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Identification of novel telogen markers underscores that telogen is far from a quiescent hair cycle phase

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To the Editor

In contrast to the dynamic and striking changes in hair follicle (HF) morphology during the anagen and catagen phases of the hair cycle, the telogen follicle appears static. However, it has been argued that the quiescent appearance of telogen HFs is deceptive (Davis 1962, Stenn, Paus 2001, Higgins, Westgate 2009). For example, the expression of some genes clearly peaks during telogen (Greco, Chen et al. 2009). Also, functionally distinct sub-phases of telogen have been identified ("competent" versus "refractory telogen") based on the ability to initiate anagen after plucking (Plikus, Mayer et al. 2008).

To gain further insights into the biological activities of telogen and to further probe the concept that telogen represents an important hair cycle-regulatory phase, we profiled skin mRNA expression patterns in mid (P54) and late (P59) second telogen in C57BL6 male mice with highly synchronized HF cycling. This data was analyzed in combination with previously published hair cycle gene expression profiling data that included an early telogen sample (P44) (Lin, Kumar et al. 2009). Microarray hybridization and data analyses are described in Supplementary Methods online. As RNA was prepared from whole skin, subtle transcriptional changes occurring in a small population of follicular cells may not be detected and opposite changes in different cell populations may cancel each other. Also, skin samples used in our study contain subcutaneous adipose tissue, recently identified as critical for extrinsic modulation of hair follicle behavior (Plikus, Baker et al. 2011, Festa, Fretz et al. 2011).

While many genes reach their highest or lowest expression in telogen skin, we defined the telogen gene expression signature more rigorously as genes with expression 1.5-fold higher

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The authors state no conflict of interest

or lower in each of the three telogen time points compared to all anagen and catagen time points and p-value less than 0.001 (Supplementary Figure S1 online). The 1.5-fold cutoff was chosen to minimize false positivity due to changes in tissue composition. Following these selection criteria, our microarray analysis revealed 425 differentially expressed genes (Supplementary Table S1 online). The 425 genes thus defined reflect the uniqueness of telogen skin, demonstrating that telogen is a highly dynamic hair cycle phase at the level of transcriptional regulation, providing new insights into previously unexplored biological processes that may drive this pseudo-quiescent hair cycle stage. Observed over the whole second hair cycle, the expression of the telogen signature genes fell into three major clusters (Figure 1a), each with unique, significant ($p<0.01$) functional categories (Figure 1b).

Cluster I contains genes upregulated in telogen; the most prominent gene ontology categories in this cluster are proteolysis, rhythmic process, cholesterol metabolic process and actinomyosin structure organization. The proteolysis category contains several proteases functioning in shedding of membrane associated cytokines and in macrophage activation. The latter is consistent with data showing hair cycle related expression of innate immunity modulators (Stenn and Paus 2001). Proteases with enhanced expression in telogen may also play a role in active hair fiber detachment during exogen (Higgins, Westgate et al. 2009).

The identification of “rhythmic process” which includes circadian target genes extends previous findings, showing that prominent circadian clock gene expression is a stable feature throughout the extended second telogen; clock genes are required for the correct timing of the initiation of the second anagen (Lin, Kumar et al. 2009). Overrepresentation of cholesterol metabolic processes is intriguing as pilosebaceous units both synthesize and metabolize steroid hormones such as estrogens, androgens and cortisol, all of which profoundly modulate hair growth. Locally produced steroid hormones like estrogens, which arrest murine HFs in telogen, may play a role in either maintaining telogen or in preparing the HF for anagen activation (Stenn and Paus 2001).

Among the genes in cluster I, the most intriguing example is Adipocyte Enhancer Binding Protein 1 (Aebp1), known to regulate cholesterol efflux and MAPK signaling (Majdalawieh and Ro, 2010), with at least 7-fold higher expression in telogen compared to all other hair cycle stages (Figure 1c). Absent in anagen skin, the Aebp1 protein is expressed most prominently in the lower telogen HF epithelium (bulge, secondary hair germ) pointing to Aebp1 as a potential novel regulator of telogen HFs (Figure 1d); Aebp1 is also expressed in basal epidermal keratinocytes. Another intriguing gene in cluster I is Keratin 24; its prominent upregulation in telogen (Figure 1c) is unexpected as most previously investigated hair keratins show minimal telogen expression. While the Keratin 24 protein is expressed at a low level in anagen VI HFs (Figure 1e), its expression in the infundibulum, isthmus, bulge and secondary hair germ of telogen HF increases dramatically compared to anagen; also epidermal Keratin 24 expression appears to increase during telogen. This telogen-enriched expression pattern suggests novel hair cycle-regulatory functions for this “structural” protein, perhaps comparable to those already identified for K17 (Tong and Coulombe 2006).

Two additional clusters encompass the downregulated telogen signature genes. Cell cycle-regulatory genes comprise the major functional category in Cluster II. Some of the

downregulated genes include key components of G2-M progression: Cdk1, Cdca2, Nek2, Cenpf, Cenpn, and SMC2. Although the cell cycle is essentially shut down in the terminal anagen hair matrix immediately prior to catagen initiation (most cell cycle regulators are already very low in catagen) (Lin, Kumar et al. 2009), it is intriguing to note that G2-M transition seems to be further inhibited in telogen. Possibly, this is due to the arrest of some bulge stem cells in G2, perhaps to enable fast anagen activation (Morris, Liu et al. 2004).

Cluster III contains genes related to general RNA processing and skin morphogenesis. Among genes involved in RNA processing are splicing factors including Ppil1 and Sfrs1, and RNA degradation factors including Eri1. Interestingly, Ppil1 has been shown to promote epithelial cellular proliferation *in vitro* (Obama, Kato et al. 2006). It's downregulation in telogen may relate to its yet unexplored effects on keratinocyte proliferation in anagen. The expression of this gene is highest in anagen, going down more than 2-fold in late catagen. Eri1, expressed most highly in the nucleolus, has an important role in ribosomal RNA processing (Ansel, Pastor et al. 2008). Its reduced telogen expression may relate to overall reduced translational activity in telogen. Sfrs1 is a splicing factor with many diverse RNA targets including mRNAs, miRNAs, snoRNAs and ncRNAs (Sanford, Wang et al. 2009). Downregulation of this gene possibly reflects overall reduction in transcriptional activity in telogen.

Several differentially regulated genes lie outside these functional categories, suggesting additional biological processes in telogen. For example, we show here that Fgf18, a recently identified inhibitor of anagen initiation secreted by K6-positive bulge residents (Hsu, Pasolli et al. 2011), is upregulated in telogen (Supplementary Table S1 online). This is consistent with its role in maintaining bulge quiescence (Hsu, Pasolli et al. 2011). In addition, we show that a member of the beta defensin family, Defb8, is upregulated more than 300-fold during the second telogen (Figure 1c). This suggests novel growth-inhibitory functions of this antimicrobial peptide, and raises the question whether Defb8 participates in the marked signaling shift from a predominantly immunoinhibitory signaling milieu of murine anagen skin to one that greatly facilitates the elicitation of type IV immune responses in telogen skin (Stenn and Paus, 2001).

In the second part of this project, we tested whether whole skin gene expression is dynamic over the second telogen; ANOVA analysis was performed to define genes exhibiting differential expression over the three telogen timepoints (P44, P54, and P59), thus identifying 376 genes (Supplementary Methods, Figure S1 and Table S2 online). These genes fell into six major clusters (Figure 1f) featuring enrichment of specific biological categories (Figure 1g). We validated the expression of three of these genes by qPCR. Placenta specific 9 (cluster 2), a placenta enriched gene with unknown functions in skin, has highest expression in early telogen (Figure 1h). Thrombospondin-2 (cluster 5), an extracellular matrix regulator, has highest expression in mid- and late telogen (Figure 1h). Interestingly, Thrombospondin-1 had already been found to be instrumental for inducing the catagen-associated regression of the HF vasculature, and *Thbs-1* knockout mice showed prolonged anagen duration (Yano, Brown et al. 2003). von Willebrand factor A domain-containing protein 2 (cluster 6), an extracellular matrix associated protein implicated in cell adhesion, has striking up-regulation in mid-telogen (Figure 1h). It is tempting to speculate

that latter may be involved in β1-integrin-dependent signaling through RGD motif containing ligands that keep bulge and SHG stem cells in a state of relative quiescence during telogen (Brakebusch, Grose et al. 2000).

In summary, it is timely to abandon the often reverberated notion that telogen constitutes the HF's "resting stage". By defining a unique gene expression signature for telogen mouse skin, the current work fully supports the concept that, despite its deceptively "quiescent" morphological appearance, telogen represents a biologically, very active phase of HF cycling (Davis 1962, Stenn & Paus 2001, Plikus et al. 2008, Higgins et al. 2009, Greco et al. 2009). More specifically, our data indicate that telogen is characterized by enhanced cholesterol and steroid metabolism, skin immune status, and circadian clock function (Supplementary Figure S2 online). We also identify AEBP1, keratin 24, Thsb-2 and Defb8 as promising new markers and potential regulators of telogen.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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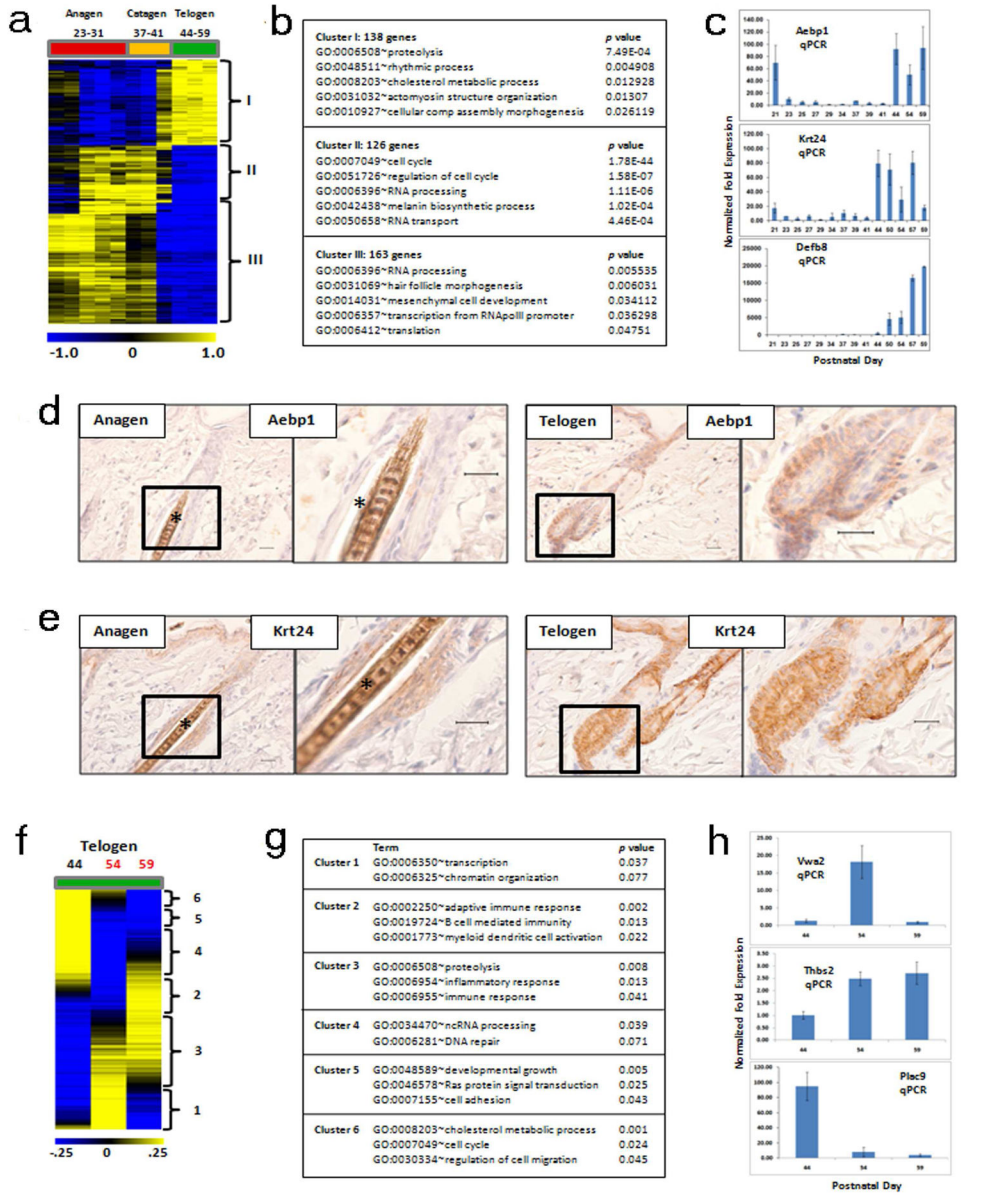

Figure 1. Definition of the telogen gene expression signature and dynamic gene expression across the second telogen

(**a**) Heatmap of the 425-gene telogen expression signature across the hair cycle. Numbers on top, postnatal days. Clusters I, II and III are indicated. (**b**) Significant ($p<0.01$) gene ontology categories for clusters in a. (**c**) qPCR validation of the expression of the indicated genes. (**d**) Immunolocalization of Aebp1 and (**e**) Keratin 24 in skin. Scale bar = 100Mm (**f**) Heatmap of the 376 genes differentially regulated across telogen. Numbers on top represent postnatal days. Clusters 1–6 are indicated. (**g**) Significant ($p<0.01$) gene ontology categories for the clusters in f. (**h**) qPCR validation of the expression of the indicated genes. Y-axes, relative mRNA levels; X-axes, postnatal days; *, Pigmented hair shaft; Aebp1, Adipocyte Enhancer Binding Protein 1; Defb8, Defensin Beta 8; Krt24, Keratin 24; Plac9, Placenta

Specific 9; Thbs2, Thrombospondin 2; Vwa2, von Willebrand factor A domain-containing protein 2.