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Genome-wide transcriptome analysis of human epidermal melanocytes ☆

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ABSTRACT

Because human epidermal melanocytes (HEMs) provide critical protection against skin cancer, sunburn, and photoaging, a genome-wide perspective of gene expression in these cells is vital to understanding human skin physiology. In this study we performed high throughput sequencing of HEMs to obtain a complete data set of transcript sizes, abundances, and splicing. As expected, we found that melanocyte specific genes that function in pigmentation were among the highest expressed genes. We analyzed receptor, ion channel and transcription factor gene families to get a better understanding of the cell signaling pathways used by melanocytes. We also performed a comparative transcriptomic analysis of lightly versus darkly pigmented HEMs and found 16 genes differentially expressed in the two pigmentation phenotypes; of those, only one putative melanosomal transporter (SLC45A2) has known function in pigmentation. In addition, we found 166 transcript isoforms expressed exclusively in one pigmentation phenotype, 17 of which are genes involved in signal transduction. Our melanocyte transcriptome study provides a comprehensive view and may help identify novel pigmentation genes and potential pharmacological targets.

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1. Introduction

Human epidermal melanocytes (HEMs) play a critical role in protecting our skin from sunburn, photoaging, and skin cancer [1]. HEMs are located on the basal layer of the epidermis and are responsible for the synthesis of the photoprotective pigment melanin [2]. Impaired melanocyte function can have severe consequences such as increased skin cancer risk, premature skin aging, or pigmentation disorders (i.e. vitiligo and albinism). Skin cancer is the most common form of cancer in the US and melanoma, resulting from melanocyte transformation, accounts for ~9000 deaths annually in the United States alone [3]. Thus, obtaining a better understanding of melanocyte function and human skin pigmentation is key to identifying novel targets for the treatment of skin cancer and pigmentation disorders.

Current insight into melanocyte function and human pigmentation has been based in part on comparative genomics studies using mouse coat color genes. Of the 378 loci that affect mouse coat color, 171 genes are cloned and 207 remain unidentified [4], suggesting that the molecular mechanisms that regulate pigmentation are far from being elucidated. With the recent advances in high-throughput sequencing technologies the identification of such pigmentation genes might become feasible.

The constitutive pigmentation of the skin is primarily determined by the amount of melanin produced in epidermal melanocytes and is closely correlated with the incidence of skin cancer, indicating that melanin has an important photoprotective function [5,6]. Indeed, in the United States, the incidence of basal and squamous cell carcinomas is 50 times lower in African Americans compared to Caucasians, while melanoma rates are 13 times lower in dark compared to light skin [7–9]. Melanin is produced and stored in organelles named melanosomes using a specific set of melanocyte-specific proteins. Some of these pigmentation proteins are enzymes involved in melanin synthesis [e.g. tyrosinase (TYR), tyrosinase-related protein 1 (TYRP1), and tyrosinase related protein 2 (DCT)], while others are structural proteins or have unknown functions. Microphthalmia transcription factor (MITF), considered the master regulator of melanocyte function, modulates the transcription of many of the pigmentation genes [10,11]. Mutations in seven of the pigmentation genes result in ocular or oculocutaneous albinism, characterized by reduced pigment levels in the skin and eye [12].

The protective function of melanin and the identification of pigmentation proteins raised the question of what accounts for the difference between dark and light skin. It was hypothesized that the main melanogenic enzyme TYR is expressed at higher levels in melanocytes from dark skin, thus producing more melanin. However, early studies showed that the mRNA and protein levels of TYR were similar in light

Abbreviations: HEMs, human epidermal melanocytes; FPKM, fragments per kilobase of exon per million fragments mapped.

☆ Sequence data from this article have been deposited with the GenBank Data Libraries under Accession No. SRP039354 http://dx.doi.org/10.7301/Z0MW2F2N.

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and dark skin, but the activity of the enzyme appeared to be different between the two skin types, being correlated with the amount of cellular melanin [13–16]. What regulates the activity of TYR is not well understood; one possibility is that it depends of the levels of TYRP1 and/or DCT, which might be higher in dark skin [17,18]. Recent studies suggested that different pigmentation phenotypes could be the result of various combinations of single nucleotide polymorphisms (SNPs) in some pigmentation genes [19]. One particular SNP resulting in a point mutation in the melanosomal protein mutated in oculocutaneous albinism IV (OCA4 or SLC45A2) shows strong correlation with skin color [20,21]. Moreover, the mRNA for the allele present in light skin was found to be higher than the allele present in dark skin [22]. Thus, the molecular mechanisms that determine and regulate skin color are yet to be revealed.

The advent of high-throughput RNA sequencing (RNA-Seq) has provided a more sensitive and dynamic way to study mRNA expression. RNA-Seq results contain less noise and have higher specificity compared with microarray experiments. Unlike microarray experiments, RNA-Seq provides quantitative data at single-base resolution, information on transcript size, and is not limited to the number of known genes and transcript isoforms at the time of the study [23,24]. In addition, RNA-Seq data can be reanalyzed as sequence databases are updated. Expression profiling of human epidermal melanocytes using Affymetrix microarrays was used as a reference point for changes in melanoma lines in a study that identified only 14,500 transcripts in normal melanocytes [25]. Another microarray study sequenced melanocytes from diverse geographical origin and found highly homogenous gene expression profiles among melanocytes from skin with different pigmentation levels [18]. The recent expansion of the transcriptome to over 30,000 known protein coding transcripts [26] and the critical role of melanocytes in normal and abnormal skin physiology, make the reassessment of the melanocyte transcriptome an important task.

In this study we performed RNA-Seq on lightly and darkly pigmented human epidermal melanocytes (HEMs) using the Illumina HiSeq 2000 platform. The vast data set obtained was used to perform a comparative transcriptomic analysis of mRNA expression levels in lightly versus darkly pigmented HEMs. This analysis provides an unbiased approach to detect novel regulators of melanin synthesis, providing insight into genes critical to melanocyte function. By compiling the gene expression data into specific families of cellular signaling molecules, we bring to light potential pharmacological targets and genes important for melanocyte signal transduction. This genome-wide trancriptome analysis may serve as a valuable resource for investigating human melanocyte function and provide insight into therapeutic targets for skin cancer and pigmentation disorders.

2. Material and methods

2.1. Sample collection, library preparation and sequencing

Lightly and darkly pigmented primary human epidermal melanocytes (HEMs) from neonatal foreskin (Life Technologies/Gibco) were cultured in Medium 254 and Human Melanocyte Growth Supplement (HMGS2, Life Technologies/Gibco). The four HEM lines used for this study (HEM-D1, -D2, -L1, -L2) were each derived from a different donor; all lines were propagated in culture under identical conditions for ≤15 population doublings. Total HEM RNA was isolated using the mirVana miRNA Isolation Kit (Ambion) and its quality was assessed using an Agilent 2100 Bioanalyzer: RNA Integrity Number (RIN) ≥8.7 for all samples, in agreement with Illumina recommended RIN ≥8. cDNA libraries were prepared with 4 μg total RNA using standard Illumina protocols (TruSeq RNA Sample Preparation Kit) and resulted in cDNA fragments with 229 bp average size, or 355 bp including adapter sequences. Each cDNA library was sequenced with 50 bp single-read chemistry using the Illumina HiSeq 2000 system. All of the sequence files have been submitted to the GenBank Sequence Read Archive for public access [Accession No. SRP039354 http://dx.doi.org/10.7301/Z0MW2F2N].

2.2. Data analysis and annotation

The computational pipeline used for data analysis is shown in Supplemental Fig. S1. The quality of the raw sequencing data was checked using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and the quality scores of the reads for each library and for each position in the read were above 30, which is defined as high quality (Supplemental Fig. S2). The sequencing data in FASTQ format was then mapped against NCBI build 37.2 of the human genome using Bowtie 2 (version 2.1.0.0) [27]. RNA sequencing metrics were obtained using Picard tools (http://picard.sourceforge.net/) (Table 1). Spliced junctions were identified using Tophat (version 2.0.8) [28] and transcript abundance estimates were performed using Cufflinks (version 2.1.1) [29]. EdgeR was used to perform differential gene expression analysis between samples with different pigmentation levels [30,31]. Genes differentially expressed were considered significant if they had a FDR adjusted p-value < 0.05 [32]. For the isoform analysis we imported the Cufflinks isoform expression data of lightly and darkly pigmented HEMs into Microsoft Excel. Using Excel functions we identified the isoforms only present in HEM-L or HEM-D and excluded those with high degree of variability (standard error mean >35% the average FPKM).

2.3. qPCR analysis

3 μg of total RNA was extracted from the same HEM lines used for RNAseq using the RNeasy Plus Kit (Qiagen) and reverse transcribed (RT) using SuperScript III kit (Life Technologies). The resulting cDNA was used for qPCR validation of each biological replicate analyzed by RNA-Seq. Reactions were prepared according to the manufacturer's protocol using SYBR Select Master Mix (Invitrogen) and cycled on a VIIA-7 Real-Time PCR System (Applied Biosystems). β-actin was used as an internal control and all reactions were run in triplicate. mRNA levels were quantified by calculating average $2^{-\Delta Ct}$ values, where Ct is the cycle number for the control and target transcript at the chosen threshold. $\Delta Ct = Ct_{target} - Ct_{\beta\text{-actin}}$ was calculated by subtracting the average Ct of β-actin from the average Ct of the target transcript. Primers were designed to span an exon–exon junction to avoid amplification of any contaminate DNA. Primers used for SLC45A2, OCA2, and SLC24A5 qPCR were designed using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/) and were as follows:

SLC45A2 (NM_016180.3) — F: CCCTGTACACTGTGCCCTTT and R: CTTCCCTCTCACGCTGTTGT,

OCA2 (NM_000275.2) — F: GTGTGCAGGGATTGCAGAAC and R: ACATCCCAACAGTGCAGGAC,

SLC24A5 (NM_205850.2) — F: GCAGCAGGAACAAGCATACC and R: ATGGAATACCAAGGCACAACA.

The relative difference in expression between the two pigmentation phenotypes (HEM-D and HEM-L) was calculated as fold change in HEM-L vs. HEM-D (HEM-L/HEM-D); for RNA-Seq it was calculated by dividing average FPKM of HEM-L by that of HEM-D and for qPCR by dividing the average $2^{-\Delta Ct}$ of HEM-L by that of HEM-D.

2.4. Melanin quantification

HEMs were grown to 70–90% confluence in 35 mm Petri dishes and melanin collected and quantified as previously described [33]. Briefly, after cell lysis soluble and insoluble fractions were separated by centrifugation. The soluble fraction was used to determine the total amount of protein in each sample using a Bradford assays (Bio-Rad Laboratories). The insoluble fraction containing melanin was resuspended in 100 μl of 1 M NaOH and incubated at 85 °C until melanin was fully dissolved

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