Androgenetic Alopecia: Identification of Four Genetic Risk Loci and Evidence for the Contribution of WNT Signaling to Its Etiology

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The pathogenesis of androgenetic alopecia (AGA, male-pattern baldness) is driven by androgens, and genetic predisposition is the major prerequisite. Candidate gene and genome-wide association studies have reported that single-nucleotide polymorphisms (SNPs) at eight different genomic loci are associated with AGA development. However, a significant fraction of the overall heritable risk still awaits identification. Furthermore, the understanding of the pathophysiology of AGA is incomplete, and each newly associated locus may provide novel insights into contributing biological pathways. The aim of this study was to identify unknown AGA risk loci by replicating SNPs at the 12 genomic loci that showed suggestive association ($5 \times 10^{-8} < P < 10^{-5}$) with AGA in a recent meta-analysis. We analyzed a replication set comprising 2,759 cases and 2,661 controls of European descent to confirm the association with AGA at these loci. Combined analysis of the replication and the meta-analysis data identified four genome-wide significant risk loci for AGA on chromosomes 2q35, 3q25.1, 5q33.3, and 12p12.1. The strongest association signal was obtained for rs7349332 ($P = 3.55 \times 10^{-15}$) on chr2q35, which is located intronically in *WNT10A*. Expression studies in human hair follicle tissue suggest that *WNT10A* has a functional role in AGA etiology. Thus, our study provides genetic evidence supporting an involvement of WNT signaling in AGA development.

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Abbreviations: AGA, androgenetic alopecia; AVG, average; chr, chromosome; CI, confidence interval; GWAS, genome-wide association study; OMIM, Online Mendelian Inheritance in Man; OR, odds ratio; SNP, single-nucleotide polymorphism

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INTRODUCTION

Androgenetic alopecia (AGA, male-pattern baldness, Online Mendelian Inheritance in Man (OMIM 109200), OMIM 3010710, and OMIM 612421) is the most common form of hair loss in humans, and affects 80% of European men by the age of 80 years. AGA is characterized by a progressive loss of hair from the scalp (Hamilton, 1951; Norwood, 1975), and changes in hair cycling are a key feature of its pathophysiology (Kaufman, 2002; Randall, 2007). Research has established that the two major etiological factors for AGA are genetic predisposition and hormonal status (Hamilton, 1942, 1951). The heritability of AGA is high, and has been estimated to be ~80% in two independent twin studies (Nyholt *et al.*, 2003; Rexbye *et al.*, 2005). Candidate gene and genome-wide association studies (GWASs), including a recent meta-analysis of seven GWASs, have identified strong associations between AGA and single-nucleotide polymorphisms (SNPs) at eight chromosomal loci (Ellis and Harrap, 2001; Hayes *et al.*, 2005; Hillmer *et al.*, 2005, 2008a,b; Levy-Nissenbaum *et al.*, 2005; Prodi *et al.*, 2008; Richards *et al.*, 2008; Brockschmidt *et al.*, 2010; Brockschmidt *et al.*, 2011; Li *et al.*, 2012). Of these, the X-chromosomal androgen receptor/ectodysplasin A2 receptor locus (*AR/EDA2R*) confers the strongest effect, and the *androgen receptor* is a highly plausible candidate gene at this locus.

However, no study has demonstrated an effect of genetic variation on the expression and function of the protein. Similarly, for the seven autosomal loci, in some cases candidate genes have been suggested, such as *histone deacetylase 9* on chromosome (chr) 7p21.1 (Brockschmidt *et al.*, 2011; Li *et al.*, 2012) and *histone deacetylase 4* on chr2q37.3 (Li *et al.*, 2012), but no direct functional evidences have been obtained so far. Despite these recent breakthroughs in our understanding of the genetics of AGA, a significant fraction of the overall heritable risk still awaits identification. The identification of additional risk factors is highly desirable, as these may provide new insights into the pathophysiology of AGA and its underlying biological pathways. A promising strategy for the identification of additional genomic loci for AGA is to replicate SNPs at the genomic loci that showed suggestive association with AGA within the meta-analysis by Li *et al.* (2012) ($P<10^{-5}$), but which fell above the threshold for genome-wide significance ($P=5\times10^{-8}$). This study involved two independent samples: first a German AGA case–control sample comprising 177 AGA cases and 272 supercontrols (>60 years, no signs of AGA), and second a sample of European descent comprising 2,582 AGA cases and 2,389 controls.

RESULTS

Analysis of the data from the MAAN (Meta-Analysis for Androgenetic Alopecia Novel Determinants) by Li *et al.* (2012) revealed a total of 12 genomic loci for which at least one SNP showed an association with AGA of $P<1\times10^{-5}$ but fell short of genome-wide significance ($P>5\times10^{-8}$; Table 1). For each of these 12 loci, the two SNPs with the best association *P*-values in the meta-analysis were genotyped in an independent German replication sample (''Replication I''). Of the 24 SNPs, 21 were genotyped successfully for 177 cases and 272 supercontrols. Of these, three SNPs at two distinct genomic loci showed nominally significant association with AGA ($P<0.05$; Table 1). However, only rs7349332 on chr2q35 showed a genome-wide significant association in a combined analysis of our data and the data of the previous meta-analysis ($P=3.32\times10^{-9}$; data not shown). We therefore attempted further replication using data from the scientific program of 23andMe (23andWe). All 24 SNPs were analyzed within the existing genome-wide data of 2,582 cases and 2,389 controls of European descent from 23andMe (''Replication II''). Of these, a total of 12 SNPs at 6 distinct genomic loci showed a nominally significant association with AGA (Table 1). A combined analysis of the two replication studies and the published meta-analysis (Li *et al.*, 2012) established genome-wide significant association with AGA for the regions on chr2q35, chr3q25.1, chr5q33.3, and chr12p12.1 (Table 1, Figure 1). The strongest association was observed for rs7349332 on chr2q35 ($P=3.55\times10^{-15}$; odds ratio (OR) = 1.34, 95% confidence interval (CI) = 1.27–1.42), which is located intronically in *WNT10A* (*wingless-type MMTV integration site family, member 10A*) (Figure 1a). This association finding on chr2q35 was further supported by rs10193725 ($P=1.46\times10^{-10}$; OR = 0.80, 95% CI = 0.74–0.87), which is located ~30 kb upstream of rs7349332 and intronically within *WNT6*, another member of the *wingless-type MMTV integration site family*. The AGA risk allele (T) for rs7349332 was associated with hair curl in two independent GWASs (Medland *et al.*, 2009; Eriksson *et al.*, 2010). To investigate a potential etiological overlap between AGA and hair curl, we analyzed whether any other genome-wide significant loci for hair curl were associated with AGA, and whether any genome-wide significant loci for AGA were associated with hair curl. Aside from rs7349332 in *WNT10A*, there were no associations with $P<0.05$ after adjusting for multiple comparisons. The second best association was observed for a locus on chr5q33.3 comprising the two SNPs rs929626 ($P=2.12\times10^{-11}$; OR = 0.84 95% CI = 0.79–0.89) and rs1081073 ($P=8.52\times10^{-9}$; OR = 1.17, 95% CI = 1.12–1.22), both of which are located intronically within *EBF1* (*early B-cell factor 1*) (Figure 1c). On chr12p12.1, the best association signal was observed for rs9668810, with $P=1.09\times10^{-10}$ (OR = 1.21, 95% CI = 1.15–1.27), followed by the 2.3-kb distal rs7975017 ($P=4.03\times10^{-10}$; OR = 1.21, 95% CI = 1.15–1.28). Both SNPs on chr12p12.1 are located in the intergenic region between the two RefSeq genes *SSPN* (*sarcospan*) and *ITPR2* (*inositol 1,4,5-triphosphate receptor, type 2*) (Figure 1d). A similarly strong association signal was identified for a locus on chr3q25.1, with *P*-values of $P=1.79\times10^{-10}$ (OR = 1.19, 95% CI = 1.13–1.24) and $P=1.20\times10^{-9}$ (OR = 1.18, 95% CI = 1.12–1.23) for rs7648585 and rs4679955, respectively. Both SNPs are located in the intergenic region between the two RefSeq genes *SUCNR1* (*succinate receptor 1*) and *MBNL1* (*muscle-blind-like splicing regulator 1*) (Figure 1b). Although the direction of effects was consistent across all studies for the region on chr12q24.33 flanked by the two RefSeq genes *TMEM132D* (*transmembrane protein 132D*) and *FZD10* (*frizzled family receptor 10*), this region failed to reach genome-wide significance in the combined analysis ($P_{min}=1.28\times10^{-6}$).

In a second step, we investigated the expression of RefSeq genes located within a 200 kb window around the SNP with the lowest *P*-value at the respective AGA risk loci within human hair follicles (Table 2). On chr2q35, eight RefSeq genes were located within this 200 kb window. Of these, two genes showed expression in human hair follicle. The highest expression values were observed for *CYP27A1* (*cytochrome P450, family 27, subfamily A, polypeptide 1*; average (AVG) signal = 377.21), which is located 76.4 kb downstream of rs7349332. Hair follicle expression was also detected for *WNT10A*—which spans rs7349332—with an AVG signal of 86.36. On chr3q25.1 and chr5q33.3, no expression was detected for the investigated genes, i.e., *SUCNR* and *EBF1*, respectively. On chr12p12.1, both of the genes that flank the association finding showed expression in hair follicles. *SSPN*, which is located ~38.7 kb downstream of rs9668810, showed an AVG signal of 70.55, and *ITPR2*, which is located ~61.9 kb downstream of rs9668810, showed an AVG signal of 119.04.

We also tested for a genotypic effect on the expression of candidate genes showing expression in hair follicles. Interestingly, we observed a genotypic effect of rs7349332 on the expression of *WNT10A*, in which AGA risk allele carriers

Table 1. Genomic regions selected for replication and results of the replication and the combined analysis

					Meta-analysis			Replication I			Replication II			Combined analysis					
Genomic region	SNP	Position	EA	OA	*P*-value	OR	95% CI	*P*-value	OR	95% CI	*P*-value	OR	95% CI	Effect direction	I^2	*P*-value	OR	95% CI	Context
1q24.2	rs928462	170290129	T	C	5.15E − 06	1.24	(1.13–1.36)	0.172	1.27	(0.89–1.80)	0.69	0.98	(0.87–1.08)	+ + −	< 0.01	4.08E − 04	1.13	(1.06–1.20)	METTL11B—[]—GORAB
	rs11589761	170771349	G	A	3.90E − 06	1.25	(1.14–1.38)	–	–	–	0.70	1.02	(0.91–1.13)	+ ? +	< 0.01	1.43E − 04	1.15	(1.08–1.22)	PRRX1–[]—C1orf129
2q35	**rs10193725**	**219726498**	**T**	**C**	**3.00E − 07**	**0.79**	**(0.72–0.86)**	**0.154**	**0.79**	**(0.57–1.10)**	**3.91E − 04**	**0.82**	**(0.72–0.93)**	− − −	**< 0.01**	**1.46E − 10**	**0.80**	**(0.74–0.87)**	**[WNT6]**
	rs7349332	**219756383**	**T**	**C**	**8.35E − 08**	**1.30**	**(1.18–1.44)**	**3.36E-03**	**1.76**	**(1.20–2.57)**	**7.63E − 08**	**1.37**	**(1.25–1.48)**	+ + +	**0.23**	**3.55E − 15**	**1.34**	**(1.27–1.42)**	**[WNT10A]**
3q25.1	**rs7648585**	**151639765**	**G**	**A**	**5.18E − 07**	**1.20**	**(1.12–1.29)**	**0.876**	**1.02**	**(0.77–1.35)**	**3.97E − 04**	**1.16**	**(1.08-1.24)**	+ + +	**< 0.01**	**1.20E − 09**	**1.18**	**(1.12–1.23)**	**SUCNR1–[]—MBNL1**
	rs4679955	**151653368**	**T**	**A**	**3.12E − 07**	**1.21**	**(1.12–1.30)**	**1.000**	**1.00**	**(0.76–1.31)**	**8.30E − 05**	**1.18**	**(1.10–1.26)**	+ + +	**0.12**	**1.79E − 10**	**1.19**	**(1.13–1.24)**	**SUCNR1–[]—MBNL1**
4p15.33	rs7699705	11467103	G	A	3.02E − 06	1.19	(1.11–1.28)	0.663	0.94	(0.71–1.23)	0.83	0.99	(0.91–1.07)	+ − −	0.01	4.80E − 04	1.10	(1.05–1.15)	HS3ST1–[]
	rs7676958	11467552	C	A	1.83E − 05	0.85	(0.80–0.92)	–	–	–	0.94	1.00	(0.92–1.09)	+ ? −	< 0.01	1.78E − 03	0.92	(0.86–0.97)	HS3ST1–[]
5q33.3	**rs929626**	**158310631**	**G**	**A**	**5.00E − 06**	**0.85**	**(0.79–0.91)**	**0.146**	**0.83**	**(0.63–1.09)**	**1.65E − 06**	**0.83**	**(0.75–0.90)**	− − −	**< 0.01**	**2.12E − 11**	**0.84**	**(0.79–0.89)**	**[EBF1]**
	rs1081073	**158381512**	**T**	**A**	**1.71E − 05**	**1.18**	**(1.10–1.27)**	**0.180**	**0.84**	**(0.64–1.10)**	**2.33E − 05**	**1.19**	**(1.11–1.27)**	+ − +	**< 0.01**	**8.52E − 09**	**1.17**	**(1.12–1.22)**	**(EBF1)**
7p22.1	rs2024122	7261751	G	A	6.41E − 06	1.19	(1.10–1.28)	1.00	1.00	(0.75–1.33)	0.91	1.00	(0.91–1.08)	+ + −	0.10	9.17E − 04	1.10	(1.04–1.15)	[C1GALT1]
	rs3807862	7277807	G	A	2.**01E** − 06	0.84	(0.78–0.90)	0.481	0.91	(0.69–1.20)	1.00	1.00	(0.92–1.08)	− − +	0.32	4.52E-04	0.91	(0.86–0.96)	[C1GALT1]
8p11.21	rs10958705	41854101	G	A	7.01E − 06	1.19	(1.11–1.29)	0.526	0.91	(0.69–1.21)	0.96	1.00	(0.92–1.08)	+ − +	< 0.01	8.89E − 04	1.10	(1.04–1.15)	(MYST3)
	rs13257590	41878091	T	C	6.23E − 06	0.84	(0.77–0.90)	0.537	1.09	(0.83–1.45)	0.94	1.00	(0.92–1.08)	− + −	< 0.01	3.93E − 03	0.92	(0.87–0.98)	[MYST3]
10p13	rs1008733	14042814	G	A	7.86E − 06	1.20	(1.11–1.29)	0.060	0.76	(0.56–1.03)	1.10E − 03	0.87	(0.78-0.95)	+ − −	0.07	0.116	1.05	(0.99–1.10)	[FRMD4A]
	rs1001201	14043452	T	G	8.47E − 06	0.84	(0.78–0.91)	0.063	1.31	(0.97–1.78)	1.41E − 03	1.15	(1.06–1.23)	− + +	0.26	0.741	0.99	(0.93–1.05)	(FRMD4A)
12p12.1	**rs9668810**	**26426420**	**T**	**C**	**6.96E − 06**	**1.20**	**(1.11–1.30)**	**0.007**	**1.55**	**(1.14–2.10)**	**8.12E − 05**	**1.19**	**(1.11–1.28)**	+ + +	**0.14**	**1.09E − 10**	**1.21**	**(1.15–1.27)**	**SSPN–[]–ITPR2**
	rs7975017	**26428793**	**T**	**C**	**5.07E − 06**	**1.21**	**(1.11–1.31)**	**0.032**	**1.44**	**(1.05–2.00)**	**1.22E − 04**	**1.20**	**(1.11–1.29)**	+ + +	**< 0.01**	**4.03E − 10**	**1.21**	**(1.15–1.28)**	**SSPN–[]–ITPR2**
12q14.2	rs342150	63157591	T	C	3.11E − 06	1.22	(1.12–1.32)	0.801	1.04	(0.780 –1.37)	0.66	0.98	(0.90–1.07)	+ + −	< 0.01	1.11E − 03	1.10	(1.04–1.16)	[PPM1H]
	rs342151	63157618	T	A	3.11E − 06	1.21	(1.12–1.32)	–	–	–	0.65	0.98	(0.89–1.07)	+ ? −	< 0.01	1.19E − 03	1.10	(1.04–1.16)	[PPM1H]
12q24.33	rs864327	130567756	C	A	9.18E − 06	1.28	(1.15–1.43)	0.507	1.14	(0.77–1.70)	0.03	1.14	(1.02–1.25)	+ + +	< 0.01	1.40E − 06	1.21	(1.13–1.29)	TMEM132D—[]–FZD10
	rs745077	130568656	G	A	1.17E − 05	1.28	(1.15–1.43)	0.406	1.18	(0.79–1.75)	0.03	1.14	(1.02–1.25)	+ + +	< 0.01	1.28E − 06	1.21	(1.13–1.29)	TMEM132D—[]–FZD10
19p13.3	rs1008076	3968605	T	G	1.35E − 06	1.22	(1.13–1.33)	0.098	0.80	(0.61–1.05)	0.30	0.96	(0.88–1.04)	+ − −	< 0.01	0.010	1.08	(1.02–1.13)	(DAPK3)
	rs2304194	3969505	G	A	2.67E − 06	1.21	(1.12–1.31)	0.118	0.81	(0.61–1.06)	0.29	0.96	(0.88–1.04)	+ − −	< 0.01	8.33E − 03	1.08	(1.02–1.13)	[DAPK3]

Abbreviations: CI, confidence interval; EA, effect allele; I^2, between-study variance; OA, other allele; OR, odds ratio; SNP, single-nucleotide polymorphism.
The replication I sample comprised 177 cases and 272 unaffected controls. The replication II sample comprised 2,582 cases and 2,389 controls. Regions that achieved genome-wide significant association with AGA in the combined analysis are printed in bold. All genomic positions are according to GRCh37/hg19.

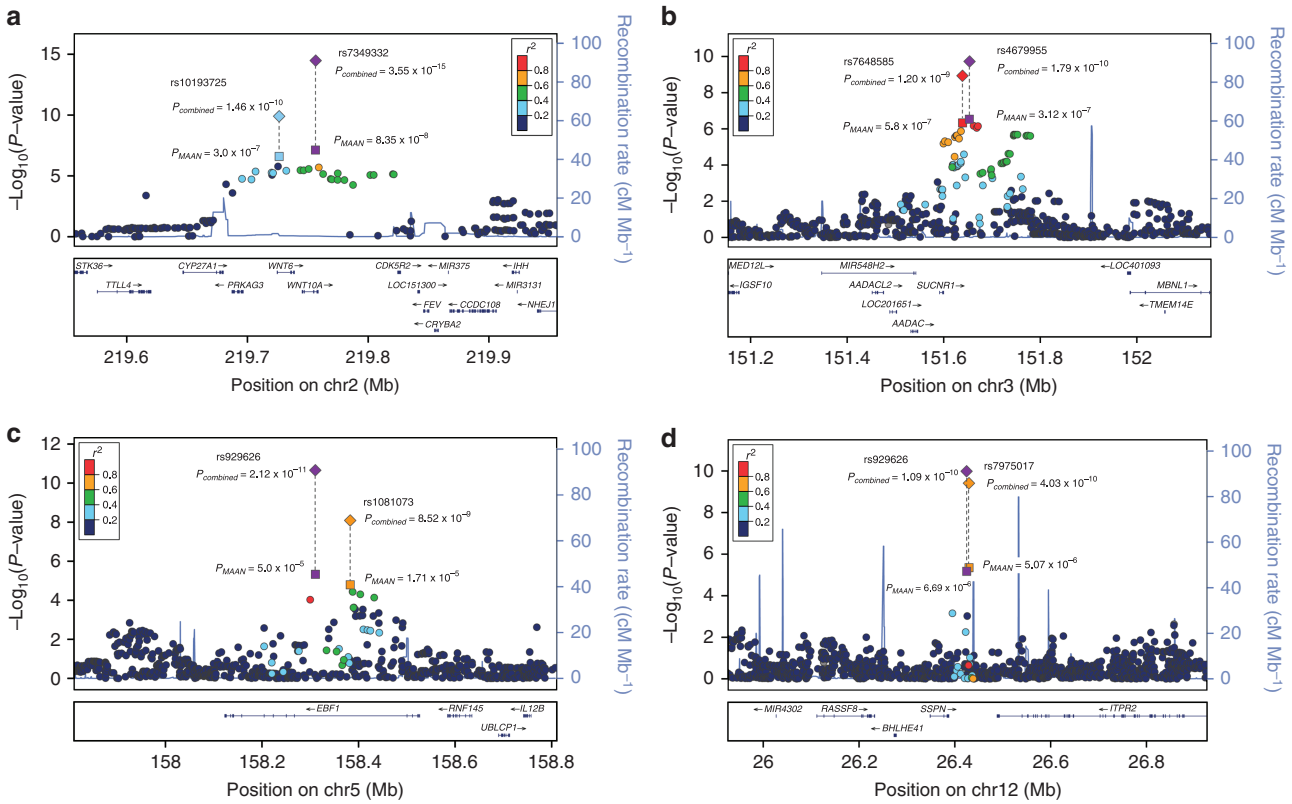

Figure 1. Local association plots for the identified androgenetic alopecia (AGA) risk loci. Regional association results for the identified AGA risk loci on chromosomes (**a**) 2q35, (**b**) 3q25.1, (**c**) 5q33.3, and (**d**) 12p12.1. Each panel shows the $-\log_{10}(P)$ of single-nucleotide polymorphisms (SNPs) against their chromosomal (chr) position. For the SNPs included in the replication analysis, association results for the published meta-analysis data (Li *et al.*, 2012) are depicted as squares (P_{MAAN}), and the results of the combined analysis are depicted as diamonds ($P_{combined}$). The lead SNP at each locus is denoted in purple; the color of other SNPs indicates the linkage disequilibrium (LD) with lead SNP as red ($0.8 \leqslant r^2 \leqslant 1$), orange ($0.6 \leqslant r^2 < 0.8$), green ($0.4 \leqslant r^2 < 0.6$), light blue ($0.2 \leqslant r^2 < 0.4$), and dark blue ($r^2 < 0.2$).

Table 2. Average expression signals for RefSeq genes located 100 kb upstream and downstream of the SNP with the lowest *P*-value at the respective AGA risk loci

Gene	Chr	Start	Stop	Illumina probe ID	AVG signal	Detection *P*-value
CYP27A1	2	219,646,471	219,680,016	ILMN_1704985	377.21	1.30E − 03
PRKAG3	2	219,687,105	219,696,512	ILMN_1716754	—	>0.01
WNT6	2	219,724,545	219,738,954	ILMN_1795706	—	>0.01
WNT10A	2	219,745,254	219,758,651	ILMN_1658426	86.36	2.60E − 03
CDK5R2	2	219,824,397	219,826,877	ILMN_1717803	—	>0.01
LOC151300	2	219,841,005	219,842,644	ILMN_3289895	—	>0.01
FEV	2	219,845,808	219,850,379	ILMN_1751460	—	>0.01
CRYBA2	2	219,854,911	219,858,127	ILMN_2260313	—	>0.01
SUCNR1	3	151,591,430	151,599,876	ILMN_1681601	—	>0.01
EBF1	5	158,122,922	158,526,788	ILMN_1778681	—	>0.01
SSPN	12	26,348,268	26,387,708	ILMN_1775486	70.55	2.60E − 03
ITPR2	12	26,488,284	26,986,131	ILMN_1736103	119.04	2.60E − 03

Abbreviations: AGA, androgenetic alopecia; AVG, average; Chr, chromosome; OR, odds ratio; SNP, single-nucleotide polymorphism.
Genes located closest to the best association finding at the respective locus are printed in bold. All genomic positions are according to GRCh37/hg19.

(CT, TT; $n=25$) showed significantly reduced expression compared with nonrisk allele carriers (CC; $n=71$; $P=0.03$; Figure 2). We detected no genotypic effect of rs7349332 or rs9668810 on the expression of *CYP27A1* or *SSPN*, respectively (data not shown). The observed tendency toward a lower expression of *ITPR2* in homozygous AGA risk allele carriers (TT) for rs9668810 was not statistically significant ($P=0.09$; data not shown). To better understand the role

of *WNT10A* and other WNT-related genes in the etiology of AGA, we also examined the interaction between the lead SNPs from loci in close proximity to genes related to WNT signaling (i.e., *WNT6*, *WNT10A*, *WNT3*, and *FZD10*). Moreover, we examined the interactions between the lead SNPs from the eight previously identified AGA risk loci (Li *et al.*, 2012), the four risk loci identified in this study, and the locus on chr12q24.33 (*FZD10*). We did not detect any interaction between rs7349332 (*WNT10A*) and any of the other loci; however, restricting the analysis to loci related to WNT signaling revealed a significant interaction between rs745077 (*FZD10*) and rs12373124 (*WNT3*) ($P=0.02$; data not shown).

DISCUSSION

This study identified four AGA risk loci with genome-wide significance on chromosomes 2q35, chr3q25, chr5q33.3, and chr12p12.1, and provides biological evidence that *WNT10A* is the gene responsible for the effect on chr2q35. *WNT10A* is a member of the family of *WNT* genes that encode small secreted signaling proteins. These proteins participate in diverse developmental processes during embryogenesis, as well as in adult tissue homeostasis (Logan and Nusse, 2004). Numerous publications have indicated the importance of WNT signaling in hair development and hair cycling (Millar *et al.*, 1999; Reddy *et al.*, 2001; Andl *et al.*, 2002; Fuchs, 2007). In particular, some authors have suggested that WNT signaling constitutes an important regulatory signal for the transition from telogen to anagen in postnatal hair follicles (Reddy *et al.*, 2001; Li *et al.*, 2011). Interestingly, *WNT10A* expression has been detected at anagen onset in mouse hair follicles (Reddy *et al.*, 2001), suggesting that *WNT10A* is implicated in anagen induction. A study by Shimizu and Morgan (2004) showed that WNT signaling is essential in the maintenance of anagen characteristics within dermal papilla cells. This finding is of particular interest, as changes in hair follicle dynamics are a key feature of AGA (Courtois *et al.*, 1994). During AGA development, the premature transition from anagen to telogen results in shorter hair growth phases and a reduction in the ratio of anagen to telogen follicles (Whiting, 1993; Courtois *et al.*, 1994; Kaufman, 2002). Moreover, the time interval between telogen and the initiation of a new hair cycle is prolonged, leading to a decrease in hair density (Ellis *et al.*, 2002). The regulation of hair cycle dynamics might be a plausible mechanism for how rs7349332 confers risk for AGA. The decrease in *WNT10A* expression observed in AGA risk allele carriers for rs7349332 might lead to a delay in telogen–anagen transition and a shortening of anagen duration, resulting in the changes in hair cycling described for AGA (Figure 3). The role of WNT signaling in AGA etiology is further supported by the fact that other genes related to WNT signaling are located in the vicinity of other AGA risk loci, e.g., *WNT3* on the previously implicated chromosomal region chr17q21.31 and *ITPR2* on chr12p12.1 implicated in our present study. Research suggests that the latter might act downstream of WNT signaling as a receptor for IP_3 (Wang and Malbon, 2004). Interestingly, another member of the IP_3 receptor family has recently been proposed to actively regulate the process of hair shedding (Sato-Miyaoka *et al.*, 2012). Moreover, the locus on chr12q24.33 that shows evidence for association in our study—although not with genome-wide significance ($P=1.28\times10^{-6}$)—harbors *FZD10,* which encodes a known WNT receptor. Observations from clinical genetics also suggest that *WNT10A* may be implicated in hair growth. Homozygous mutations in *WNT10A* are a known cause of autosomal recessive odonto-onycho dysplasia (OMIM 257980 and OMIM 606268), which may present with sparse scalp and body hair (Adaimy *et al.*, 2007; Bohring *et al.*, 2009). Additional support for a role of WNT signaling in the etiology of AGA, although preliminary, was provided by our interaction analysis suggesting an interaction of *FZD10* and *WNT3*.

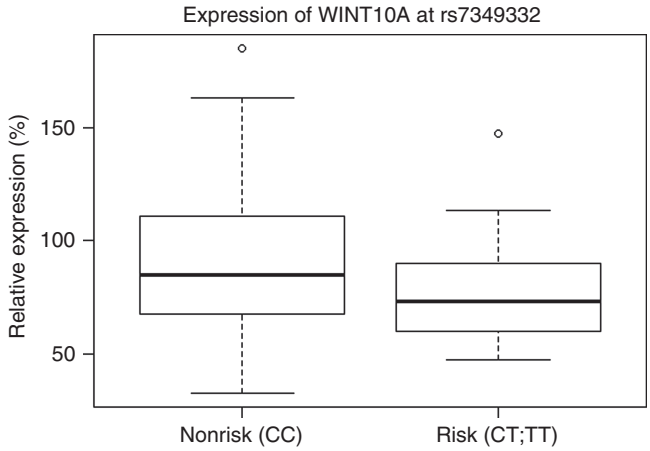

Figure 2. Genotype-specific expression analysis of *WNT10A* at rs7349332. Boxplots indicate the distribution of the normalized expression data for the respective genotype group (CC, $n=71$; CT, $n=24$; TT, $n=1$). Genotype-specific expression levels were calculated from the normalized fluorescence-intensity signals for *WNT10A* (ILMN_1658426) relative to the average expression level of rs7349332 nonrisk genotype (CC) carriers, which was set to 100%. The bold horizontal line indicates the median value; limits of the rectangle indicate lower and upper quartiles. End points of the dashed vertical lines indicate the minimum and maximum expression values. Outliers (i.e., values deviating >1.5-fold the interquartile range from the median value) are depicted by circles. Androgenetic alopecia (AGA) risk allele carriers for rs7349332 show significantly lower expression of *WNT10A* ($P=0.03$).

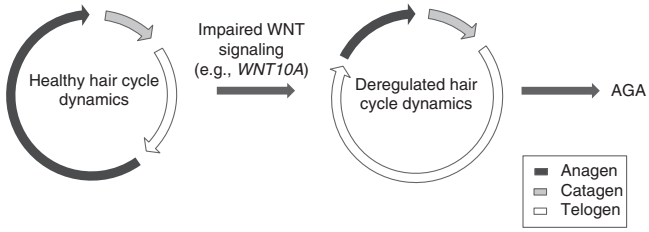

Figure 3. Proposed model for the functional role of *WNT10A* in the regulation of hair cycle dynamics. *WNT10A* promotes anagen induction and maintenance of anagen state. A reduction in *WNT10A* expression leads to deregulation of the healthy hair cycle, a delay in telogen to anagen transition, and shortening of the anagen phase, as observed in androgenetic alopecia (AGA) hair follicles.

Although our data and evidence from the literature suggests that WNT10A is involved in the etiology of AGA, any such role needs to be substantiated by additional studies including detailed functional studies or, at the genetic level, by identifying independently associated *WNT10A* alleles through resequencing and high-resolution association studies.

The association finding on chr2q35 also suggests an unexpected link between AGA and hair structure. The AGA risk allele (T) of rs7349332 was associated with curly hair in two independent GWAS studies (Medland *et al.*, 2009; Eriksson *et al.*, 2010). To our knowledge, no previous study has reported an association between AGA and curly hair at either a clinical or an epidemiological level. GWA studies enable investigation of whether this identified link is restricted to the *WNT10A* locus or whether it is more widespread, affecting additional genomic loci. As a preliminary step in this direction, we investigated whether the other genome-wide significant loci for AGA and curly hair were associated with the risk for the other trait. As this was not the case, the etiological overlap between the two traits may specifically involve the WNT signaling pathway. This hypothesis requires comprehensive investigation in large GWAS data sets.

In summary, this study provides genetic evidence for a contribution of WNT signaling to AGA etiology. We demonstrated that the AGA risk variant rs7349332 had a regulatory effect on *WNT10A* expression in the human hair follicle. This effect might be of functional relevance in the regulation of hair cycle dynamics. As WNT signaling has been implicated in a number of human disorders (Clevers and Nusse, 2012), this pathway might account for some of the unexpected associations of AGA with common traits as described by Li *et al.* (2012). The candidate genes for the genetic loci 3q25.1 and 5q33.3 remain unknown. Further studies using complementary strategies, such as re-sequencing and functional studies, are needed to identify these candidate genes and to further elucidate the actual functional causes that underlie these disease associations. As exemplified by the *WNT10A* finding, the identification of candidate genes on the basis of genetic association studies may provide new insights into AGA pathophysiology.

MATERIALS AND METHODS

Samples and DNA extraction

This replication study included two independent samples of European descent. For the ''Replication I'' sample, peripheral blood samples were collected from 177 unrelated AGA-affected males and 272 AGA-unaffected controls, all of German descent. The hair status of each participant was assessed by a dermatologist according to the Hamilton/Norwood (HN) classification. Affected men were aged <30 years with HN grades IV–VII, or <40 years with HN grades V–VII, and were thus representative of the most severely affected 10% of the distribution for the respective age classes (Hamilton, 1951; Norwood, 1975). The controls were aged >60 years and showed no signs of AGA, and were thus representative of the 20% least affected individuals in the population. DNA from lymphocytes was isolated by salting out with saturated NaCl solution (Miller *et al.*, 1988), or using Chemagic Magnetic Separation Module I (Chemagen, Baesweiler, Germany) according to the manufacturer's instructions. The second sample (''Replication II'') comprised unrelated male individuals of European descent, selected from users of the 23andMe Personal Genome Service (Eriksson *et al.*, 2010). The affection status for AGA was measured by self-assessment through a web-based survey. Individuals with HN grade $\geqslant$III and age of onset <40 years were defined as cases ($n=2{,}582$), and individuals with HN grade I and age $\geqslant30$ or HN grade II and age $\geqslant50$ were considered as controls ($n=2{,}389$). Individuals related to any individual included in our previous AGA meta-analysis (Li *et al.*, 2012) were also excluded. A summary of the case–control samples used in this study is provided in Supplementary Table S1 online. All studies were approved by the institutional ethics review committees of the respective organizations. The studies were conducted in accordance with the Declaration of Helsinki Principles, and all participants provided written informed consent.

Genotyping

Genotyping for the ''Replication I'' sample was carried out on Sequenom's Mass Array System (Sequenom, San Diego, CA) using the iPlex Gold assay in a multiplex reaction. On the basis of the association signals obtained in the meta-analysis (Li *et al.*, 2012), 12 loci were selected, for which at least one SNP had attained an association signal of $5\times10^{-8}<P<1\times10^{-5}$. For each locus, the two SNPs with the best association signals were selected for replication. SNP call rates of 90% were required for the analysis, and samples with call rates of $<90\%$ were excluded. Genotyping was successful for 21 of the 24 SNPs. All samples had genotyping call rates of $>90\%$. Primer sequences and standard assay conditions are available upon request. Genotyping for the ''Replication II'' sample was performed using Illumina (San Diego, CA) genome-wide SNP arrays as previously described (Li *et al.*, 2012). A total of 315 individuals were genotyped using a custom version of the Illumina HumanHap550 array, and 4,656 were genotyped using a custom version of the Illumina OmniExpress. All individuals were phased using Beagle 3.3.1 (Browning and Browning, 2007) and imputed against 1,000 Genomes haplotypes (August 2010 release) using Minimac (2011-10-27) (Howie *et al.*, 2012). For the SNPs included in Table 1, all had imputation $r^2>0.8$. Minor allele frequencies for the 24 SNPs in the replication samples are given in Supplementary Table S2 online.

Statistical analysis

The Armitage trend test (Armitage, 1955) was used for the single-marker analysis within the ''Replication I'' sample as implemented in the INTERSNP software package (Herold *et al.*, 2009). Only those SNPs that passed the following quality criteria were included in the analysis: minor allele frequency $>1\%$, and deviation from Hardy–Weinberg equilibrium *P*-values (P_{HWE}) in cases >0.001 and in controls >0.05. Single-marker analysis for the ''Replication II'' sample was performed using logistic regression with allele dosages from imputation and including five principal components as covariates to control for differences in ancestry. One SNP, rs7349332, was directly genotyped but not present in the 1,000 Genomes imputation reference panel; for this SNP, we performed logistic regression using the observed genotypes. All tested SNPs had $P_{HWE}>0.01$ across the tested individuals, rounding imputed allele dosages to the nearest genotype. A fixed-effects model (de Bakker *et al.*, 2008) and a random-effects model (Higgins *et al.*, 2003) were used to perform a meta-analysis of the data of all three studies (meta-analysis,

''Replication I,'' and ''Replication II''). To obtain OR estimates for the combined analysis, the allelic OR of the single studies were weighted according to their standard errors, and significance was assessed using the standard normal distribution (de Bakker *et al.*, 2008). The total variance Q was computed according to Cochrane (1954), and the between-study variance I^2 was calculated as suggested by Higgins *et al.* (2003). I^2 was used to determine whether a fixed-effects model or a random-effects model was appropriate. In case I^2 was significant after adjustment for the number of SNPs tested ($n=24$), a random-effects model was used instead of the fixed-effects model. However, we did not find evidence for heterogeneity across studies for the significant loci. We used the established significance threshold for GWAS of 5×10^{-8} (Frazer *et al.*, 2007). Interaction analysis was carried out within an extended case–control sample of 4,737 AGA patients and 4,159 controls from the 23andMe genomic service. A model with a ''g1*g2'' term was fitted and then tested for significance of the g1:g2 interaction by analysis of variance.

Expression analysis

DNA from the whole blood and RNA from the occipital hair follicles of 98 population-based German controls (age 20–40 years) were collected. Total RNA was extracted from human hair follicles (RNeasy Micro Kit, Qiagen, Hilden, Germany). The quality and quantity of the RNA were analyzed on a NanoDrop ND-1000 spectrophotometer (Peqlab Biotechnologie, Erlangen, Germany). RNA samples were additionally checked for degradation via gel electrophoresis in a BioAnalyzer 2100 (Agilent Technologies, Waldbronn, Germany) using RNA 6000 nano lab chips, according to the manufacturer's instructions. The Illumina TotalPrep-96 RNA Amplification Kit (Illumina) was used for the amplification and biotinylation of the RNA. Subsequent array-based gene expression analysis of individual tissue samples was performed on Illumina's human HT-12v4 Expression BeadChips using standard protocols (Illumina). The obtained fluorescence expression data were background subtracted and quantile normalized using the GenomeStudio software (Illumina). In the analysis of gene expression within human hair follicle, the AVG signal and the detection *P*-value were taken into account. Genes were assumed to be reliably expressed if the detection *P*-value was <0.01. If a gene or different transcripts of a gene were detected by more than one Illumina probe, the probe with the best expression values based on all samples tested was analyzed. Nonpresent fluorescence intensity after background subtraction or expression with detection *P*-value >0.01 was marked as ''—'' and defined as not expressed (Table 2). To calculate genotype-specific expression, only those samples that showed detection *P*-values of <0.01 were taken into account. DNA extraction and genotyping was performed as described above. Genotype-specific expression levels were calculated from the normalized AVG signals for the respective gene-specific probe relative to the expression levels of individuals with the AGA nonrisk genotype, which was set to 100%. The R software (version 2.10.1; http://www.R-project.org) was used for the statistical analysis, and *P*-values were calculated using a one-sided *t*-test.

Association with hair curl

To analyze for a potential etiological overlap between AGA and hair curl, we investigated all known AGA susceptibility loci for association with hair curl and vice versa. Hair curl was assessed and association tests were performed as previously described by Eriksson *et al.* (2010) using data from 29,353 unrelated individuals of European ancestry. We tested lead SNPs from a total of 12 AGA loci—the 8 loci identified in our previous meta-analysis (Li *et al.*, 2012) and the 4 additional loci identified in this study—for association with hair curl.

CONFLICT OF INTEREST

VM is a full-time employee of GSK; JYT, NE, and DAH have stock options in 23andMe; AKK is an employee of 23andMe.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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