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***Crisp1* and Alopecia Areata in C3H/HeJ Mice**

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Running Head: *Crisp1* and alopecia areata

Abbreviations: AA, alopecia areata; *Ala1*, alopecia areata quantitative trait locus 1; *Crisp1*/CRISP1, cysteine-rich secretory protein 1, *gene*/PROTEIN; *Crisp1*^{tm1Pasc}, *Crisp1* targeted (knockout) mutant mouse line; *Foxq1*, forkhead box q1 gene; HSM, hair shaft medulla; *Hoxc13*, homeobox C13 gene; ISH, *in situ* hybridization; QTL, quantitative trait locus; *Soat1*, sterol-acyltransferase 1, gene

Abstract

Alopecia areata (AA), a cell mediated autoimmune disease, is the second most common form of hair loss in humans. While the autoimmune disease is responsible for the underlying pathogenesis, the alopecia phenotype is ultimately due to hair shaft fragility and breakage associated with structural deficits. Quantitative trait genetic analyses using the C3H/HeJ mouse AA model identified cysteine-rich secretory protein 1 (*Crisp1*), a hair shaft structural protein, as a candidate gene within the major AA locus. *Crisp1* transcripts in the skin at various times during disease development were barely detectable. *In situ* hybridization identified *Crisp1* expression within the medulla of hair shafts from clinically normal strains of mice but not C3H/HeJ mice with AA. Follow-up work with 5-day-old C3H/HeJ mice with normal hair also had essentially no expression of *Crisp1*. Other non-inflammatory based follicular dystrophy mouse models with similar hair shaft abnormalities also have little or no *Crisp1* expression. Shotgun proteomics, used to determine strain difference in hair proteins, confirmed there was very little CRISP1 within normal C3H/HeJ mouse hair in comparison to 11 other strains. However, mutant mice with hair medulla defects also had undetectable levels of CRISP1 in their hair. *Crisp1* null mice had normal skin, hair follicles, and hair shafts indicating that lack of the CRISP1 protein does not translate directly into defects in the hair shaft or hair follicle. These results suggest that CRISP1 may be an important structural component of mouse hair and that its strain-specific dysregulation may indicate a predisposition to hair shaft disease such as AA.

Introduction

Alopecia areata (AA), a cell mediated autoimmune disease, is the second most common form of alopecia in humans. The C3H/HeJ mouse model has been instrumental in dissecting the pathophysiology of this disease (King et al., 2008), but little is known about the cause or structural defects in the hair shafts that ultimately result in breakage (clinically evident alopecia) other than that hair keratins and melanin associated proteins have been proposed as the inciting antigens (Gilhar et al., 2001; Tobin et al., 1997). Transcriptome studies suggested that epitope spreading was a nonspecific, secondary effect of the cell mediated autoimmune aspect of AA, thereby explaining why a variety of autoantibodies were found to melanin associated and keratin proteins in both patients and controls (Carroll et al., 2002). Through studies in both mouse models and human AA patients, natural killer cell subsets are emerging as the underlying effector cells causing abnormalities in the hair follicle with secondary disruption of the normal hair shaft structure (Duncan et al., 2013; Xing et al., 2014). However, the cellular and molecular mechanisms underlying this structural damage to the hair shaft are obscure. For example, it is currently not clear whether pre-existing structural abnormalities (e.g. changes in protein composition) are involved either with the initiation of the disease process or with the severity and extent of AA.

The *Hoxc13* transcription factor regulates formation of the hair shaft medulla (HSM). Dysregulation of this gene, either by overexpression in transgenic mice or lack of expression in null mice, results in defective hair shafts that break at the skin surface similar to AA. Cysteine-rich secretory protein 1 (*Crisp1*) expression is lost within the HSM in both of these *Hoxc13* mutant mice (Peterson et al., 2005; Potter et al., 2011). *Crisp1* is located on mouse chromosome 17 within the major quantitative trait locus for AA (*Alaa1*) (Sundberg et al., 2004). Therefore, it is possible that *Crisp1* plays a role in the pathogenesis of AA in the C3H/HeJ mouse model.

This study describes the lack of CRISP1 protein and *Crisp1* transcripts in the skin and hair follicles of mice that develop spontaneous AA, both mice with the disease and those that are young and clinically normal in comparison to other strains which do not develop AA. These results suggest CRISP1 is either involved in the pathogenesis predisposing to AA or its presence or absence may affect severity variability among individual patients.

Materials and methods

Mice.

All procedures were approved by The Jackson Laboratory Animal Care and Use Committee. Mice were obtained from production colonies at The Jackson Laboratory (Bar Harbor, ME; <http://jaxmice.jax.org/>). The strains evaluated were AKR/J-*Soat1*^{ald/ald} (strain abbreviation: AK; JR#648), BALB/cByJ (CBy; JR#1026), C3H/HeJ (C3; JR#659), C57BL/6J (B6; JR#664), CAST/EiJ (CAST; JR#928), DBA/1J (D1; JR#670), LP/J (LP; JR#676), MRL/MpJ (MRL; JR#486), MRL/MpJ-*Fas*^{lpr/lpr}, *Foxq1*^{sa-/sa-J} (MRL-FX; JR#3896), NOD/ShiLtJ (NOD; JR#1976), NZW/LacJ (NZW; JR#1058), RF/J (RF; JR#682), SB/LeJ-*Lys1*^{bg/bg}, *Foxq1*^{sa/sa} (SB; JR#269), STOCK-*a/a*, *Tmem79*^{ma/ma}, *Flg*^{ft/ft}/J (MAFT; JR#281), STOCK- *Sgk3*^{fz-icalfz-ica}/MciJ (FZ; JR#6135), and WSB/EiJ (WSB; JR#1145) (strain abbreviations obtained from: http://www.informatics.jax.org/external/festing/search_form.cgi; <http://jaxmice.jax.org/>).

Mice were maintained at The Jackson Laboratory in a humidity-, temperature-, and light cycle (12:12) controlled vivarium under specific pathogen-free conditions and were allowed free

access to autoclaved food (NIH 31, 6% fat; LabDiet 5K52, Purina Mills, St. Louis, MO) and acidified water (pH 2.8-3.2).

Dorsal skin was collected from adult C57BL/6N-*Crisp1*^{tm1Pasc/tm1Pasc} (1 female and 5 male), *Crisp1*^{tm1Pasc/+} (1 male and 1 female), and *Crisp1*^{+/+} (4 male) mice (DaRos et al., 2008) at the Instituto de Biología y Medicina Experimental in Buenos Aires, Argentina. Skin was fixed in Fekete's acid-alcohol-formalin, processed routinely, embedded in paraffin, sectioned at 6 µm, stained with hematoxylin and eosin (H&E). Hair was also collected by plucking from these same mice, mounted on glass slides with mounting media and coverslips as previously described (Silva and Sundberg, 2012). All samples were reviewed by an experienced board certified pathologist (JPS).

The microarray study was previously reported (Duncan et al., 2013; McPhee et al., 2012). Full thickness skin grafts from old C3H/HeJ mice with AA to young, histocompatible, unaffected mice were performed to induce AA in a very reproducible manner in terms of time of onset and progression (McElwee et al., 1998; Silva and Sundberg, 2013). Only female mice were used to reduce fighting, and females have a slightly higher frequency of spontaneous AA with greater clinical severity than do males (Sundberg et al., 1994a). Skin was collected and fixed by immersion in Fekete's acid-alcohol-formalin solution for histopathology and in RNAlater (Ambion, Austin, TX) for transcriptome analyses. Skin was collected from 3 different AA graft and 3 different normal graft recipient mice at 5, 10, 15, and 20 weeks post grafting, as well as 3 different mice with spontaneous (natural) AA and 3 different normal C3H/HeJ mice.

For the *in situ* hybridization studies, skin was collected from 5 C3H/HeJ mice at 5 days of age and 5 at 12-18 months of age. For comparison, skin was also collected from age and gender matched FVB/NTac (Taconic, Hudson, NY).

Transcriptome analysis.

Five age groups were analyzed consisting of 3 mice each with AA (15 arrays) versus age and gender matched controls (15 arrays), for a total of 30 arrays. Briefly, skin samples were stored in RNAlater (Ambion, division of Life Technologies, Grand Island, NY). Total RNA was isolated using TRIzol and reverse transcribed with an oligo(dT)-T7 primer (Affymetrix, Santa Clara, CA), followed by double-stranded cDNA synthesis with the Superscript (Invitrogen, division of Life Technologies, Grand Island, NY). The cDNA was amplified using T7 RNA polymerase, labeled with biotinylated nucleotides (Enzo Diagnostics, Farmingdale, NY) and was hybridized onto the MOE430v2.0 GeneChip™ arrays (Affymetrix, Santa Clara, CA). The arrays were scanned with a GeneChip™ Scanner 3000 laser confocal slide scanner. Images were quantified using GCOS 1.0 software GeneChip™ Operating Software, Affymetrix, Santa Clara, CA). Data were transformed using RMA (Robust Multi-Array) normalization. An analysis of variance (ANOVA) model was applied to the data. Differentially expressed genes with q values < 0.05 were considered significant. Significantly different genes were then analyzed using Ingenuity Pathways Analysis® and Ariadne Genomics Pathway Studio® tools.

Shotgun Proteomics.

Shotgun proteomic studies were previously reported (Rice et al., 2012) and details are available on the Mouse Phenome Database (<http://phenome.jax.org/>). Pelage hair from 16 mouse strains and mutant stocks were obtained by cutting hair with electric clippers from mice

ethanized by CO₂ asphyxiation. Hair samples were rinsed in SDS, reduced and alkylated, recovered by ethanol precipitation, and proteolyzed with stabilized bovine trypsin. The clarified digest was acidified with trifluoroacetic acid and applied to online reverse phase chromatography connected to a Thermo-Finnigan LTQ ion trap mass spectrometer. Mass spectra were extracted with Xcalibur version 2.0.7, X!Tandem was used to search the mouse Uniprot database, and Scaffold (version 3.5.1) was used to validate the peptide and protein identifications.

In situ hybridization.

In situ hybridization (ISH) for *Crisp1* was done as previously described (Peterson et al., 2005). For the collection of skin from 5 day postpartum, clinically normal, C3H/HeJ female and FVB/NTac mice were euthanized by decapitation. Older, 12/18 month old female C3H/HeJ mice with alopecia areata and FVB/NTac normal mice were euthanized by CO₂ asphyxiation. Skin from the scapular region was fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4°C overnight, followed by dehydration through a series of 5%, 15% and 30% sucrose in PBS prior to embedding in OCT compound and stored at -80°C. *In situ* hybridization of adult skin with *Crisp1*-specific digoxigenin (Roche Applied Science, Indianapolis, Indiana)-labeled RNA probes (Peterson et al. 2005) to 10 µm cryosections was performed as described (Abzhanov et al., 2003; Murtaugh et al., 1999; Pruett et al., 2004). Hybridization signals were visualized using the standard NBT/BCIP detection system (Roche).

ISH analysis of *Crisp1* expression in dorsal skin of mice at 5 days postpartum was performed with a probe generated from plasmid *pCrisp1-ISH*. This plasmid was cloned by inserting into the EcoRI site of pSafyre vector (Bieberich et al., 1990) a central segment of 676 bp of the *Crisp1* coding region that was prepared by PCR-amplification from mouse *Crisp1* cDNA (GenBank: BC011150.1) using 5'CCTTGCATCATGGTCTTCTGC and 5'TGGGCTAGACTTGACTCCGA forward and reverse primer sequences, respectively.

Results and discussion

Shotgun proteomic analysis revealed low (females) to undetectable (males) levels of CRISP1 protein in normal C3H/HeJ hair shafts (Fig. 1). This finding was revealed during analysis of 11 inbred strains and 5 mutant stocks, showing that these strains were distinguishable by their protein profiles (Rice et al., 2012). CRISP1 was also undetectable in mutant mice in which the HSM did not form properly (mutations in the *Foxq1* and *Soat1* genes) and do not develop AA spontaneously (Wu et al., 2010; Wu et al., 2013). Targeted mutant mice, in which the *Crisp1* gene had been inactivated, did not have any lesions affecting the hair follicles or hair shafts other than scattered follicular dystrophy, mild ulceration, or subepidermal fibrosis consistent with B6 alopecia and dermatitis, a common strain specific background disease in the C57BL/6 substrains (Sundberg et al., 2011).

In situ hybridization to detect *Crisp1* transcript expression patterns within the HSM in C3H/HeJ adult female mice with AA, due to either full thickness skin engraftment (McElwee et al., 1998) or spontaneous disease (Sundberg et al., 1994b), indicated nearly undetectable expression within the hair follicle (**Fig. 2a, b**). Using methods previously described (Peterson et al., 2005), no *Crisp1* expression was found in C3H/HeJ adults with AA compared with normal FVB/NTac control HSM. In follow-up studies in 5 day old C3H/HeJ mice with clinically and histologically normal HSM, *Crisp1* mRNA expression was also undetectable compared to age- and gender-matched FVB/NTac mouse hair where it was evident.

Gene expression studies using both the full thickness skin graft (at 5, 10, 15, and 20 weeks after engraftment) and spontaneous disease C3H/HeJ mouse models revealed average intensity values close to background (data not shown), thereby confirming the proteomic and ISH studies.

Quantitative trait locus (QTL) analysis genetic studies of C3H/HeJ females with AA crossed with C57BL/6J mice, analyzing the F2 progeny, identified one major and three minor QTLs. (Sundberg et al., 2004) *Crisp1* was located within the major QTL on chromosome 17. As we showed previously, if *Hoxc13*, a transcription factor involved in hair medulla formation, was overexpressed (transgenic mice) or not expressed (null mutation) the hair shafts were severely dystrophic resulting in alopecia, similar to that seen in human and mouse AA. We also demonstrated that several mouse strains with single mutations in genes downstream of *Hoxc13* had hair medulla defects combined with low to no expression of CRISP1 protein (Rice et al., 2012). This was further validated in adult C3H/HeJ mice with AA in which there were essentially no detectable levels of *Crisp1* within the medulla. These results suggested that *Crisp1* may be one of the target genes/proteins involved in the autoimmune process ultimately causing the hair shaft defect seen in AA. However, lack of *Crisp1* expression was also evident in hair of 5 day old female C3H/HeJ mice as determined by ISH. This finding is consistent with data at the protein level obtained by shotgun proteomics of adult C3H/HeJ mice that had no clinical or histologic evidence of AA. Collectively, these results indicate that CRISP1 expression within the HSM of C3H/HeJ mice is greatly reduced or totally absent. Different *Crisp1* alleles contain single nucleotide polymorphisms and some of these result in amino acid changes in the predicted protein sequence when compared to C57BL/6J (<http://www.sanger.ac.uk/cgi-bin/modelorgs/mousegenomes/snps.pl>), the normal reference strain crossed with C3H/HeJ for QTL studies (Sundberg et al., 2004), confirming these data. While C3H/HeJ mice with clinically normal hair have what appears to be a normal HSM, the medulla and entire hair shaft disintegrate when mice develop AA at specific stages of the disease leading to hair shaft breakage and alopecia. Initial studies found that male and female *Crisp1* null mice exhibited no differences in fertility compared to controls, although there were functional differences in the ability of sperm to penetrate both zona pelucida-intact and zona pelucida-free eggs (DaRos et al., 2008) suggesting that hair defects might also go unnoticed unless stressed by AA or environmental influences. These results suggest that in addition to serving as a structural component of the HSM, *Crisp1* is likely to have roles that are currently unknown. Abnormalities in CRISP1 expression may predispose patients to AA or affect severity of disease.

Conclusions

While alopecia areata is a cell mediated autoimmune disorder, the ultimate cause of the clinical hair loss is follicular dystrophy with breakage of the hair shaft. This change is evident in humans and mice that develop alopecia areata. The inbred mouse strain which is prone to alopecia areata, C3H/HeJ and related substrains, have an inherent deficiency of CRISP1 protein in the hair shaft with low to undetectable levels of mRNA by gene array and *in situ* hybridization. These findings suggest this may predispose this strain of mice to develop alopecia or may be a complicating factor affecting severity of disease among affected individuals.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Figure 1. Shotgun proteomic peptide spectral counts for CRISP1 protein in female and male mice from 10 inbred strains (C3H/HeJ, C57BL/6J, CAST/EiJ, DBA/1J, FVB/NJ, LP/J, MRL/MpJ-*Fas*^{lpr/lpr}, NOD/ShiLtJ, NZW/LacJ, and WSB/EiJ wild type mice) and inbred strains carrying filaggrin (STOCK- *a/a*, *Tmem79*^{ma/ma}, *Flg*^{fl/fl}), satin (SB/LeJ-*Lyst*^{bg/bg}, *Foxq1*^{sa/sa}), Satin-J (MRL/MpJ-*Fas*^{lpr/lpr}, *Foxq1*^{sa-J/sa-J}, and sterol O-acyltransferase 1 (AKR/J-*Soat1*^{ald/ald}) mutations. Note that C3H/HeJ males have no CRISP1 and females have very little as also seen for mutant mice with hair medulla defects (*Foxq1*^{sa}, *Foxq1*^{sa-J}, and *Soat1*).

Figure 2. *In situ* hybridization for *Crisp1*. There is no *Crisp1* expression in representative anagen follicles from an adult AA affected C3H/HeJ mouse (A) in contrast to the blue signal in the medulla of an FVB/NTac albino mouse (B). Hair shafts were clipped from 2 adult females and 2 adult males. Note the dystrophic hair shaft in the bulb of the C3H/HeJ mouse hair follicle (A, arrow). *Crisp1* expression was not detectable in normal hair shafts from 5 day old C3H/HeJ mice (C) in contrast to control 5 day old FVB/NTac mice (D). Size bar = 40µm.

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