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Long noncoding RNA expression in dermal papilla cells contributes to hairy gene regulation

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ABSTRACT

Dermal papilla (DP) cells may be the source of dermal-derived signaling molecules involved in hair-follicle development and postnatal hair cycling. Early-passage DP cells can induce hair growth in vivo, but, on further culture, this ability is lost. The cellular mechanisms underlying the hair-follicle induction property of early-passage DP cells are unclear. Long noncoding RNAs (lncRNAs) are an important class of genes involved in various biological functions. They are aberrantly expressed and play roles in the regulation of the Wnt signaling pathway, a critical point in maintaining hair-induction activity. LncRNA microarray revealed 1683 upregulated and 1773 downregulated lncRNAs in passage-4 DP cells compare with passage-10 DP cells. To investigate the relation between lncRNAs and coding genes in WNT signaling, we constructed a coding–noncoding gene co-expression network using lncRNAs and coding genes that were differentially expressed between the passage-4 and -10 DP cells. RP11-766N7.3, H19 and HOTAIR are specific lncRNAs that were aberrantly expressed in DP cells and played an important role in regulating Wnt signaling. This study may provide potential targets for discovering the hair-follicle induction mechanism of early-passage DP cells.

1. Introduction

Dermal papilla (DP) cells are specialized mesenchymal cells located in the base of hair follicles [1]. They control hair follicle development and the postnatal hair cycle as well as regeneration of hair follicles. The hair-follicle induction property of DP cells has been clearly demonstrated [2–5].

In 1986, Oliver and colleagues found that early-passage DP cells have aggregative behavior and can induce hair growth in vivo, but, on further culture, this ability is lost [3,6,7]. Versican and alkaline phosphatase are typical indicators of hair inductivity of DP cells [6,8,9]; however, the expression of these markers is diminished with increasing of DP cell passage [8,10]. Various approaches to maintaining in vivo characteristics in cultured DP cells, included co-culture with keratinocytes [11] and sphere formation [12]. Alternatively, Wnt3a or Bmp6 mixed into the culture medium helped DP cells could maintain hair-follicle induction ability even in later passages [6]. However, little is known about the significance of the specific regulatory factors in DP cells in terms of hair-follicle induction ability [7].

Many studies have attempted to identify the DP cell signature genes associated with hair induction [13,14]. Renda et al. identified 8 receptors and 8 ligands, including Wnt5a, Wif1, and bone morphogenic proteins (BMPs), expressed specifically in DP cells [10]. Ohyama and colleagues found high expression of Wnt, BMP, and fibroblast growth factor (FGF) pathway members expressed in fresh DP by bioinformatic analysis [6]. Researchers have investigated the secreted proteins involving hair induction of DP cells. More than 70 proteins related to the aggressive behavior of DP cells, include fibronectin and thrombospondin 1 [15,16]. Synthesizing proteins undergo a series of complicated processes, including transcriptional and posttranscriptional regulation, so discovering the critical factors of the hair follicle induction ability of DP cells is a challenge.

Long noncoding RNAs (lncRNAs) (200 nt) have only recently emerged as a major class of eukaryotic transcripts. LncRNAs may regulate protein-coding gene expression at both posttranscriptional and transcriptional levels [17,18]. The mechanisms mediated by lncRNAs include chromatin modification, genomic imprinting, and functioning as molecular cargos to target protein

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subcellular localization [19,20]. LncRNAs have been implicated in various important biological processes: cell differentiation and developmental patterning, translation regulation, diseases, subcellular localization and cellular structural maintenance [19,21].

In recent years, several classes of lncRNAs have been discovered, for tremendous impact on genome regulation and revealing the complexity of protein post-translational modifications in Wnt/β-catenin signaling. Many lncRNAs regulating Wnt/β-catenin signaling include mrhl-RNA [22], LALR1 [23], HOTAIR [24], H19 [25] and UCA1 [26]. However the roles of lncRNAs in regulating hair-follicle reconstruction are unknown. Whether lncRNAs control the Wnt pathway, then regulate the development and function of DP cells remains unclear.

In this study, we profiled the expression of lncRNAs and mRNAs in 6 DP cells samples. LncRNA and mRNA expression profiles differed significantly between early- and late-passage DP cells. We also found some key lncRNAs with induction properties, and investigated the relationship between lncRNAs and Wnt signaling.

2. Materials and methods

2.1. Animals

NU/NU mice (6 weeks old) were obtained from the Center of Research of Animals, Shantou University Medical College. All experiments were approved by the Ethics Committee on Research Animal Care at Shantou University Medical College.

2.2. Isolation and cultivation of DP cells

Intact DP was obtained from human scalp follicles as described [27]. Briefly, the connective tissue was sliced with use of scissors until the hair-bulb regions were visible, then pulped, mixed with collagenase I (2 mg/ml, Gibco) and incubated at 37 °C for 2–3 h. The mixture was sucked into a pipette to free most of the DP, which was then collected by use of a pipette gun under a binocular dissecting microscope. The DP was cultured in Dulbecco's modified Eagle's medium (Gibco) at 37 °C in a humidified atmosphere containing 5% CO_2. DP cells were cultivated continuously (we used passage-4 and -10 DP cells only).

2.3. Function study of different passage DP cells in animals

NU/NU mice ($n = 24$) were divided into 2 groups: the experimental group was injected with passage-4 DP cells, and the control group with passage-10 DP cells. About 8×10^5 DP cells with 0.8 ml DMEM containing 10% fetal serum were subcutaneously injected into each mice dorsum. Mice were killed after 1 week. The implantation sites were biopsied for histology.

2.4. Histology

Specimens were treated with 4% paraformaldehyde, dehydrated through a graded series of ethanol, washed with xylene, and embedded in paraffin wax. Treated specimens were cut into 4 μm sections and stained with hematoxylin and eosin for routine histology evaluation.

2.5. Transmission electron micrography

Biopsy specimens were fixed with glutaraldehyde and postfixed with OsO_4, dehydrated by ethanol and placed in a 1:1mixtur of absolute acetone, then final Spurr-resin mixture for 1 h at room temperature, then transferred to absolute acetone and the final resin mixture and to final Spurr resin mixture for overnight. Sections were stained with uranyl acetate and lead citrate and observed in by transmission electron microscopy (TEM) with a JEM1400 microscope (JEOL, Japan).

2.6. RNA extraction, microarray analysis and data analysis

Total RNA of DP cells was isolated by using TRIzol (Invitrogen) and the RNeasy mini kit (QIAGEN). RNA quality and quantity was measured by use of a nanodrop spectrophotometer (ND-1000, Nanodrop Technologies) and RNA Integrity was determined by gel electrophoresis.

Arraystar Human lncRNA Microarray V3.0 contained about 30,586 lncRNAs and 26,109 coding transcripts was used for global profiling of human lncRNAs and protein-coding transcripts.

After slides were washed, arrays were scanned by the Agilent Scanner G2505. Data were extracted by use of Agilent Feature Extraction software (v11.0.1.1). Quantile normalization and data processing involved use of GeneSpring GX v11.5.1 (Agilent Technologies). The analysis was performed by KangChen Biotech (Shanghai).

2.7. RT-PCR

The RNA from the samples was reversed transcribed by use of PrimeScript RT reagent Kit with gDNA Eraser (Perfect Real Time) (TaKaRa, Dalian, China). We randomly selected 4 lncRNAs (NR_038905, ENST00000436097, ENST00000577198 and ENST00000413768) that were upregulated and 3 lncRNAs (ENST00000540024, uc001uih.2 and ENST00000488192) that were downregulated in all samples to validate the microarray data by real-time PCR with SYBR Green assay (PCR master mix, Superarray). GAPDH expression was an internal control. We also selected some mRNAs (FGFR I, FGFR II, BMP4, WIF1 and FZD8) for validation. The primers are in Supplementary Table 1. The expression of each lncRNA was represented as fold change by the $2^{-\triangle\triangle Ct}$ method.

2.8. Bioinformatics analysis of differentially expressed gene profiles

We used gene ontology (GO) analysis associating differentially expressed mRNAs with GO function categories (http://www.geneontology.org). With the latest Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg) database, we analyzed the pathway for differentially expressed mRNAs.

2.9. Construction of the coding non-coding (CNC) gene co-expression network

To investigate the relation between lncRNAs and coding genes in Wnt signaling, we constructed a CNC network using lncRNAs and their coding genes that were differentially expressed between the early- and late-passage DP cells as previously described [28]. We selected correlation coefficient 0.9953 as a threshold and drew the co-expression network using Cytoscape.

2.10. Statistical analysis

Differential expression was defined by fold change up- or downregulated expression (>2.0, $P < 0.05$). Data are mean ± SD and were analyzed by the two-tailed Student's t test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Cell morphology and functional study of passage-4 and -10 DP cells

Isolated DPs (Fig. 1A) were purified; a large number of cells migrated from the collagenase I-treated DP, and were loosely shaped after 7–9 days. These cells displayed a flattened, polygonal morphology (Fig. 1C). TEM of isolated DP confirmed the retention of an intact basal lamina that was multi-laminated in places (Fig. 1B). With increasing numbers, cells associated into multi-layered aggregates and clumps. Furthermore, some cell aggregations looked like an intact DP. As was reported, only early-passage DP cells showed aggregative behavior. The early-passage DP cells resembled fibroblasts and generally exhibited a multilayer aggregative behavior in culture, forming small clumps of cells overlapping each other (Fig. 1D). However, late-passage (up to 6 passages) DP cells greatly different from early-passage DP cells and were large and plump, and lost the aggregative behavior and proliferative capacity.

To elucidate the role of follicle-inducing ability by early- and late-passage DP cells, we subcutaneously injected passage-4 cells (DP4) and passage-10 cells (DP10) into the mice's back (Fig. 1E). For DP4 cells, white hair coat emerged about 5 days after injection. Until 7 days, the fibers grew to 1 cm, followed by hair loss after 3 or 4 days. In contrast, control mice backs were still hairless. Skin histology revealed hair follicle structures DP4-cell-injected in mice compared with controls.

3.2. Global profiling of lncRNAs and mRNAs in different passage DP cells

We determined the globally expressed lncRNAs of RefSeq_NR, UCSC_knowngene, Ensembl, H-invDB, Fantom, Fantom_stringent, NRED, RNAdb, misc_lncRNA, UCR, and lincRNA. In the lncRNA expression profiling data, we found 14,211 lncRNAs (Supplementary Table 2) and 20,436 coding genes (Supplementary Table 3) expressed in passage 4 and passage 10 DP cells. Scatter plot was used to assess the variation in expression of lncRNAs and mRNAs in passage 4 and passage 10 DP cells (Fig. 2A and B). To identify significant differentially expressed lncRNAs and coding genes, we performed volcano plot filtering for the two group (Fig. 2C and D).

3.3. Validation of microarray data by RT-PCR

To validate the microarray assay findings, we examined a random selection of 8 lncRNAs. Consistent with microarray data, RT-PCR results showed NR_038905, ENST00000436097, ENST00000577198 and ENST00000413768 upregulated and ENST00000540024, uc001uih.2, ENST00000488192 downregulated in DP4 cells compared with DP10 cells ($P < 0.05$, Fig. 2E).

3.4. Differentially expressed lncRNAs and mRNAs

From the lncRNA microarray data, we compared the lncRNA expression level between DP4 and DP10 cells, and identified 1773 upregulated and 1638 downregulated lncRNAs that were

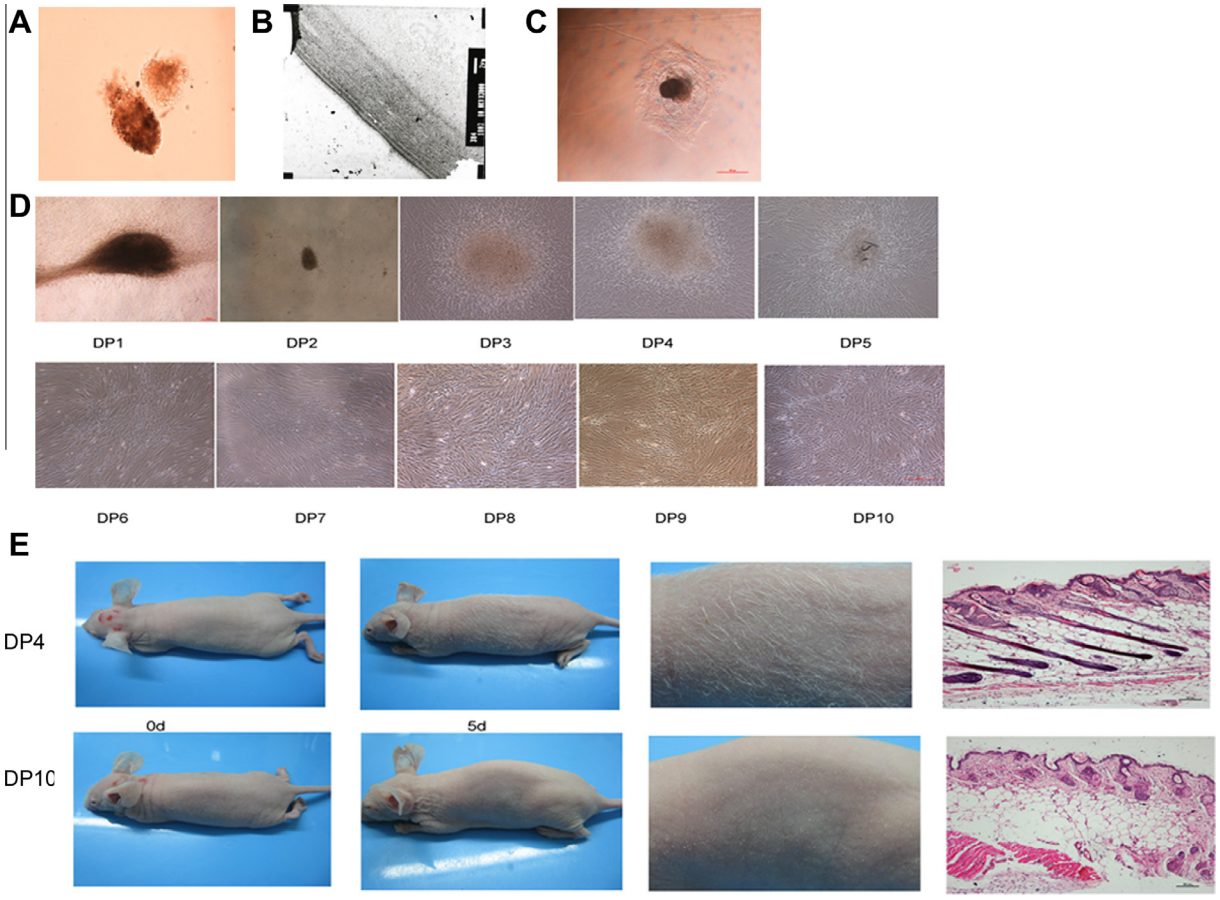

Fig. 1. Different hair follicle inductivity between early- and late- passage dermal papilla (DP) cells. (A) Isolated DP. (B) Transmission electron micrograph of an isolated DP showing no epithelial cell contamination, and retention of an intact basal lamina. (Bar = 2 μm). (C) DP cells migrated from collagenase I-treated DP at about 1 week. (Bar = 500 μm) (D) DP cells gradually lost aggregative behavior from early to late passages. (E) Early passage DP cells (DP4) induced hair generation on the mice backs after 5 days, while late-passage DP cells (DP10) lost the hair induction ability. Skin histology revealed hair-follicle structures in mice injected withDP4 cells compared with the mice injected with DP10 cells.

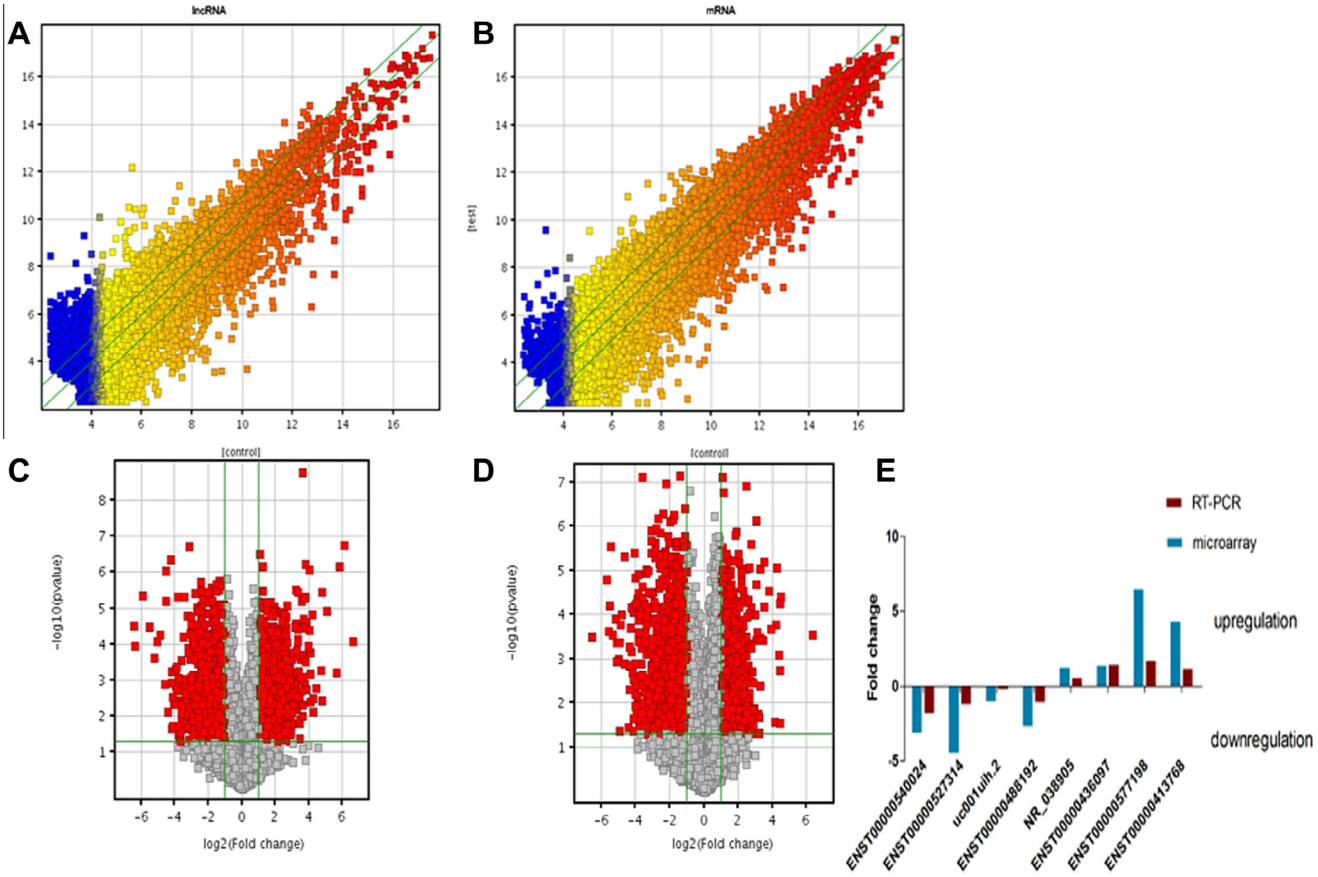

Fig. 2. Global profiling of lncRNAs and mRNAs in DP cells at different passages. Scatter-plot of variation in expression of long non-coding RNAs (lncRNAs) (A) and mRNAs (B) between early- and late-passage DP cells. *X* and *Y* axes are the mean normalized signal values (log2 scaled). Green lines are fold change in expression (default fold change is 2.0). Volcana plots was used to visualize differential expression lncRNAs (C) and mRNAs (D) between DP4 and DP10 cells. The vertical lines correspond to 2.0-fold up- and downregulation and the horizontal line represents $P = 0.05$. Red dots represent lncRNAs and mRNAs with significant differential expression. (E) Microarray data were further confirmed by RT-PCR. LncRNA expression was normalized to that of GAPDH. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

significantly differentially expressed (Supplementary Table 4). We found 1703 upregulated and 2020 downregulated mRNAs differentially expressed in the 2 cells groups (Supplementary Table 5). Hierarchical clustering analysis revealed distinct lncRNA and mRNA expression profiling among samples (Fig. 3A and B). RT-PCR was used to analyzed the expression of some genes (FGFR I, FGFR II, BMP4, HHIP, WIF1 and FZD8) which related to hair induction deregulation. The results are consistent with the microarray data. In DP4 cells, BMP4, FGFR I, WIF1 and FZD8 were downregulated (Fig. 3C–F) and FGFRII was upregulated (Fig. 3G).

3.5. lncRNA classification and subgroup analysis

According to their genomic relationship with coding genes, differentially expressed lncRNAs were grouped into intergenic, intronic antisense, intron sense-overlapping, exon sense-overlapping, natural antisense, bidirectional. LncRNA subgroups was shown in Fig. 3H.

Recent focus has been on long intervening noncoding RNAs (lincRNAs, also called long "intergenic" ncRNAs, even though the lincRNAs derive from genes and are thus genic), which do not overlap with exons of protein-coding or other non-lincRNA types of genes. We provided the lincRNAs calculated by genomic coordinates, and differentially expressed, with lincRNAs and nearby coding gene pairs (distance < 300 kb) (Supplementary Table 6).

The profiling data for all probes targeting lncRNAs and coding transcripts in the 4 human HOX loci are in Supplementary Table 7. We found about 79 lncRNAs transcribed; 29 were found differently expressed in the human HOX loci.

In this study, lncRNAs with enhancer-like function excluded transcripts mapping to the exons and introns of annotated protein coding genes, the natural antisense transcripts, overlapping with protein coding genes and all known transcripts. The profiling data for all probes with enhancer-like lncRNAs revealed 1225 lncRNAs (Supplementary Table 8); 380 were differentially expressed. Previous study showed that activated lncRNAs feature cis-mediated function. We also provide differentially expressed enhancer-like lncRNAs and nearby coding gene pairs (distance < 300 kb) (Supplementary Table 9).

3.6. GO analysis and pathway analysis

The highest enriched GO terms for upregulated transcripts were cellular macromolecule metabolic process (biological process), intracellular (cellular component) and nucleic acid binding (molecular function) (Supplementary Table 10) and the highest enriched GO terms for downregulated transcripts were multicellular organismal process (biological process), cell periphery (cellular component) and receptor binding (molecular function) (Supplementary Table 10). According to BP classification, the highest enriched GO terms for upregulated transcripts were metabolic process, cell cycle, biosynthetic process and for downregulated mRNAs were cell differentiation, developmental process and signaling, which was fairly consistent with the Sleeman et al.' study [29].

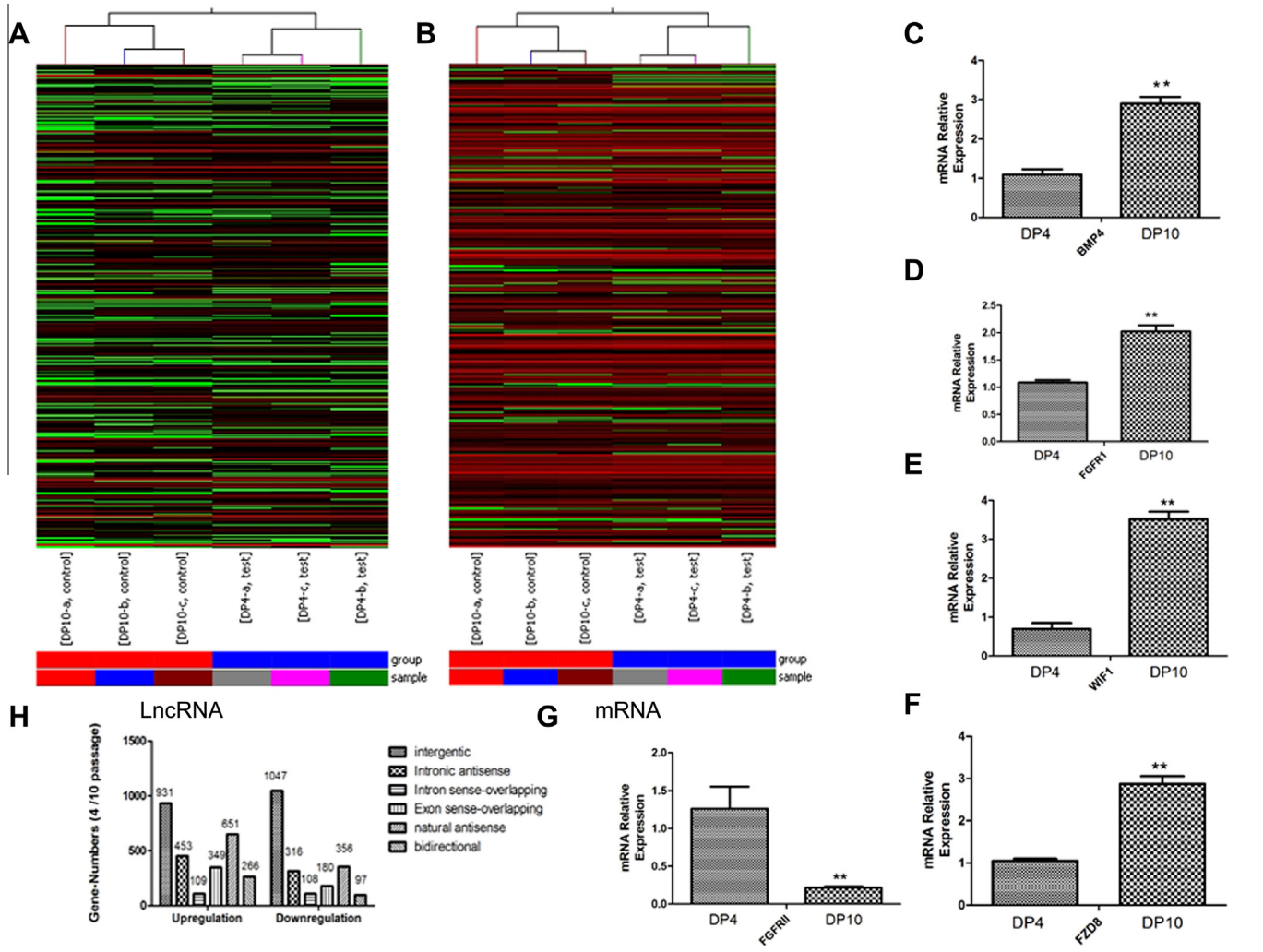

Fig. 3. Differentially expressed lncRNAs and mRNAs. Heat map presentation of the expression profile of lncRNAs (A) and coding genes (B). Each column represents a sample and each row a gene. High relative expression is indicated by red and low relative expression by green. RT-PCR analysis of the expression of DP-cell signature genes in passaged-4 and -10 DP cells: BMP4, FGFR I, WIF1 and FZD8 downregulated (C–F), and FGFR II was expressed upregulated (G). ($^{**}P < 0.01$, $^{*}P < 0.05$). (H) Categorization of differentially expressed lncRNAs in early- and late-passage DP cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Pathway analysis revealed that 14 pathways corresponding to upregulated transcripts; the most enriched network was "ribosome-Homo sapiens (human)" with 135 targeted genes. In total, 34 pathways corresponded to downregulated transcripts, and the most enriched network was "insulin secretion-Homo sapiens (human)," with 87 targeted genes (Supplementary Table 11).

3.7. Construction of the CNC gene co-expression network with Wnt signaling

To investigate the relation between lncRNAs and their coding genes in Wnt signaling, we constructed a CNC network based on correlation between the differentially expressed lncRNAs and mRNAs involved in Wnt signaling. The CNC network node consisted of 469 lncRNAs and 17 mRNAs, for 601 network pairs of co-expressing lncRNAs and mRNAs. Many pairs presented positive correlation (Supplementary Table 12). The CNC network showed that one mRNA could correlate with 1–20 of lncRNAs and so were the lncRNAs. The fold change of differentially expressed lncRNAs ranged from 83-fold (ENST00000458519) to 2-fold. The fold change of differentially expressed lncRNAs with fold change > 10-fold are listed in Supplementary Table 12.

4. Discussion

In the present study, we detected 3456 lncRNAs and 3723 mRNAs differentially expressed in DP4 and DP10 cells by lncRNA microarray. A random small number of lncRNAs and mRNAs were validated by quantitative RT-PCR. To identify lncRNAs and their corresponding genes, lncRNAs were grouped into 6 categories. Recent focus has been on lincRNAs. In this study, lincRNAs accounted for the highest proportion of lncRNAs. LincRNA research is at an interesting juncture—thousands of lincRNA genes have been identified, and the diverse functional and mechanistic underpinnings of a few well-studied examples suggest that many of these might participate in important and diverse aspects of biology [20].

The activity of Wnt pathway is critical in maintaining the hair-follicle induction ability. Considering the important biological role of lncRNAs, we wondered whether lncRNAs regulate Wnt signaling by transcription, post-transcription or epigenetic regulation.

Further research demonstrated that some lncRNAs are involved in the Wnt signaling regulation. H19 was found associated with enhancer of zeste homolog (EZH2), resulting in Wnt/β-catenin activation [25]. We found 3 lncRNAs that could be regulators of the

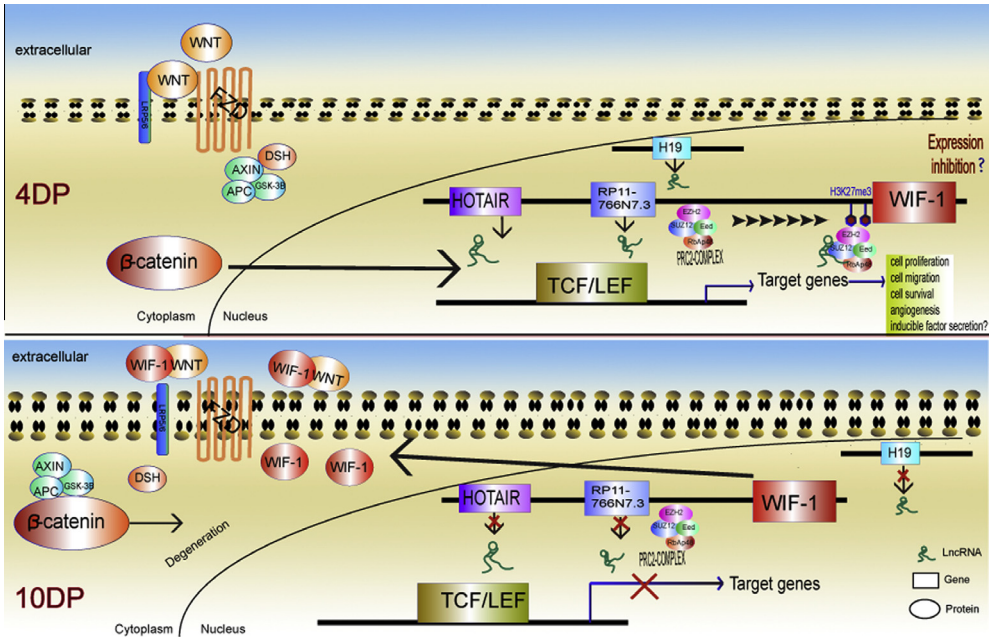

Fig. 4. WIF-1 gene promoter methylation by lncRNAs may decrease WIF-1 mRNA expression. Wnt signaling is maintained in the early-passage DP cells, whereas in late-passage DP cells, the expression of some lncRNAs (H19, HOTAIR, RP11-560A15.3) is increased, which could be associated with the expression of enhancer of zeste homolog 2 (EZH2), and result in Wnt/β-catenin activation and depressed expression of Wnt members such as WIF-1. Then DP cells would loss the ability to induce hair-follicle reconstruction.

Wnt pathway, with significant difference in expression between DP4 and DP10 cells. The lncRNAs (ENST00000442037, uc001lva.4, uc021qbz.1, ENST00000439725 and ENST00000417089), with upregulated expression, are transcripted from the H19 gene located on chromosome 11.

Wnt inhibitory factor-1 (WIF-1) is a key Wnt antagonist [30]. Downregulation of WIF-1 mRNA and protein levels by WIF-1 gene promoter methylation has been reported in human astrocytoma, and Kim thought this could be an important mechanism of aberrant Wnt/β-catenin pathway activation [31]. We validated the downregulated expression of WIF-1 by RT-PCR (fivefold change in expression), downregulated the most among Wnt/β-catenin antagonists. WIF-1 promoter methylation may have decreased WIF-1 mRNA level. WIF-1 promoter methylation is mediated by ncRNAs [24,32]. HOTAIR directly decreased WIF-1 expression by promoting its histone H3K27 methylation in the promoter region and then activating the Wnt/β-catenin signaling pathway [24]. We found 2 genotypes of HOTAIR genes, NR_047518 and NR_047517. The lncRNA-P11-766N7.3 is from a gene located downstream of WIF-1 on chromosome 12, which is 10 kb from the WIF-1 gene. P11-766N7.3 is downregulated along with WIF-1. Depletion of multiple lncRNAs decrease the expression of neighboring protein-coding genes. Whether P11-766N7.3 could regulate WIF-1 expression mediated by cis should be validated by further study.

In addition to Wnt as a promoters of hair induction, BMP signaling has an inhibitory role [2]. AL163953.3 is located downstream of BMP4 on chromosome 14, and RP11-560A15.3 is located downstream of BMP7 on chromosome 20; the 2 lncRNAs were downregulated together with Bmp4, Bmp7 in early-passage DP cells. AL163953.3 and RP11-560A15.3 belong to enhancer-like RNAs, which act over long distances and across chromosomes to activate transcription at distal promoters [32]. The enhancer-like RNA mentioned above may indirectly regulate the downregulated expression of BMPs in early DP cells with WNT/β-catenin signaling activation.

In summary, our data reveal differentially expressed lncRNAs in DP4 and DP10 cells and discuss the potential role of lncRNAs contributing to hairy gene regulation. We speculated their functions by connecting them with Wnt pathway. In late-passage DP cells cultured in vitro, some differentially expressed lncRNAs could be indirectly associated with increased lncRNAs in the Wnt pathway, including WIF-1 and BMPs by epigenetic regulation, then depressing WNT signaling in late-passage DP cells. Then the DP cells would loss the ability to induce hair follicle reconstruction (Fig. 4). Further functional study is critical to confirm the hypothesis. LncRNAs may regulate hair-follicle reconstruction by reacting the reduced ability of late-passage DP cells.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.09.119.

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