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Research paper

Expressed miRNAs target feather related mRNAs involved in cell signaling, cell adhesion and structure during chicken epidermal development

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ABSTRACT

MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression at the post-transcriptional level. Previous studies have shown that miRNA regulation contributes to a diverse set of processes including cellular differentiation and morphogenesis which leads to the creation of different cell types in multicellular organisms and is thus key to animal development. Feathers are one of the most distinctive features of extant birds and are important for multiple functions including flight, thermal regulation, and sexual selection. However, the role of miRNAs in feather development has been woefully understudied despite the identification of cell signaling pathways, cell adhesion molecules and structural genes involved in feather development. In this study, we performed a microarray experiment comparing the expression of miRNAs and mRNAs among three embryonic stages of development and two tissues (scutate scale and feather) of the chicken. We combined this expression data with miRNA target prediction tools and a curated list of feather related genes to produce a set of 19 miRNA-mRNA duplexes. These targeted mRNAs have been previously identified as important cell signaling and cell adhesion genes as well as structural genes involved in feather and scale morphogenesis. Interestingly, the miRNA target site of the cell signaling pathway gene, Aldehyde Dehydrogenase 1 Family, Member A3 (*ALDH1A3*), is unique to birds indicating a novel role in Aves. The identified miRNA target site of the cell adhesion gene, Tenascin C (*TNC*), is only found in specific chicken *TNC* splice variants that are differentially expressed in developing scutate scale and feather tissue indicating an important role of miRNA regulation in epidermal differentiation. Additionally, we found that β-keratins, a major structural component of avian and reptilian epidermal appendages, are targeted by multiple miRNA genes. In conclusion, our work provides quantitative expression data on miRNAs and mRNAs during feather and scale development and has produced a highly diverse, but manageable list of miRNA-mRNA duplexes for future validation experiments.

1. Introduction

Regulation of gene expression contributes to phenotypic differences between different structures (Lockhart and Winzeler, 2000). Although many other mechanisms such as alternative splicing and polyadenylation are involved in modulating the expression of genes on the mRNA level (Chen and Rajewsky, 2007), microRNA (miRNA) regulation has been recognized as one of the key mechanisms in the mRNA transcript regulatory network in eukaryotes (He and Hannon, 2004). MicroRNAs are small endogenous noncoding RNAs between 19 and 24 nucleotides (nt) in size (Bartel, 2004; Carrington and Ambros, 2003). A mature miRNA is generated from a pre-miRNA, also referred to as a miRNA gene (Kamanu et al., 2013), which is a ~70 nt hairpin precursor. Mature miRNAs can complementarily bind target mRNA sequences to inhibit translational initiation or lead to mRNA degradation. In animals, it is reported that miRNAs can bind with the coding sequence region (CDS), 3′ or 5′ untranslated regions (UTR) of target mRNAs (Duursma et al., 2008; Lytle et al., 2007; Wu et al., 2006).

MicroRNAs have been found to widely regulate many biological processes such as development, metabolism, organogenesis, differentiation and proliferation in animals (Betel et al., 2008) including the spatio-temporal patterns important for development (Miska et al., 2004). The

Abbreviations: *ALDH1A3*, Aldehyde Dehydrogenase 1 Family Member A3; BLAST, Basic Local Alignment Search Tool; BLASTN, Basic Local Alignment Search Tool nucleotide-nucleotide; *BMP2*, Bone morphogenic protein 2; *CDH1*, Cadherin 1, type 1; CDS, coding sequence region; CNEEs, conserved non-exonic elements; DF, dorsal feather; *EDAR*, Ectodysplasin A receptor; *EPHA4*, Ephrin type-A receptor 4; *FGF20*, Fibroblast Growth Factor 20; *GBX2*, Gastrulation Brain Homeobox 2; mfe, minimum free energy; miRNA, microRNA; MRCA, most recent common ancestor; nt, nucleotides; *RALDH*, retinaldehyde dehydrogenases; SC, scutate scale; *SHH*, Sonic Hedgehog; *SLC45A2*, solute carrier family 45 member 2; *TNC*, Tenascin C; UTR, untranslated region; WF, wing feather.

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chicken (*Gallus gallus*) embryo develops and hatches in just ~20–21 days (Hamburger and Hamilton, 1951) and in this short time span, many unique and fascinating structures such as scales and feathers are formed during this complex and amazing transformation from an embryo into chick. Only 23 miRNA target genes have been validated through experimentation in the chicken (Hsu et al., 2014) and none of these are related to chicken embryonic development. However, Zhang et al. (2013) conducted a miRNA profiling analysis of the duck feather follicle and skin using high-throughput sequencing data. Using only miRNA differential expression data and only genes involved in a few signaling pathways (Wnt/β-catenin, *SHH-BMP2* and Notch signal pathways) for miRNA target prediction, they found that seven miRNAs are involved in feather morphogenesis. However, there are >700 predicted miRNAs in the chicken and each miRNA may target multiple mRNAs at multiple sites (Lewis et al., 2005) thereby increasing the complexity of the miRNA regulation network. Therefore, identification of miRNA targets coupled with mRNA and miRNA expression data is critical to advancing our understanding of the function of miRNAs during chicken epidermal appendage development.

Recently, it has been found that a high number of β-keratins are expressed during the development of scutate scales and feathers in the chicken using high-throughput microarray technology (Greenwold et al., 2014; Ng et al., 2014). However, at the protein level, only a few β-keratin species can be detected from scale and feather tissues on 2-dimensional gels at the same developmental stages (Knapp et al., 1991; Knapp et al., 1993; Rice et al., 2013; Shames and Sawyer, 1986; Shames et al., 1988), suggesting that β-keratin mRNAs are being regulated possibly by miRNAs.

This study profiled differentially expressed chicken mRNA and miRNA genes among 14 comparison groups from three important embryonic stages of development (day 8, 17 and 19) and between two tissue types (scutate scale and feather). Although feather development begins as early as day 6, the formation of barbs and barbules does not begin until ~day 12 of embryogenesis (Lucas and Stettenheim, 1972; Sengel, 1976) and in the case of scutate scales, epidermal placodes do not begin to develop until day 9 of embryogenesis making day 8 of embryogenesis an ideal control for feather and scale tissue (Shames and Sawyer, 1986). The scutate scale of the chicken abundantly expresses β-keratin at embryonic day 17 (Shames and Sawyer, 1986). Therefore, day 8 is the stage before epidermal tissue development, day 17 is a peak stage of epidermal development and day 19 is a late development stage close to hatching (~20–21 days).

In order to study the role of miRNAs in the development of scutate scales and feathers, miRNA target prediction tools were customized to produce a list of top ranked differentially expressed target mRNA genes from the differentially expressed miRNA genes of our microarray experiment. These identified target genes are known as important cell signaling (Fibroblast Growth Factor 20 [*FGF20*] and Aldehyde Dehydrogenase 1 Family, Member A3 [*ALDH1A3*]), cell adhesion (Cadherin 1, type 1 [*CDH1*] and Tenascin C [*TNC*]) and structural genes (α- and β-keratins) involved in avian epidermal appendage morphogenesis. Cell signaling molecules are important for gross tissue patterning, cell adhesion molecules are important in the intercellular interactions that result in the complex pattering of the feather rachis, barbs and barbule components while the structural proteins lend unique biomechanical properties to scales and feathers (Fraser and Parry, 1996; Lin et al., 2006; Maderson et al., 2009; Pabisch et al., 2010; Weiss and Kirchner, 2011). Therefore, our findings provide a mechanism of miRNA regulation during embryonic scutate scale and feather differentiation.

2. Material and methods

2.1. RNA isolation and purification

Dorsal feather (DF) and scutate scale (SC) tissues were taken at day 8, 17 and 19 of chicken embryonic development, while the wing feather (WF) tissue was taken at day 17 and 19. The tissue samples were of the whole skin which includes the epidermis and dermis. All tissues after the dissection were fixed immediately in RNA later (Qiagen; Germantown, MD). Chicken tissue samples were generously provided by Dr. Richard Goodwin at the School of Medicine of the University of South Carolina. Total RNA was isolated by using Trizol (Life Technologies; Carlsbad, CA) and Qiagen miRNeasy Mini Kit (Qiagen; Germantown, MD) with the manufacturer's instructions. The quantity and quality of extracted RNA were evaluated by both the Agilent Technologies 2100 Bioanalyzer (Agilent Technologies; Palo Alto, CA) and Thermo Scientific NanoDrop 2000c spectrometer (Thermo Scientific; Waltham, MA).

2.2. Microarray experiment

A customized version of the chicken 44K Agilent microarray was used in this study. This 60-mer oligonucleotide microarray has been validated and tested in different tissues and demonstrated to have high sensitivity and specificity (Li et al., 2008). We removed non-relevant genes such as avian viruses, immune genes, and sensory receptor genes. Using Agilent's (Agilent Technologies; Palo Alto, CA) eArray web based tool (http://www.genomics.agilent.com/en/Custom-Design-Tools/eArray), we created unique 60-mer oligonucleotide sequences for 639 chicken specific stem loop pre-miRNAs (miRNA genes) on July 10th 2012, as well as 27 α-keratins and 102 β-keratins for the microarray.

Total RNA was reversed transcribed into cDNA and then the cDNA was fragmented, labelled and hybridized to the single color custom Agilent microarray gene chip at the South Carolina College of Pharmacy Microarray Core Facility (University of South Carolina, Columbia, SC). After hybridization and washing, the processed slides were scanned with the Agilent High-Resolution Microarray Scanner (Agilent Technologies; Palo Alto, CA). All 8 samples were analyzed with 4 biological replicates, except for the scutate scale day 8 sample which was in triplicate thereby resulting in a total of 31 individual microarray expression samples.

Raw gene expression level files were imported into Agilent GeneSpring software 12.5 (Agilent Technologies; Palo Alto, CA) and normalized by the quantile method, enabling 14 sample comparisons with the 8 samples. Hierarchal clustering with Euclidean distance and normalized intensity values of mRNA genes was also conducted by GeneSpring to analyze overall sample relationships (Fig. 1). Differentially expressed genes were determined using the Mann-Whitney *U* test with p-values ≤0.05 and the fold change expression ≥2. We also performed a hierarchical cluster analysis for the 31 individual microarray expression samples using the R package pvclust (Suzuki and Shimodaira, 2006) and the Approximately Unbiased (AU) p-value and Bootstrap Probability (Supplementary Fig. 1).

Kane et al. (2000) found that 50 mer microarray oligonucleotide probes will cross hybridize with non-specific targets with an equal to or greater similarity of 80%. Therefore, we performed a BLASTN search (Basic Local Alignment Search Tool nucleotide-nucleotide v. 2.2.23 +; Altschul et al., 1990) of the chicken genome (v. 4.0) with the 226 differentially expressed 60 mer oligonucleotide miRNA probes. We found that ~93% (209 of 226) of the probes had only one unique hit against the chicken genome indicating that the majority of our differentially expressed miRNAs expression values were specific to the intended miRNA gene.

2.3. Genome searches and sequence alignments

737 total chicken miRNA gene sequences (miRNA primary transcript) were retrieved from miRBase database (Griffiths-Jones et al., 2008). A BLASTN (Basic Local Alignment Search Tool nucleotide-nucleotide v. 2.2.23 +; Altschul et al., 1990) search with default settings was conducted using these 737 chicken miRNA genes as query for the genomes of an amphibian (*Xenopus Silurana tropicalis*) (Hellsten et al.,