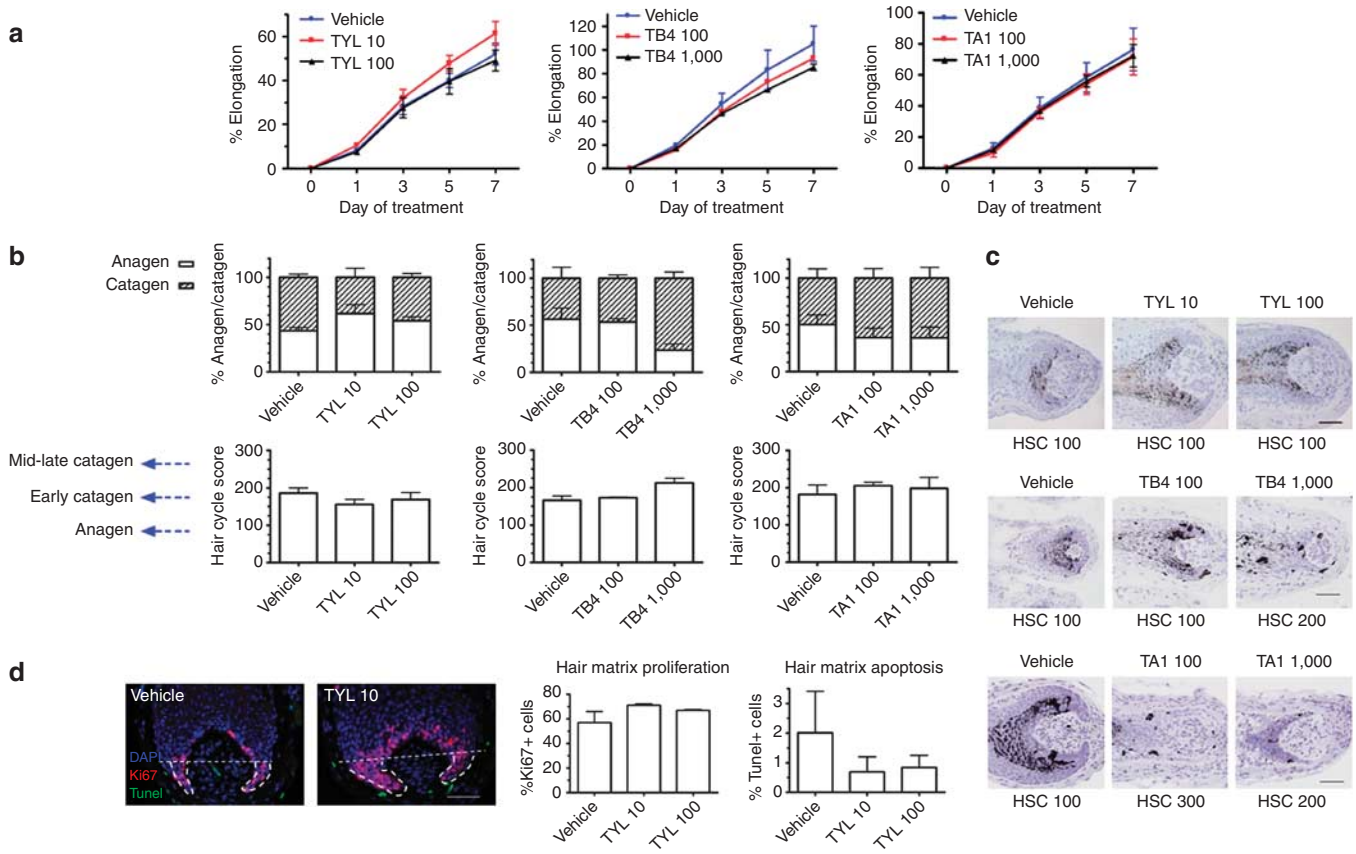

Figure 2. Thymic peptides (TPs) differentially modulate human hair growth *in vitro*. (**a**) Growth curves of hair follicles treated with two different doses of each TP (thymulin (TYL) 10: 10 pg ml^{-1} TYL; TYL 100: 100 pg ml^{-1} TYL; thymosin alpha 1 (TA1) 100: 100 ng ml^{-1} TA1; 1,000 ng ml^{-1} TA1; thymosin β4 (TB4) 100: 100 ng ml^{-1} TB4; TB4 1,000: 1,000 ng ml^{-1} TB4). Treatment with 10 pg ml^{-1} TYL led to an increase of hair elongation compared with the control condition during the treatment period, whereas prothymosin alpha and TB4 treatment slowed down hair growth. Data points in each curve represent mean ± SEM. (**b**) The hair cycle effects of TPs were assessed by quantitative hair cycle histomorphometry, which included the histochemical analysis of hair cycle–associated hair pigmentation changes (Masson–Fontana). Note that the anagen–catagen transformation stage of the human hair cycle is characterized by cessation of proliferation in the matrix and the rapid onset of hair bulb keratinocyte and melanocyte apoptosis (Kloepper *et al*, 2010). Top row: all experimental conditions except treatment with 10 pg ml^{-1} TYL led to a shortening of the anagen stage compared with control hair follicles ($P = 0.06$). In the case of 1,000 ng ml^{-1} TB4 and 100 ng ml^{-1} TA1, this effect was the strongest. Bottom row: only treatment with both concentrations of TYL led to a reduction of the hair cycle score (HSC) compared with vehicle control hair follicles, i.e., the duration of anagen was prolonged. Hair follicles incubated with 1,000 ng ml^{-1} TB4 and 100 and 1,000 ng ml^{-1} TA1 had an average HSC of 200, i.e., the onset of catagen was accelerated. (**c**) Photographs of exemplary hair follicles submitted to different experimental conditions as indicated above each photograph. The corresponding HSC values assigned to each hair follicle are below each photograph. All hair follicles shown in a row are from the same experiment. Bars = 50 μm. (**d**) Immunofluorescence for the detection of Ki67/TUNEL (Kloepper *et al*, 2010) was performed on sections of anagen hair follicles after 7 days of culture under the different treatment conditions. Ki67 and TUNEL + cells were counted in the marked areas with the highest mitotic activity below Auber's line (white dotted line, see also Kloepper *et al*, 2010). In two of three experiments, 10 pg ml^{-1} TYL led to a 20% increase of Ki67 + cells. In two experiments conducted with 100 pg ml^{-1}, TYL increased the number of Ki67 + cells. Treatment with 10 and 100 pg ml^{-1} TYL reduced the number of apoptotic cells in the hair matrix. Bar = 50 μm. (**a–d**) Number of independent experiments: 10 pg ml^{-1} TYL and 100 and 1,000 ng ml^{-1} TA1: $n = 3$ (in total, 36 HFs per treatment condition). Number of independent experiments: 100 pg ml^{-1} TYL, 100 and 1,000 ng ml^{-1} TB4: $n = 2$ (in total, 24 HFs per treatment condition).

catagen (Figure 2b and c), yet did not affect the number of Ki67 + hair matrix cells, if only anagen HFs were compared between test and control groups (Supplementary Figure S2 online).

In conclusion, our pilot study presents evidence that selected TPs are produced by human HF epithelium and operate as direct modulators of human HF growth; this is—to our knowledge—previously unreported. In addition, these initial findings in human scalp HFs suggest that selected TPs may be exploited therapeutically for stimulating, and also for inhibiting, human hair growth. Namely, we identify TYL as a clinically interesting candidate hair growth stimulator. The varying response of the limited number of donors from whom HFs were available for study suggests substantial interindividual variations in the response of human HFs to TP stimulation. Although the mechanisms of action through which selected TPs exert their hair growth–modulatory properties remain to be dissected (Bock-Marquette *et al.*, 2004; Mosoian *et al.*, 2010), our data support the idea that the tested TPs are

indeed important regulators of human HF biology (see Supplementary Information S3 online).

CONFLICT OF INTEREST

FPA is the CEO of Immundiagnostik AG, which has a commercial interest in kits for the measurement of TPs. The remaining authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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