1 **Full title:** Coordinated periodic expression of the imprinted network genes in the hair follicle growth
2 cycle.

3 **Short title:** Imprinted network genes in hair follicle cycling

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Abstract

Imprinted genes play a critical role in the proliferation and differentiation of embryonic cells and somatic stem cells. They also participate in the development of a wide spectrum of clinical manifestations when they are dysregulated. In this study, we analyzed expression profiles of the network-forming 16 imprinted genes (imprinted gene network: IGN) in three phases of the hair follicle growth cycle by analyzing publicly available datasets deposited in the Gene Expression Omnibus (GEO). We found elevated expression of IGN genes including *H19* in the telogen quiescent phase compared to the anagen proliferative and catagen regression phases in the transcriptomic dataset created from the mouse skin biopsy samples. Our findings suggest a novel role of the 16 IGN genes in the regulation of the hair follicle growth cycle, that manifests possibly through altering the transition between proliferation, quiescence and/or differentiation of the follicular stem cells.

Keywords: follicular stem cells, genomic imprinting, growth cycle, skin hair follicle, telogen phase

Introduction

Genomic imprinting is an epigenetic regulatory mechanism that confers the expression of selected genes from one parental allele, and thus, transfer of its effects to descendants does not follow the classic Mendelian pattern (1). Genomic imprinting is established in the parental germline cells, and is maintained throughout mitotic cell divisions in somatic cells (2). Thus far, 85 and 95 murine imprinted genes are reported in the COXPRESdb and the Gemma database, respectively (1). The process of gene imprinting involves coordinated DNA and histone methylation, whereas the mechanisms underlying its selective targeting to a particular set of genes is largely unknown (3). Altered expression of imprinted genes has been associated with the development of various pathological conditions in humans, including obesity, diabetes mellitus, muscular dystrophy, mental disability and neoplasms (1).

Imprinted genes are composed of functionally distinct members, but most are involved in controlling the transition of cells between their quiescent, proliferation and/or differentiation states (1). These genes cooperatively participate in the regulation of specific biological pathways by forming a gene cluster called the imprinted gene network (IGN), which is further divided into three subgroups based on their functional and regulatory connectivity (1). One of these subgroups is comprised of 16 imprinted genes [namely Cyclin Dependent Kinase Inhibitor 1C (*Cdkn1c*), Decorin (*Dcn*), Delta Like Non-Canonical Notch Ligand 1 (*Dlk1*), Glycine Amidinotransferase (*Gatm*), *GNAS* Complex Locus (*Gnas*), Growth Factor Receptor Bound Protein 10 (*Grb10*), Imprinted Maternally Expressed Transcript (*H19*), Insulin Like Growth Factor 2 (*Igf2*), Insulin Like Growth Factor 2 Receptor (*Igf2r*), Maternally Expressed 3 (*Meg3*), Mesoderm Specific Transcript (*Mest*), Necdin (*Ndn*), Paternally Expressed 3 (*Peg3*), PLAGL1 Like Zinc Finger 1 (*Plagl1/Zac1*), Sarcoglycan Epsilon (*Sgce*), Solute Carrier Family 38 Member 4 (*Slc38a4*)], all of which are known to be involved in the control of embryonic, fetal and/or postnatal growth (3). Their targeted deletion also develops over-growth phenotypes in mice (4). Expression of this subset of 16

imprinted genes (hereafter called the IGN genes) is generally elevated at the transition from cell proliferation to quiescence or differentiation, as observed during the fibroblast cell cycle withdrawal, adipogenesis and muscle regeneration (1). It was shown that the Plag1/Zac1 influences the expression of some member genes (*Igf2*, *H19*, *Cdkn1c* and *Dlk1*) (3), while H19 long non-coding RNA fine-tunes the expression of other genes (4). These pieces of evidence suggest that biological input from upstream signaling pathways to one or some of the network genes triggers coordinated expression of other IGN genes by activating their mutual regulatory cascades, which ultimately influences the transition of cells in which they are expressed from proliferation to quiescence or differentiation state (or *vice versa*) (4).

Hair growth and renewal are organized by the cycling activity of the hair follicles. During their life span, hairs undergo the process of proliferation, degeneration and regeneration in concert with the activation and quiescence of the epidermal stem cells located in the bulge of the hair follicles (5, 6). The cyclical activity of hair follicles is divided into three phases, referred to as the anagen (growth), catagen (regression), and the telogen (resting) phases (7). The follicular stem cells are maintained in a quiescent state during the telogen phase. Once they receive the activating signals from upstream regulatory systems, they initiate a new round of hair growth (anagen phase) (6, 8, 9). After the active growth phase, proliferating matrix cells of the hair follicles are induced to undergo coordinated apoptosis (catagen phase) (7). Following catagen, the hair follicles move eventually to the telogen phase in which hairs are no longer produced due to inactivation of the follicular stem cells (7).

The aim of this study was to examine the involvement of the IGN genes in the process of hair cycling organized in hair follicles by analyzing publicly available data resources. We identified one dataset with transcriptome profiles of the different cycling stages of hair follicles in mouse skin. Using *in silico* data analysis, we found that the IGN genes show characteristic expression patterns with an elevated expression in the telogen phase compared to the anagen phase of the hair follicles. We provide evidence that the IGN genes play important roles in the skin/hair follicle biology in after-birth life in

addition to their well-known activity during embryonic/fetal growth. Our results also suggest the possibility that these genes are involved in certain pathological processes of hair follicles.

Materials and Methods

To identify the publicly available data in which our genes of interest are differentially expressed, a specific query or single keyword (see Results section) was used in the GEO Profiles database, which stores gene expression data derived from the curated GEO DataSets (10). Identified transcriptomic datasets present the expression levels of the selected gene across all samples within the dataset. We then curated all filtered datasets for differential expression of our genes of interest. To compare gene expression profiles in two or more groups of samples in a dataset, the interactive web tool GEO2R was used (11). One-way ANOVA analysis on quantile normalized data with Bonferroni post-testing was used for multiple comparisons, whereas two-tailed t-test was performed on the quantile normalized data for two group comparisons.

Results

Identification of datasets harboring differential expression of H19 in skin

To identify publicly available datasets in which imprinted genes of the IGN could be examined in the skin, we queried for 'H19[*gene symbol*] AND skin' in the NCBI GEO Profiles (10). We selected *H19* as a representative for imprinted genes in this initial search, as it is known to influence the expression of several other imprinted genes in IGN (4). This search revealed 156 datasets, which were manually curated for differential expression of *H19* across all samples within a dataset, based on the visual gene

expression level displayed in the GEO Profiles. Using this strategy, we finally identified one dataset (GSE11186 (12)), which contains transcriptomic profiles of the different stages of the first and the second synchronized natural and a depilation-induced growth cycles of hair follicles from mouse skin biopsies. In this dataset, *H19* expression is significantly elevated in the first telogen phase (day 23) and second telogen phase (day 44) compared to the anagen (day 27) or catagen (days 37 and 39) phases (Fig 1).

Fig 1. Time-course profile of *H19* expression during the synchronized first- and second postnatal hair growth cycle. Values shown are quantile normalized absolute expression data from GSE11186 for telogen (day 23, n=2), mid anagen (day 27, n=3), catagen (day 37 and day 39, n=6) and telogen (day 44, n=3). P-value shown in the upper right corner of the graph was determined by ordinary one-way ANOVA corrected for multiple comparisons using Bonferroni test. n.s.: not statistically significant, *: $p \leq 0.05$, ****: $p \leq 0.0001$

IGN gene expression is elevated during the telogen phase of hair follicle growth cycle

A previous report demonstrated that *H19* forms an IGN together with 15 additional co-regulated imprinted genes (3). Thus, we examined the expression of these 15 IGN genes associated with *H19* in the identified dataset. Using GEO2R, we identified differential expression of the 16 imprinted genes (*Cdkn1c*, *Dcn*, *Dlk1*, *Gatm*, *Gnas*, *Grb10*, *H19*, *Igf2*, *Igf2r*, *Meg3*, *Mest*, *Ndn*, *Peg3*, *Plagl1*, *Sgce*, *Slc38a4*) across all experimental conditions in the dataset by comparing the quantile normalized data of GSE11186. Notably, we observed a significant difference in the mean expression ratio of all 16 IGN member genes when comparing telogen phase (day 23) over catagen phase (day 37 and day 39), which was not the case in the comparison of the mid anagen phase (day 27) over the catagen phase (day 37 and day 39) (Fig 2A).

Fig 2. Expression of all 16 IGN genes in the telogen and the anagen phases. (A) The fold expression values of all pooled 16 IGN genes identified in the telogen phase (T, day 23) and the anagen phase (A, day 27) were normalized to those of the catagen phase (C, day 37 and day 39) in the dataset GSE11186. T/C- and A/C values that correspond to the same gene are connected with a line. The A/C fold value is smaller than the T/C fold value for all IGN genes except for *Dcn* and *Igf2r* (marked in red). Statistical comparison of T/C- and A/C values was made using a paired Student t test, **: $p < 0.001$. (B) Dot plot showing the absolute expression (after quantile normalization) of the IGN genes and control genes in telogen (day 23) and anagen (day 27) from GSE11186. Each dot (telogen: blue and anagen: red) corresponds to a subject from dataset GSE11186. IGN genes tend to be higher expressed in telogen compared to anagen. Known telogen activated genes (*Ar*, *Esr1*, *Lhx2*, *Nr1d1*, *Sox18*, *Stat3*) and telogen repressed genes (*Elf5*, *Foxn1*, *Grhl1*, *Lef1*, *Msx2*, *Vdr*) (12) were used as controls.

Next, we examined the absolute expression levels of each IGN gene separately in telogen (day 23) and mid anagen (day 27) from the synchronized second postnatal hair growth cycle from dataset GSE11186. For comparison, we also examined the absolute expression of six known telogen-activated genes (*Ar*, *Esr1*, *Lhx2*, *Nr1d1*, *Sox18*, *Stat3*), and six known telogen-repressed genes (*Elf5*, *Foxn1*, *Grhl1*, *Lef1*, *Msx2*, *Vdr*) (12). Again, we observed that IGN gene expression was elevated in telogen compared to mid anagen, with some of the IGN genes (i.e. *H19*, *Gnas*, *Meg3*) even expressed at higher levels than the known telogen-activated genes (Fig 2B).

Most IGN genes are periodically expressed, thus they are considered as hair cycle-regulated genes

To assess whether the IGN genes are hair cycle-regulated, we took advantage of a public available dataset that was obtained after processing mRNA microarray data from mouse skin at eight time points

corresponding to the first synchronously (days 1, 6 and 14: anagen, day 17: catagen, day 23: telogen) and the asynchronously (9th week, 5th month, 1st year) postnatal hair cycling, with the latter three time point samples containing skin patches in different phases of the hair-growth cycle (13). After excluding genes that were not expressed in the mouse skin and applying a computational approach including replicate variance analysis (F test), Lin *et al.* identified a dataset of 2,461 probe sets corresponding to 2,289 potential hair cycle-associated genes (hereafter called 'Lin1-dataset') (13). The P -value cut-off for the F test was set previously to 0.05, as it was found that > 80% of known hair cycle-dependent expressed genes had a P -value of < 0.05 using this computational approach (13). As the pool of these 2,289 hair cycle-associated genes are restricted to protein-coding genes, we included only the 14 protein-coding IGN genes (*Igf2*, *Cdkn1c*, *Dcn*, *Dlk1*, *Gatm*, *Gnas*, *Grb10*, *Igf2r*, *Ndn*, *Mest*, *Peg3*, *Plagl1*, *Sgce* and *Slc38a4*) in our analysis and excluded the two non-coding RNA IGN genes (*H19* and *Meg3*). We identified ten IGN genes (*Cdkn1c*, *Dcn*, *Dlk1*, *Gatm*, *Gnas*, *Igf2r*, *Ndn*, *Peg3*, *Sgce*, *Slc38a4*), corresponding to 71% of all protein-coding IGN genes, among the pool of 2,461 probe sets categorized as periodically expressed, hair cycle-regulated genes in the mouse dorsal skin (Fig 3A). The Lin1-dataset had been previously characterized by cluster analysis and was divided into three general profile patterns ('anti-hair growth', 'hair growth', and 'catagen-related' pattern), and further subdivided into 30 clusters of co-expressed genes which differed by their expression peaks at different stages of the hair growth cycle (13). Seven of our identified hair cycle-associated IGN genes (*Cdkn1c*, *Dlk1*, *Gnas*, *Peg3*, *Gatm*, *Ndn*, *Slc38a4*) fell into the 'anti-hair growth' category and showed a decline in expression levels during anagen. One IGN gene (*Igf2r*) fell into the 'hair growth' pattern with peak expression at early anagen. On the contrary, *Sgce* was categorized as 'catagen-related' gene with a drop in expression level at catagen and finally, one IGN gene (*Dcn*) belongs to a gene cluster that does not fall into the three main profile patterns (Table 1).

Fig 3. Most GN genes are periodically expressed, hair-cycle regulated genes. (A) Venn Diagram

illustrating that 71% of protein coding IGN genes (10 out of 14) are hair cycle-regulated. The ten protein coding-, hair cycle-regulated IGN genes identified are *Cdkn1c*, *Dcn*, *Dlk1*, *Gatm*, *Gnas*, *Igf2r*, *Ndn*, *Peg3*, *Sgce*, and *Slc38a4*. **(B)** Time course profiles of hair cycle regulated IGN genes during hair follicle cycling. Shown are the normalized expression levels of eight hair cycle regulated IGN genes (*Igf2*, *Cdkn1c*, *Dcn*, *Dlk1*, *Gnas*, *Mest*, *Peg3*, *Plagl1*) in red, eleven control genes (telogen upregulated genes (12): *Dbp*, *Tef*, *Nr1d1*, *Per1*, *Per2*, and anagen/catagen upregulated genes (12): *Dlx3*, *Elf5*, *Foxn1*, *Foxq1*, *Hoxc13*, and *Ovol1*) in black. Normalized gene expression values are provided in supplemental table S1 from (12). **(C)** Time-course profile of *Meg3* expression during the synchronized first- and second postnatal hair growth cycle. Values shown are quantile normalized absolute expression data from GSE11186 for telogen (day 23, n=2), mid anagen (day 27, n=3), catagen (day 37 and day 39, n=6) and telogen (day 44, n=3). P-value shown in the upper right corner of the graph was determined by ordinary one-way ANOVA corrected for multiple comparisons using Bonferroni test. n.s.: not statistically significant, *: $p \leq 0.05$, ****: $p \leq 0.0001$.

Table 1. Potential hair cycle-regulated IGN genes (identified in Lin1-dataset)

Gene Symbol	Gene name	P-value*	cluster description*
<i>Cdkn1c</i> **	cyclin-dependent kinase inhibitor 1C (P57)	0.0004	anti-hair growth pattern with decline expression level during anagen
<i>Dcn</i> **	Decorin	0.0392	does not fall into any of the three main profile patterns
<i>Dlk1</i> **	delta-like 1 homolog (Drosophila)	0.0166	anti-hair growth pattern with decline expression level during anagen
<i>Gatm</i>	glycine amidinotransferase (L-arginine:glycine amidinotransferase)	0.0009	anti-hair growth pattern with decline expression level during anagen
<i>Gnas</i> **	GNAS (guanine nucleotide binding protein, alpha stimulating) complex locus	0.0040	anti-hair growth pattern with decline expression level during anagen
<i>Igf2R</i>	insulin-like growth factor 2 receptor	0.0004	hair growth pattern including genes that peak at early anagen
<i>Ndn</i>	necdin	0.0171	anti-hair growth pattern with decline expression level during anagen
<i>Peg3</i> **	paternally expressed 3	0.0001	anti-hair growth pattern with decline expression level during anagen

<i>Sgce</i>	sarcoglycan, epsilon	0.0117	catagen-related expression patterns with drop in expression level at catagen
<i>Slc38a4</i>	solute carrier family 38, member 4	0.0013	anti-hair growth pattern with decline expression level during anagen

186 * Defined by Lin *et al.* (13)

187 ** Gene was independently identified as potential hair cycle-regulated gene in Lin2-dataset (see Table 2)
188

189 Next, we analyzed an independent, published dataset (hereafter called ‘Lin2-dataset’) comprising a set
190 of previously identified 6,393 mRNA probe sets (corresponding to 4,704 genes) (12). This pool of 4,704
191 genes was identified by Lin *et al.* by processing expression data obtained from mRNA profiles of the
192 mouse dorsal skin at multiple time points during: 1) the postnatal completion of hair follicle
193 morphogenesis, including the first catagen and telogen; 2) the synchronized second postnatal hair
194 growth cycle; and 3) a depilation-induced hair growth cycle (12). By applying a matrix model, 8433
195 periodically expressed probe sets (6,010 genes) were identified, of which 2,040 probe sets (1,306 genes)
196 were excluded from this subset since these genes changed their expression due to the cell-type
197 composition that occur in the skin during the hair follicle cycling (such as cornified cells, suprabasal cells,
198 mesenchymal cells and myocytes) (12). The final set of 6,393 probe sets (4,704 genes, Lin2-dataset)
199 exhibited periodic expression patterns that cannot be explained by cell-type specific alterations of the
200 skin that occur during the hair follicle cycling and were thus defined as hair cycle-regulated genes (12).
201 Similar to the Lin1-dataset, this set of 4,704 hair cycle-regulated genes are restricted to protein-coding
202 genes. Thus, we included only the 14 protein-coding IGN genes in our analysis, excluding the two non-
203 coding RNA IGN genes. We identified eight IGN genes (*Igf2*, *Cdkn1c*, *Dcn*, *Dlk1*, *Gnas*, *Mest*, *Peg3*, *Plagl1*)
204 (corresponding to 57% of all protein-coding IGN genes) among the pool of 6,347 probe sets of the Lin2-
205 dataset that were categorized as periodically expressed, hair cycle-regulated genes in the mouse dorsal
206 skin (Table 2) (12). 3,180 genes from the Lin2-dataset were grouped earlier according to their
207 expression peak during the hair growth cycle with 1,169 genes in early anagen, 1,017 genes in mid

anagen, 243 genes in late anagen, 208 genes in early catagen, 253 genes in mid catagen and 290 genes in telogen (12). The eight IGN genes identified in this study being among the hair cycle-regulated genes of the Lin2-dataset were categorized as genes with expression peak in telogen (*Dcn*, *Gnas*) and early anagen (*Igf2*, *Cdkn1c*, *Dlk1*, *Mest*, *Peg3*, *PlagL1*) (Table 2). Furthermore, we examined the gene expression levels of the eight hair cycle-regulated IGN genes (listed in table 2) during the nine time points provided in the 'Lin2-dataset' (12). The gene expression levels of the eight hair cycle-regulated IGN genes show elevated gene expression profiles in telogen and early anagen compared to mid/late anagen and catagen (Fig 3B), similar to *Dbp*, *Nr1d1*, *Per1*, *Per2*, and *Tef* which were shown earlier to have prominent expression during telogen (12). In contrary, their expression differs from *Dlx3*, *Elf5*, *Foxn1*, *Foxq1*, *Hoxc13*, and *Ovol1*, a group of transcriptional regulators that have an expression peak from mid anagen to late catagen (12).

Table 2. Potential hair cycle-regulated IGN genes (identified in Lin2-dataset)

Gene Symbol	Gene name	cluster description*
<i>Cdkn1c</i> **	cyclin-dependent kinase inhibitor 1C (P57)	Expression peaks at early anagen
<i>Dcn</i> **	Decorin	Expression peaks at telogen
<i>Dlk1</i> **	delta-like 1 homolog (Drosophila)	Expression peaks at early anagen
<i>Gnas</i> **	GNAS (guanine nucleotide binding protein, alpha stimulating) complex locus	Expression peaks at telogen
<i>Igf2</i>	insulin like growth factor 2	Expression peaks at early anagen
<i>Mest</i>	mesoderm specific transcript	Expression peaks at early anagen
<i>Peg3</i> **	paternally expressed 3	Expression peaks at early anagen
<i>Plag1</i>	PLAG1 like zinc finger 1	Expression peaks at early anagen

* Defined by Lin *et al.* (12)

** Gene was also identified as potential hair cycle-regulated gene in Lin1-dataset (see Table 1)

Finally, as non-coding RNA genes in general were not included in the Lin1- and Lin2-datasets, we established the time course profile of *Meg3* expression during telogen (day 23), mid anagen (day 27), catagen (day 37 and day 39) as well as telogen (day 44) with expression values provided in dataset GSE11186 (12). In this analysis we identified a periodic expression pattern of the non-coding RNA *Meg3*

during the hair cycle with significantly reduced expression levels during mid anagen and catagen (Fig 3C), similar as we showed in this study for the non-coding IGN gene H19 (Fig 1).

Discussion

The 16 IGN genes examined in this study are known to play important roles in the embryonic development (3, 14). However, involvement of this particular set of imprinted genes in other biological processes and regulatory mechanisms associated with these genes were largely unknown. Here we report a coordinated elevation in the telogen and early anagen phase compared to the mid anagen phase of the hair follicle growth cycle. Thus, the majority of the protein-coding IGN genes is considered as the hair cycle-regulated genes. Hair cycle-associated changes in the expression of members of the IGN genes have not been previously described. Thus, our results emphasize the importance of these imprinted genes for the transition from cell quiescence (telogen phase) to proliferation (anagen phase) possibly by acting as negative regulatory factors, and further suggest their vital roles in skin/hair homeostasis (15). This is consistent with previous reports indicating the involvement of IGN genes in muscle regeneration and haematopoietic stem cell biology (3, 14).

The IGN genes we examined in this study are downregulated postnatally, but are continuously expressed in pluripotent stem cells and/or progenitor cells of the hematopoietic system, skin and skeletal muscles with a significant lower expression levels in their differentiated progeny (15). Somatic stem cells, such as haematopoietic stem cells, epidermal stem cells and satellite cells of the skeletal muscles are generally considered to be quiescent, dividing infrequently, but are driven into active proliferation/differentiation cycles upon tissue regeneration or for self-renewal (15). Our results suggest a periodic expression cycle of the IGN genes during the follicular growth cycle with zenith in the telogen

and early anagen phase and nadir in the mid anagen and catagen phase, which is in agreement with the consensus that hair follicle stem cells receive activating or inhibitory signals at distinct stages of the hair growth cycle, allowing them to either remain quiescent or become proliferative (16). During the transition from the telogen to the anagen phase, biological signals from the dermal papilla stimulate the quiescent follicular stem cells to proliferate (17). At the same time, melanocytes are also activated, supplying multiplied progeny to the hair matrix, where most of them mature into differentiated melanocytes (18, 19). As most of the IGN genes are known as tumor suppressors, it is likely that the decrease in the expression of these IGN genes from the telogen to the anagen phase triggers the biological cascades stimulating cell proliferation (20-30). Further, the dysregulation of IGN genes might participate in the autonomic proliferation of their transformed cells observed in malignant melanoma, and might be involved in certain congenital syndromes characterized by impaired hair growth cycles such as the short anagen hair syndrome (31), or the synchronized pattern of scalp hair growth (32).

Acknowledgements

The authors would like to thank Dr. N. Marr for critically reading the manuscript.

Data availability

The datasets underlying the results are available in the NCBI GEO DataSets (GSE11186) at ncbi.nlm.nih.gov/gds/ as well as in the supplemental material of (12) and of (13).

Competing interests

No competing interests were disclosed.

Grant information

The study was supported by Sidra Medicine Internal Research Fund to TK. The authors declared that no grants were involved in supporting this work.

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Figure 1

A**GSE11186****Fold regulation of pooled IGN** **B** **Figure 2**

Figure 3