DIFFERENTIAL GENE EXPRESSION PROFILES IN FOETAL SKIN OF REX RABBITS WITH DIFFERENT WOOL DENSITY

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Abstract: This study investigated the mechanisms controlling hair follicle development in the Rex rabbit. The Agilent rabbit gene expression microarray was used to determine differentially expressed genes in Rex rabbit foetuses with different wool densities. The expression patterns of selected differentially-expressed genes were further investigated by quantitative real-time PCR. Compared to low wool density rabbits, 1342 differentially expressed probes were identified in high wool density rabbits, including 950 upregulated probes and 392 downregulated probes. Gene ontology analysis revealed that the most upregulated differentially expressed probes belonged to receptors and the most downregulated differentially expressed probes belonged to DNA binding molecules. Kyoto Encyclopedia of Genes and Genomes pathway analysis revealed that the differentially expressed probes were mainly involved in the sonic hedgehog (Shh) and Eph signalling pathways. The results also suggest that transforming growth factor-beta 1, growth hormone receptor, and the keratin-associated protein 6.1 genes, as well as the Shh and Eph signalling pathways, may be involved in the regulation of hair follicle developmental in Rex rabbits.

Key Words: Rex rabbit foetus, wool density, gene expression, gene chip.

INTRODUCTION

The quality of Rex rabbit hair is largely dependent on hair density. Paus and Cotsarelis (1999) proved that hair follicle density determines hair density while Chase (1954) found that hair follicles are formed during foetal development and that hair density was directly determined by follicle density in the late foetus. Thus, structural features of the skin and hair in the Rex rabbit foetus are not only important to the biological profile but may also be a direct determinant of clothing-hair quality.

Hair follicles represent a complex system of multiple layers of various cell types (Roges, 2004). Development of the hair follicle can be divided into 3 phases; anagen, the growth phase, catagen, the cessation phase, and telogen, the rest phase. Hair follicles exist in large quantities and exhibit a unique cyclical regenerative behaviour (Schneider et al., 2009), as hair follicle cells in telogen phase may resume anagen development after stimulation by certain factors. These cycles continue repetitively throughout the entire lifetime of mammals. However, foetal hair follicle development occurs in different periods across various species.

In the present study, we investigated changes in gene expression and signalling pathways by transcriptomic analysis of Rex rabbit foetal skin at certain development stages to determine any relationship between these genes and signalling pathways with hair follicle development.
MATERIALS AND METHODS

**Animals and Diets**

Buttock skin samples from Rex rabbits at a gestational age (in days [d]) of 19, 20, 21, 22, 23, 24, 25 and 26 d were obtained and stored in 4% formaldehyde. Haematoxylin and eosin (H&E) staining of hair follicle slices was used to determine the stage of hair follicle development. Eight adult female Rex rabbits with high (>14000/cm²) or low wool density (<10000/cm²) were chosen and divided into 2 groups. An adult male rabbit was selected for mating with female rabbits within each group. Twenty-four days after mating, female rabbits were slaughtered and 1 foetus was randomly selected from each female rabbit. The group with high wool density was designated A, and the group with low wool density was designated B. Buttock skin was obtained and preserved in liquid nitrogen for extraction of total RNA. Rabbits were caged in pairs (size of cage: 60×40×40 cm) and reared in an environmentally controlled room (15-25°C) with *ad libitum* access to food and water.

**Probe Design**

The Agilent rabbit single standard microarray (4×44K) and a one colour design were used in the experiments; 8 microarrays were completed for 8 specimens.

**Haematoxylin and Eosin Staining**

The developmental stage of skin specimens was determined by H&E staining as described in Wang *et al.* (2000). Briefly, the skin specimens were fixed conventionally in 4% formaldehyde, dehydrated and embedded in paraffin. Deparaffinated sections of 4 μm thickness were stained with Harris haematoxylin and eosin (Sigma-Aldrich, St. Louis, MO) and examined using an Olympus CX-41 phase contrast microscope (Olympus, Tokyo, Japan).

**Microarray Hybridisation**

Microarray hybridisation was conducted by Shanghai Ouyi Science and Technology Limited Company (Shanghai, China) as follows: Total RNA was extracted from rabbit foetal skin using Trizol reagent following the manufacturer’s instructions (Invitrogen, Carlsbad, CA, USA), with each group representing 4 samples. RNA was purified and assessed using a QIAGEN RNeasy Mini kit (Qiagen). Samples were then prepared using single-cycle cDNA amplification and biotin labelling methods (LowInput Quick-Amp Labeling Kit and Gene Expression Hybridization Kit, Agilent) and used for microarray hybridisation.

**Microarray Imaging and Data Analysis**

The microarray results were captured using an Agilent scanner and the data was read and processed using Feature Extraction software (scan resolution, 5 μm; photoelectric multiplication tube, 100%). GENESPRING12.0 software was used for quantile normalisation. The collected microarray data were analysed by SAM software using P<0.05 and a difference ratio of >2-fold as the screening criteria (Patterson *et al.*, 2006). Cluster analysis was conducted using selected differentially expressed probes from Rex rabbits with different wool densities using Cluster 3.0 software. Enrichment analysis of gene ontology (GO) and pathways related to the differentially expressed probes was completed using FunNet software, providing information which can be used to understand the functions of these genes and their influences on signalling pathways.

**RESULTS**

**Development of Hair Follicles**

As shown in Figure 1, hair follicles develop continuously from a gestational age of 19-26 d. Moreover, the size of hair follicles enlarges rapidly from the gestation age of 24 d, which suggests that hair follicle development speeds up at the gestation age of 24 d.
Figure 1: Rex rabbit hair follicle slices at a gestational age of (A) 19 d, (B) 20 d, (C) 21 d, (D) 22, (E) 23 d, (F) 24, (G) 25 d and (H) 26 d (H&E staining, original magnification: ×100, Arrows indicate primary hair follicles).
Gene expression in Rex rabbit foetuses with high (group A) or low wool density (group B) was analysed and compared. Overall, 1342 probes related to wool density were identified \((P<0.05, \text{fold change}>2)\). Thus, 950 probes in group A were overexpressed compared to group B \((P<0.05, \text{fold change}>2)\) and by blasting the 950 Agilent probes to rabbit transcripts, 395 functional genes were identified (e.g. growth hormone receptor (GHR) and keratin-associated protein 6.1 \([\text{KAP6.1}]\)). However, 555 probes did not blast to rabbit transcripts.

In addition, 392 probes were overexpressed in group B compared to group A \((P<0.05, \text{fold change}>2)\) and these probes related to 164 functional genes (e.g. transforming growth factor-beta 1 \([\text{TGF-β1}]\), and Erythropoietin producing hepatocyte receptor A6 \([\text{EphA6}]\) \([\text{Table 1}]\)). However, 228 of these probes did not blast to rabbit transcripts.

### Gene Ontology (GO) Analysis

As shown in Figure 2 and Figure 3, according to the gene annotation of GO and Agilent, the 1342 differentially expressed probes were involved in various molecular functions and biological processes.

The molecular analysis results indicated that the most upregulated differentially expressed probes belonged to receptors (37.3%), signal sensing molecules (27.6%), and G-protein-coupled receptor molecules (27.6%) (Figure 2A).

**Table 1: Parts of differentially expressed probes**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Agilent probe ID number</th>
<th>A/B ratio</th>
<th>(P)-value</th>
</tr>
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<tbody>
<tr>
<td>Krt28</td>
<td>A_04_P043950</td>
<td>+32.92</td>
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</tr>
<tr>
<td>GHR</td>
<td>A_04_P059357</td>
<td>+18.76</td>
<td>&lt;0.001</td>
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<td>+12.03</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Vmn3</td>
<td>A_04_P072778</td>
<td>+9.01</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Adora3</td>
<td>A_04_P006997</td>
<td>+8.40</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mrps33</td>
<td>A_04_P042317</td>
<td>-6.84</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Smx31</td>
<td>A_04_P092863</td>
<td>-5.14</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>A_04_P045372</td>
<td>-5.12</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>EphA6</td>
<td>A_04_P053337</td>
<td>-4.82</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Atp5b</td>
<td>A_04_P027198</td>
<td>-4.19</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

A: high wool density group; B: low wool density group. +: represents upregulation; –: represents downregulation.

**Figure 2:** Gene Ontology analysis of all differentially expressed probes. A, Upregulated genes in molecular function/Total upregulated genes \((A/B)\times 100\); B, Downregulated genes in molecular function/Total downregulated genes \((A/B)\times 100\).

- A: receptor activity; B: signal transducer activity; C: G-protein coupled receptor activity; D: olfactory receptor activity; E: ion channel activity.
- a: sequence-specific DNA binding transcription factor activity; b: sequence-specific DNA binding; c, transporter activity; d, hydroxylase activity, acting on acid anhydrides, catalysing transmembrane movement of substances; e, potassium channel activity.
Most of the downregulated differentially expressed probes belonged to sequence-specific DNA binding transcription factors (20.4%), sequence-specific DNA binding proteins (16.3%), and reactive carrier molecules (8.2%) (Figure 2B).

The biological process studies indicated that most of the upregulated differentially expressed probes were involved in the G-protein-coupled receptor signalling pathway (28.8%), signal transduction (27.4%), and transportation (23.1%) (Figure 3A). Most of the downregulated differentially expressed genes mainly participated in transportation (32.8%), ion transport (16.4%), and cross-membrane transport (13.1%) (Figure 3B).

**Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Analysis**

The KEGG database was also used to analyse differentially expressed probes. As illustrated in Figure 4, the genes found in this study were involved in the Shh and Eph signalling pathways, tryptophan metabolism, linoleic acid metabolism, vitamin digestion and absorption, and calcium metabolism.

**Quantitative Real-Time PCR Tests**

Primers for glyceraldehyde-3-phosphate dehydrogenase (Gapdh), Krt28, Vnn3, Adora3, Mrps33, Epha6, and Atp5b were designed using Genbank data and DNAMAN software. Primer sequences are shown in Table 2. The genes, including three upregulated genes (Krt28, Vnn3, and Adora3) and three downregulated genes (Mrps33, Epha6, and Atp5b), were randomly chosen for quantitative real-time PCR (Q-RT-PCR) to verify the results of the gene chip. Rabbit Gapdh was used as an internal standard.

The Q-RT-PCR results show that the expression levels of Krt28, Vnn3, and Adora3 in group A with high wool density were significantly higher than group B with low wool density. The expression levels of Krt28, Vnn3, and Adora3 genes in group A were 24.54-, 6.15-, and 6.64-fold greater than group B, respectively (Figure 5A). Alternatively, Mrps33, Epha6, and Atp5b mRNA levels in group A with high wool density were significantly decreased (by 6.85-, 4.83-, and 4.20-fold respectively) relative to group B with low wool density.
DISCUSSION

In the present study, we show that hair follicles speed the development at gestational day 24, and so we assessed Rex rabbit foetal skin samples with high and low wool densities at this time point. We identified 1342 probes with significantly different expression values between the 2 foetal skin groups by gene chip technology. These included 950 upregulated probes (A/B) and 392 downregulated probes (A/B). The RT-PCR results were consistent with the gene chip results suggesting that the results generated are accurate and reliable.

Upregulation of GHR and KAP6.1 from the KAP (Keratin Associated Protein) family and downregulation of TGF-β1 from the TGF-β family have been previously associated with hair follicle development (Wynn et al., 1988; Zhang et al.,

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Table 2: Primer Sequences.

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank number</th>
<th>Sequence</th>
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<tr>
<td>GAPDH</td>
<td>NM_001082253</td>
<td>F: TGCCACCCACCTCCTACCTGGGCTCTTTACT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: CCGGTGATTGAGGGCTCTTACT</td>
</tr>
<tr>
<td>Krt28</td>
<td>XM_002719365</td>
<td>F: ATGGTGATGAGATTCATGCT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: CCTTTGCTCTCTACCTGTGGGA</td>
</tr>
<tr>
<td>Vnn3</td>
<td>XM_002714826</td>
<td>F: CGCAGCTGGGAGACACCTTTACTG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: AATATGTGCACCCTGCTGGGGA</td>
</tr>
<tr>
<td>Adora3</td>
<td>NM_001082058</td>
<td>F: TTGGCCATTGTCATCAGGCT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: ACCCCTTCTGATCAGGCG</td>
</tr>
<tr>
<td>Mrps33</td>
<td>XM_002722065</td>
<td>F: AGAGAGAGCTGCTCAGGGACA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: AGGTGGCTCCCTTGAGGAGA</td>
</tr>
<tr>
<td>Epha6</td>
<td>XM_002716627</td>
<td>F: GATGTGTGACACAGATGCAGG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: CGGTGCAAGAGCCCAAAAG</td>
</tr>
<tr>
<td>Atp5b</td>
<td>CU465574</td>
<td>F: CGAAGGGATTGTCGCCCCAG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: AAGTAGCCTGGGTTCAGG</td>
</tr>
</tbody>
</table>

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Figure 4: Kyoto Encyclopedia of Genes and Genomes analysis of the differentially expressed genes. A Upregulated genes in signalling pathway/Total upregulated genes (A/B)×100 (A: Neuroactive ligand-receptor interaction; B: Shh signalling pathway; C: Tryptophan metabolism; D: Linoleic acid metabolism; E: Vitamin digestion and absorption); B Downregulated genes in signalling pathway/Total downregulated genes (A/B)×100 (a: Eph signalling pathway; b: Calcium signalling pathway; c: TGF-beta signalling pathway; d: Adipocytokine signalling pathway; e: PPAR signalling pathway).
Gene chip on wool density of foetus in Rex Rabbits

2001, Bickel et al., 2008). Additionally, the Small proline-rich protein 3 (SPRP3) has also been associated with hair follicle development (Hohl et al., 1995). According to our KEGG analysis, Shh and Eph signalling pathways may play an important role in the development of hair follicles in the Rex rabbit foetus.

Growth hormone (GH) plays a critical role in the regeneration and maintenance of various tissues and contributes to the synthesis of proteins (Müller et al., 1999). GH exerts its regulatory property via a functional interaction with the GHR (Su and Zhu, 1999). Oakes et al. (1992) and Lobie et al. (1990) found that GHR was widely distributed in skin fibroblasts, the internal and external root sheath, and dermal papillae etc. Wynn et al. (1988) found GHR expression in the primary and secondary hair follicles of goats using in situ hybridisation while Mu et al. (2006) also found GHR expression in sheep hair follicles. In the present study, GHR gene expression was upregulated in the higher-hair-density group, which suggests that GHR controls hair follicle development in Rex rabbit foetuses. Furthermore, GHR can induce the expression of insulin-like growth factor 1 (Kajimura et al., 2002) and its receptors, which all play an important role in follicle development in sheep (Harris et al., 1993). However, we did not find any significant change in the gene expression of insulin-like growth factors 1 or its receptors between Rex rabbit foetuses with high and low wool density, and we suggest that these conflicting results may be caused by species-specific factors.

In mammals, hair is primarily composed of keratins, which account for 65-95% of total hair fibres and constitutes the framework of the hair fibre itself (Zhang et al., 2001). KAPs, divided into 23 sub-families (KAP1. n-KAP23. N), are the major part of keratins. KAP expression is closely related to hair growth, although structure and content levels are highly variable among different species and within the same species. A previous study showed that KAP gene was involved in the formation process of hair (Powell and Rogers, 1997). Cockett et al. (2001) reported an association between KAP6.n and the diameter of fleece, although information on the KAP gene family in Rex rabbits remains scarce. In the present study, we found significantly increased KAP6.1 gene expression in high wool density rabbits compared to the low wool density rabbits, implying that KAPs may control clothing hair development of the Rex rabbit.

TGF-β1, a member of TGF-β family, is involved in cellular apoptosis and epidermal regeneration (Sporn and Roberts, 1992; Myers et al., 2007). Hibino and Nishiyama (2004) found TGF-β2 mRNA expression in the internal root sheath of mature human hair follicles. Catagen (hair follicle regression) development was significantly delayed in the TGF-β1-knockout mice compared to wild-type mice (Myers et al., 2007), and, in contrast, TGF-β1 treatment of the back skin of mice induced premature catagen development (Foitzik, 2000). Therefore, TGF-β1 may induce catagen development in mice by inhibiting cellular proliferation and promoting cellular apoptosis. In our present study, TGF-β1 gene expression was paradoxically higher in the low-hair-density group than the high-hair-density group,
which is inconsistent with studies in mice. Therefore, the relationship between TGF-β1 expression and follicular development in Rex rabbit foetuses remains unclear and warrants further investigation.

In the present study, we also identified some novel genes, such as small proline-rich protein 3 (SPRP3) and alpha (S2)-casein gene (CSN1S2). Most of these novel genes are associated with the metabolism of nutrients essential to hair follicle development, and can, therefore, directly or indirectly regulate the growth and development of hair follicles.

In addition, KEGG analyses were also applied to study the differentially expressed genes. The Shh and Eph signalling pathways and some new pathways involving amino acid (proline, arginine, and tryptophan) metabolism, linoleic acid metabolism, vitamin synthesis and metabolism, and calcium metabolism were identified in our study.

In mammals, sonic hedgehog (Shh) is the only member of hedgehog signalling protein family and exists largely in the hair follicle to accelerate the transition of follicle development from telogen phase to anagen phase (Wang et al., 2000). Mill et al. (2005) showed that the Shh protein was highly expressed in epithelial cells, and Shh gene expression increased in telogen phase and accelerated in anagen phase. Shh signalling can also control the speed and pattern of epithelial progenitors through convergent Gli-mediated regulation (Mill et al., 2005). Previous studies demonstrated that Shh was not required for hair follicle development in foetuses, but could stimulate the proliferation of epithelial and dermal cells (Ting-Berreth et al., 1996). In the present study, genes of the Shh signalling pathway were upregulated in the high-hair-density group, suggesting that the Shh signalling pathway may control hair follicle development in Rex rabbits, and thus affect clothing-hair density.

Eph factors belong to the family of tyrosine kinase receptors which directly regulate angiogenesis (Helbling et al., 2000). However, a link between the Eph signalling pathway and hair follicle developmental has yet to be described in the literature. Chong et al. (2000) first discovered the relation of Eph receptor family and EGF receptor family, while Warne et al. (2009) found that EGF receptors were directly involved in the growth of hair follicles. Compared to the low-hair-density group, many Eph signalling pathway genes were significantly downregulated in the high-hair-density group, which suggests the Eph signalling pathway is involved in the regulation of hair follicle developmental of Rex rabbits. However, we note that further studies are required to verify this inference.

In the present study, a large number of pathways associated with the metabolism of amino acids (e.g. proline, arginine, tryptophan and etc.), linoleic acid, and vitamins were also identified. None of these pathways had been reported previously, thus further studies are required to ascertain their exact contributions.

CONCLUSION

In summary, we investigated differentially expressed genes in Rex rabbit foetuses with different wool densities. We identified 1342 differentially expressed probes related to wool density, including 950 upregulated probes (A/B) and 392 downregulated probes (A/B). Genes involved in nutrient metabolism (e.g. SPRP3 and CSN1S2) might play an important role in the supply of nutrient for hair follicle development, while Shh and Eph signalling pathways may also be involved in the regulation of hair follicle developmental in Rex rabbits.

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Gene chip on wool density of foetus in Rex Rabbits


