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Manuscript Title: Differential expression between human dermal papilla cells from balding and non-balding scalps reveals new candidate genes for androgenetic alopecia

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Study conducted in Singapore, Singapore and London, UK.

Short title: Expression differences in balding and non-balding human DPC

Abbreviations: AGA -- androgenetic alopecia, DP – dermal papilla, DPC -- dermal papilla cells, BAB – immortalised balding dermal papilla cell, BAN – immortalised non-balding dermal papilla cell, DHT – dihydrotestosterone, EC – endothelial cell

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ABSTRACT

Androgenetic alopecia (AGA) is a common heritable and androgen-dependent hair loss condition in men. Twelve genetic risk loci are known to date but it is unclear which genes at these loci are relevant for AGA. Dermal papilla cells (DPC) located in the hair bulb are the main site of androgen activity in the hair follicle. Widely used monolayer-cultured primary DPC in hair-related studies often lack dermal papilla (DP) characteristics. In contrast, immortalised DPC have high resemblance to intact DP. We derived immortalised human DPC lines from balding (BAB) and non-balding (BAN) scalp. Both BAB and BAN retain high proportions of DP signature gene and versican protein expression. We performed expression analysis of BAB and BAN and annotated AGA risk loci with differentially-expressed genes. We found evidence for *AR* but not *EDA2R* as the candidate gene at the AGA risk locus on chromosome X. Further, our data suggest *TWIST1* and *SSPN* to be the functionally relevant AGA genes at the 7p21.1 and 12p12.1 risk loci, respectively. Down-regulated genes in BAB compared to BAN were highly enriched for vasculature-related genes, suggesting that deficiency of DPC from balding scalps in fostering vascularisation around the hair follicle may contribute to the development of AGA.

INTRODUCTION

Androgenetic alopecia (AGA) is a prevalent hair loss condition that affects up to 80% of European males by age eighty (Hamilton, 1951) and is characterised by bitemporal regression of hair growth in combination with hair loss at the vertex (Trueb, 2002). AGA development is attributed to androgen dependence (Hamilton, 1942) and genetic predisposition (Nyholt *et al.*, 2003; Rexbye *et al.*, 2005). The X-chromosomal androgen receptor (*AR*)/ectodysplasin A2 receptor (*EDA2R*) locus was the first described and replicated risk locus for AGA (Ellis *et al.*, 1998; Prodi *et al.*, 2008). In recent years, a total of 12 AGA risk loci have been described (Brockschmidt *et al.*, 2011; Heilmann *et al.*, 2013; Hillmer *et al.*, 2008; Li *et al.*, 2012; Richards *et al.*, 2008). However, the genes causative for increased AGA risk at the majority of the risk loci are still unclear. Functional understanding of AGA biology is lacking as it is not trivial and scalable to dissect the different compartments of hair follicles. Appropriate model systems for AGA are also unavailable. Medical treatments for AGA include minoxidil, which is hypothesized to promote hair growth by increasing vascular endothelial growth factor (*VEGF*) expression in dermal papilla cells (DPCs) during the growth (anagen) phase of the hair cycle. DPCs, located at the base of the hair follicle, are essential in the hair growth process. DPCs have the most consistent *AR* expression among all compartments of the hair follicle (Hodgins *et al.*, 1991) and thus are the postulated sites of androgen response, where *AR* activation by androgens affect gene expression regulating hair growth and cycle (Itami *et al.*, 1994; Kitagawa *et al.*, 2009). To date, only a macroarray-based comparative study interrogating 1,185 genes has been conducted on primary DPCs from balding and non-balding scalps (Midorikawa *et al.*, 2004). The limited life span of primary cell lines hampers complex and long term experiments.

In this study we present a robust and practical model system for AGA. We immortalised two human DPC lines, one derived from a balding vertex scalp area (BAB) and one derived from a non-balding occipital scalp area (BAN) and determined that both cell lines possess DP character. The utility of single cell lines are supported by their usage in discovery (Edwards *et al.*, 1980; Elenbaas *et al.*, 2001) and functional

studies (Boukamp *et al.*, 1988) for various disease conditions. We analysed BAB and BAN gene expression alterations in response to DHT treatment by microarray technology interrogating 26,000 genes and used the expression information to interpret described AGA risk loci. Further, we identified differentially-expressed genes between balding and non-balding DPCs revealing down-regulation of vasculature-related genes in DPCs of balding hair follicles.

RESULTS

Immortalised balding and non-balding DPC retain DP character

Immortalised balding DPC (BAB; **Figure 1a**) and non-balding DPC (BAN; **Figure 1b**) established from biopsies of balding and non-balding scalps of male patients by hTERT (human telomerase reverse transcriptase) expression were observed to have different cell morphologies. Both BAB and BAN retained DP characteristics after *in vitro* two-dimensional (2D) culturing, as indicated by the high expression of VCAN (versican) protein (**Figure 1c and d**), and the expression of human DP signature genes (Ohyama *et al.*, 2012), with 83 out of 117 (70.9%, $p < 0.001$) and 75 out of 117 (64.1%, $p < 0.05$) of DP signature genes expressed in BAN and BAB, respectively (**Figure 1e and S2, Table S1a**). DP signature genes have previously been defined as significantly up-regulated genes in freshly-dissected DP compared to dermal fibroblasts (Ohyama *et al.*, 2012). We observed similar fractions of DP signature gene expression in primary non-balding DP ($n = 2$) and primary balding DP ($n = 3$) cells which expressed 79 out of 116 (68.1%, $p < 0.01$) and 69 out of 116 (59.5%, $p < 0.05$) DP signature genes, respectively (**Table S1b**). Furthermore, we found that DP signature gene expression profile of both BAN and BAB were more similar to primary occipital DPC (HFDPC) than to primary fibroblasts (L5-F and NHDF) (**Supplementary Results**, **Figure S3**, **Table S1a** and **S1c**).

Differential gene expression analysis between BAB and BAN

Both inherent and DHT-dependent gene expression differences were found to be present between BAB and BAN (**Figure 2a**). Among the differentially expressed genes in BAB compared to BAN under 1 nM and 10 nM of DHT stimulation, 1,482 and 1,508 genes were found to be commonly up-regulated and down-regulated, respectively (**Figure 2c** and **d**; **Table S2a** and **S2b**). Subsequent analysis was carried out with commonly up- and down-regulated genes between both DHT concentrations. The trend of expression of qPCR validated genes (*CAV1* (caveolin 1), *EDNRA* (endothelin receptor type A), *IGFBP5* (insulin-like growth factor binding protein 5), and *SCG2* (secretogranin II)) was consistent with that from the microarray data (**Figure 2e-h**).

Annotation of AGA risk loci with differentially-expressed genes in BAB compared to BAN

Genome-wide association studies (GWAS) have identified genomic loci which confer risk to develop AGA. However, it is largely unclear which genes at these loci are causal for the associations. Our tissue and phenotype-specific expression data allowed us to infer which genes at the risk loci most likely contribute to AGA development. Twelve differentially-expressed genes in BAB compared to BAN overlapped within 500 kb of seven lead SNPs at established AGA risk loci (**Table 1** and **S8**). On Xq12, the major AGA risk locus, *AR* was up-regulated in BAB compared to BAN suggesting that *AR* rather than the centromeric *EDA2R* contributes to AGA. At the 2q37 risk locus, only *PER2* and *TWIST2* showed expression differences within 500 kb from the lead SNP rs9287638, and therefore are more likely candidate genes at this locus than non-differentially expressed *HDAC4*. At the 5q33.3 risk locus, down-regulated *RNF145* is a potential candidate gene. Up-regulation of *TWIST1* was found at the 7p21.1 risk locus. Furthermore, our finding of 7q11.22 candidate gene *AUTS2* being down-regulated in BAB compared to BAN is in agreement with a previous study (Li *et al.*, 2012) which suggested *AUTS2* as the candidate gene causing the association of this locus with AGA. On 12p12.1, *SSPN* showed down-regulation and is the more likely candidate gene at this locus instead of non-differentially expressed *ITPR2*. On 17q21.31, down-regulated gene *MAPT* is the likely candidate gene instead of non-differentially expressed *SPPL2C*.

Gene ontology analysis of differentially-expressed genes in BAB as compared to BAN

Down-regulated genes in BAB compared to BAN under DHT treatment were found to be most enriched in gene ontology (GO) clusters related to vasculature (max enrichment score = 10.516; 65 genes) and included the sub-terms of vasculature development, blood vessel development and blood vessel morphogenesis (**Figure 3a**; **Table 2**, **S3a** and **S4a**). We found support for vasculature-related differences between BAB and BAN by immunofluorescence (**Figure 3c** and **d**) and migration assay experiments (**Figure S5 and Supplementary Results**). We tested the down-regulated vasculature-related genes *CAV1*, *CYR61* (cysteine-rich, angiogenic inducer, 61) and *MMP14* (matrix metallopeptidase 14) by immunofluorescence and observed protein down regulation for MMP14 ($p = 0.0004$) and CAV1 ($p = 0.0002$) in BAB compared to BAN (**Figure 3c** and **d**). A significant number of the identified vasculature-related genes (**Table 2 and S3a)** were also found to be down-regulated in independent samples of primary balding (n = 3) compared to non-balding DPC (n = 2; $p < 0.001$; 16 out of 65: **Table S3c, Supplementary Results**). Up-regulated genes were most enriched in GO clusters related to cell cycle and mitosis (max enrichment score = 38.551; 139 genes; **Figure 3b** and **Table S4b**).

Motif analysis in promoters of differentially-expressed genes in BAB compared to BAN

We performed a transcription factor (TF) motif enrichment analysis in promoters of differentially-regulated genes in BAB compared to BAN to investigate potential TF implicated in characteristic differences between the cell types. The SIX1 motif which was enriched in promoters of down-regulated genes (**Table S2b** and **S9a**) is implicated in hair placode development (Rhee *et al.*, 2006). While enriched motifs in the promoters of up-regulated genes (**Table S2a** and **Table S9b**) include E2F and CHR motifs which correlate with biological function in cell cycle and mitosis (**Figure 3b; Table S4b**). We have also identified genes which could be synergistically regulated by multiple TF due to the co-occurrence of E2F, NFY and CHR sites (**Table S9d**) (Tabach *et al.*, 2005) (**Supplementary Results)**.

DISCUSSION

In this study, we have demonstrated that both 2D-cultured immortalised balding DPC and non-balding DPC maintained DP characteristics and therefore provide a model for studying AGA and hair biology. First, both BAB and BAN retained significant expression of a majority of DP signature genes (BAB: 64.1%, $p < 0.05$; BAN: 70.9%, $p < 0.001$; **Figure 1e** and **Table S1a**) defined from expression profiling of freshly-dissected human DPs (Ohyama *et al.*, 2012). DP signature genes interrogated for include classical DP signature genes such as *ALPL, WIF1, LEF1* (Kratochwil *et al.*, 1996) and *VCAN* (Ohyama *et al.*, 2012; Soma *et al.*, 2005), of which *ALPL, LEF1,* and *VCAN* were expressed in BAB and BAN (**Figure 1e** and **Table S1a**). Second, both DPC lines stained positively for VCAN protein which expression is correlated with DP inductivity and hair morphogenesis (Kishimoto *et al.*, 1999; Soma *et al.*, 2005). The BAB and BAN cell lines serve as a useful model system for AGA as they are simple to maintain in culture, requiring only FBS-supplemented media. In contrast, primary DPC which are widely used in hair-related studies are less ideal models, as primary DPC lose DP character upon 2D-culturing (Higgins *et al.*, 2013) and are challenging to maintain in culture over extended periods. We were only able to immortalise balding and non-balding DPC from non-matched patient biopsies as primary balding DPC were prone to senescence during the initial culturing process and had low success rates for immortalisation. The difficulty of establishing immortalised DPC lines are further evidenced by the lack of such cell lines to date, to the best of our knowledge. Further, we would like to emphasize that single cell lines such as HaCaT (Boukamp *et al.*, 1988), MCF7 (Edwards *et al.*, 1980) and HMEC (Elenbaas *et al.*, 2001) have contributed significantly to our understanding of cell type-specific behaviour and serve as disease models. It is desirable to establish more balding and non-balding DP cell lines to validate the transcriptomic signatures. Further, cell lines from vertex with full hair should be included to dissect balding-specific from vertex-specific signatures.

We annotated AGA risk loci with differentially-expressed genes in BAB compared to BAN and found differential gene expression in seven out of the twelve interrogated genomic regions (**Table 1** and **S8**).

Notably, we found *AR* to be the likely candidate gene at the major risk locus for AGA (Xq12, rs2497938) instead of *EDA2R*. This is consistent with previous observations of higher AR levels in balding scalps (Hibberts *et al.*, 1998) and reinforced the importance of the androgen/AR interaction in AGA development. Furthermore, the identification of *MAPT* as the relevant AGA candidate gene at 17q21.31 instead of *SPPL2C* suggests that changes in *MAPT* function connect the risk for AGA with the risk for Parkinson's disease (Li *et al.*, 2012).

None of the candidate genes at the 20p11 susceptibility locus (rs6047844), *FOXA2* (forkhead box A2) and *PAX1* (paired box 1), were differentially expressed between BAB and BAN. *PAX1*, but not *FOXA2*, was expressed in BAB and BAN. The lack of differentially-expressed genes within 500 kb from rs6047844 suggest (i) that this susceptibility region may be involved in long-range chromatin interaction that result in the regulation of distant candidate genes, or (ii) that DPCs are not the cell type which confers the AGA-related functional effect of this locus or (iii) that the BAN/BAB model cell lines do not preserve this characteristic.

At the 1p36 locus, non-differentially-expressed candidate gene *TARDBP* (TAR DNA binding protein) is unlikely to be causative. Instead, *SRM*, previously mentioned as a potential candidate gene due to its proximity to rs12565727 (Li *et al.*, 2012), was up-regulated in BAB compared to BAN. SRM is involved in the synthesis of spermidine which acts on matrix keratinocytes to promote hair elongation and prolong anagen (Ramot *et al.*, 2011). However, the effect of SRM activity and the resultant spermidine synthesized in DPC is unknown. Further, we found *CASZ1*, *EXOSC10*, *FRAP1*, and *UBIAD1* to be differentially-expressed at this locus. They provide new potential candidate genes for hair loss/growth modulation.

The candidate genes *HDAC4*, *EBF1* and *HDAC9* at susceptibility loci 2q25, 5q33.3 and 7p21.1, respectively, were not differentially-expressed between BAB and BAN and thus are unlikely to be the causative genes at these loci. Instead, we found four other differentially-expressed genes in BAB compared to BAN as potential candidates. *RNF145*, located at 5q33.3, is implicated in endoplasmic

reticulum-associated protein degradation (Kikkert *et al.*, 2004) and apoptosis (Ho *et al.*, 2014). At the 7p21.1 locus, we found *TWIST1*, a DP signature gene expressed in both BAB and BAN (**Figure 1e** and **Table S1**) being up-regulated in BAB (**Table 1**). Twist1, a bHLH (basic helix-loop-helix) protein, is crucial for anagen-to-catagen transition during the hair growth cycle as Twist1 protein ablation in adult mice DP result in prolonged anagen (Xu *et al.*, 2013). Hence, *TWIST1* up-regulation in DPC of balding scalps as compared to non-balding scalps may result in accelerated transition from anagen to catagen and thus shortened period of anagen during the hair cycle; a phenomenon in balding scalps that leads to the formation of short vellus hairs instead of long terminal hairs (Paus and Cotsarelis, 1999). Furthermore, bHLH proteins such as Twist1 bind to the consensus 5'-NCANNTGN-3' E-box motif, thus the down-regulation of genes in BAB compared to BAN with E-box motif in their promoter regions (**Table S9a**; **Supplementary Results**) may be attributed to repression by TWIST1. Interestingly, we identified another TWIST protein, *TWIST2*, as a potential candidate gene at the 2q37 risk locus. *TWIST2* has been implicated in mesenchymal cell lineage development (Li *et al.*, 1995) but little is known about its function in the DP. The combinatorial binding and interaction of TWIST1 and/or TWIST2 with other bHLH proteins (Franco *et al.*, 2010) at the promoters of target genes may result in gene regulation in DPC. TWIST1 also interacts and binds to HDAC4 (Danciu and Whitman, 2009) to regulate gene expression (Gong and Li, 2002; Lee *et al.*, 2003). In addition, binding of TWIST1 at E-boxes in the *AR* promoter region result in up-regulated *AR* expression (Shiota *et al.*, 2010). Our observation of both *TWIST1* and *AR* up-regulation in BAB as compared to BAN could be attributed to increased regulation of *AR* expression by the increased TWIST1 levels in BAB compared to BAN. This potential relationship between *AR* and *TWIST1* in balding DPC, which to our knowledge has not been considered previously, provides support for these two candidate genes to be the causative AGA genes at the 7p21.1 and Xq12 susceptibility loci.

Down-regulated genes in BAB compared to BAN were significantly enriched in vasculature-related GO cluster (**Figure 3a**; **Table 2** and **S3a**), with 41.5% (27 genes) of the vasculature-related genes (**Table S3a**) being down-regulated in BAB compared to BAN through DHT treatment (**Table S3b**). Moreover, a

significant number of vasculature-related genes identified (**Table 2 and S3a)** were also found to be down-regulated in independent samples of primary balding DPC as compared to non-balding DPC ($p < 0.001$; 16 out of 65: **Table S3c**, **Supplementary Results**). The Maz motif, which has been found in promoters of angiogenesis-related genes (Yang *et al.*, 2008), was enriched in down-regulated genes in BAB compared to BAN (**Table S9a**). Furthermore, a trend analysis suggested less coherent vasculature-related gene expression changes in BAB compared to BAN over time (**Figure S8**). Hair follicle vascularisation affects follicle size and length (Yano *et al.*, 2001), with arrangement and density of vasculature altering during anagen (Durward and Rudall, 1958) and catagen (Ellis and Moretti, 1959). Reduced vasculature has also been observed around DP of balding vertex scalps as compared to non-balding scalps (Cormia and Ernyey, 1961). The ability to attract endothelial cells for follicular vasculature network regeneration is therefore closely tied to hair follicle cycling and growth.

In summary, the immortalised DPC lines, BAB and BAN, introduced in this study provide a useful resource for AGA-related studies. We identified a set of vasculature-related genes which were down-regulated in balding DPC under the influence of DHT. With the limitation that our finding is based on two individual cell lines only, we suggest that DPC in balding scalps may be deficient in fostering vasculature development as compared to DPC in non-balding scalps. Further in-depth studies are required for validating this finding, deciphering the contribution of identified molecular elements in altering vasculature in the hair follicle and whether this underlies the development of AGA. Our annotation of AGA risk loci with (differentially) expressed genes in BAB compared to BAN has shown that it is insufficient to rely solely on expression of proximal genes to lead association SNPs for candidate gene discovery at susceptibility loci. Our findings point to *AR* as the candidate gene at the lead AGA risk locus on chromosome X. We also identified *TWIST1* as a functionally relevant gene for AGA at the 7p21.1 susceptibility locus. In depth studies are required to ascertain the causative role of these candidate genes in the development of AGA.

MATERIALS AND METHODS

Isolation and immortalisation of human balding and non-balding primary DPC

Between one and three DP were isolated from each matched 2 mm punch biopsies of balding (frontal) and non-balding (occipital) scalps of male AGA patients who were undergoing hair transplant surgery and not currently on hair loss medications, as described previously (Bahta *et al.*, 2008; Philpott *et al.*, 1994; Upton *et al.*, 2015). Ethics approval was obtained from East London and City health authority (T/98/008) and all biopsies were taken with full patient written consent. All experiments adhered to the Declaration of Helsinki Principles. Isolated primary DPCs were cultured up to passage 3 (**Supplementary Materials and Methods**) and immortalised with hTERT using pBABEhygro-hTERT. Hygromycin-resistant clones with stable hTERT expression were then cultured as described in the **Supplementary Materials and Methods**. We were able to derive one immortalized balding (BAB) and one non-balding (BAN) cell line originating from two different male individuals due to limitation in tissue materials, difficulties in establishing pure primary DPC cultures and low transformation efficiencies. Both BAB and BAN have been established from Anglo-Saxon males with Hamilton scale 4 AGA.

Microarray and processing

Gene expression in BAB, BAN, primary occipital DPC (HFDPC) and primary fibroblasts (L5-F and NHDF) were interrogated with HumanHT-12 v4 BeadChip arrays (Illumina, St. Diego, CA). Gene expression of 3 primary balding DPC (Passage 2) and 2 primary non-balding DPC (Passage 2) were interrogated with HG 133A GeneChips arrays (Affymetrix Inc., Santa Clara, CA) (**Figure S1, Supplementary Materials and Methods)**. All 5 primary DPC samples and the immortalised BAB and BAN were isolated from independent subjects.

Characterisation of BAB and BAN

Immunostaining for versican was conducted on BAB and BAN to determine expression of DP marker (**Supplementary Materials and Methods**). Expression of 117 DP signature genes was interrogated in

BAB, BAN, primary balding and non-balding DPC from AGA individuals, primary occipital DPC (HFDPC) and primary fibroblasts (L5-F and NHDF) (**Supplementary Materials and Methods**). Gene expression of DP signature genes was also validation by RT-qPCR in BAB and BAN (**Supplementary Materials and Methods**).

Differential gene expression analysis between BAB and BAN

BAB and BAN were conditioned in phenol red-free DMEM media for 24 hr, and treated with 1 nM or 10 nM DHT for 15 min, 30 min, 1 hr, 3 hr, 6 hr, 12 hr, 18 hr, 20 hr, 24 hr, 36 hr and 48 hr prior to microarray analysis. GO analysis was then carried out on differentially expressed genes found in BAB as compared to BAN under both 1 and 10 nM DHT treatment (**Figure S1, Supplementary Materials and Methods**). The concentration of DHT applied on DPC was within the adult human male physiological DHT serum levels (Cailleux-Bounacer *et al.*, 2009; Feldman *et al.*, 2002). Expression of differentially-expressed genes *CAV1*, *EDNRA*, *IGFBP5* and *SCG2* were also validation by RT-qPCR (**Supplementary Materials and Methods**). Immunostaining for CYR61, MMP14 and CAV1 was conducted to validate expression of differentially-expressed genes (**Supplementary Materials and Methods**).

Annotation of AGA risk loci with differentially-expressed genes in BAB as compared to BAN

As differentially-expressed genes at the risk loci were likely to be causative genes that contribute to AGA development, we overlapped differentially expressed genes with 12 lead SNPs with genome-wide association significance using upstream and downstream windows of 500 kb, 100 kb and 50 kb (**Supplementary Materials and Methods**).

CONFLICT OF INTEREST

There are no conflicts of interest for declaration.

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Table 1: Overlap of differentially-expressed genes identified in balding DPC (BAB) compared to non-balding DPC (BAN) with AGA risk loci of genome-wide significance

					500kb		100kb		50kb		Median fold change	
Chr	**Position[1]**	**SNP[2]**	**Genomic region**	**Genes[3]**	**Diff genes[4]**	**Up/ down[5]**	**Diff genes[4]**	**Up/ down[5]**	**Diff genes[4]**	**Up/ down[5]**	**1nM DHT**	**10nM DHT**
1	11,033,082	rs12565727[6]	1p36	[]--*TARDBP*	*CASZ1*[15]	down	-	-	-	-	2.010	1.945
					EXOSC10[15]	up	*EXOSC10*[15]	up	-	-	1.918	1.998
					FRAP1[15]	up	-	-	-	-	1.740	-
					SRM	up	*SRM*	up	-	-	2.254	2.122
					UBIAD1[15]	up	-	-	-	-	2.154	1.945
2	219,756,383	rs7349332[7]	2q35	[*WNT10A*]	-	-	-	-	-	-	-	-
2	239,694,631	rs9287638[6]	2q37	[]--*HDAC4*	*PER2*[15]	down	-	-	-	-	1.933	1.909
					TWIST2[15]	down	*TWIST2*[15]	down	-	-	1.949	2.305
3	151,653,368	rs4679955[7]	3q25.1	*SUCNR1*--[]---*MBNL1*	-	-	-	-	-	-	-	-
5	158,310,631	rs929626[7]	5q33.3	[*EBF1*]	*RNF145*[15]	down	-	-	-	-	1.730	-
7	18,877,874	rs2073963[6,8]	7p21.1	[*HDAC9*]	*TWIST1*[15]	up	-	-	-	-	2.548	2.244
7	68,611,960	rs6945541[6]	7q11.22	[]---*AUTS2*	*AUTS2*	down	-	-	-	-	2.801	2.868
12	26,426,420	rs9668810[7]	12p12.1	*SSPN*--[]--*ITPR2*	*SSPN*	down	*SSPN*	down	*SSPN*	down	2.197	2.133
17	43,924,219	rs12373124[6]	17q21.31	[*SPPL2C*]--*MAPT*	*MAPT*	down	*MAPT*	down	*MAPT*	down	1.930	1.835
18	42,800,148	rs10502861[6]	18q21.1	*SETBP1*---[]	-	-	-	-	-	-	-	-
20	22,037,575	rs6047844[6,9,10]	20p11	*PAX1*--[]--*FOXA2*	-	-	-	-	-	-	-	-
X	66,563,018	rs2497938[6,1,12,13,14]	Xq12	*EDA2R*---[]--*AR*	*AR*	up	-	-	-	-	1.751	1.737

[1] - Chromosomal position in GRCh37/hg19 assembly; [2] - references include first descriptions of associations of the respective locus that not necessarily have the same SNP; [3] - Candidate genes harbouring SNP or in close proximity to SNP; [4] - Differentially-expressed genes in balding DPC (BAB) compared to non-balding DPC (BAN) that are within window stated to SNP - up: differentially-expressed gene is up-regulated in BAB compared to BAN, down: differentially-expressed gene is down-regulated in BAB compared to BAN; [6] - (Li *et al.*, 2012); [7] - (Heilmann *et al.*, 2013); [8] - (Brockschmidt *et al.*, 2011); [9] - (Hillmer *et al.*, 2008); [10] - (Richards *et al.*, 2008); [11] - (Ellis *et al.*, 2001); [12] - (Hillmer *et al.*, 2005); [13] - (Brockschmidt *et al.*, 2010); [14] - (Prodi *et al.*, 2008); [15] - Novel candidate genes not considered by previous publications

Table 2: List of vasculature-related genes that are down-regulated in balding DPC (BAB) compared to non-balding DPC (BAN)

Gene Name	Median fold change		Gene Name	Median fold change	
	1nM DHT	10nM DHT		1nM DHT	10nM DHT
ACTC1	2.628	2.532	GNA13	-	1.885
ADORA2A	1.924	1.752	GUCY1A3	1.832	1.846
ADRA1B	1.833	1.749	HEY1	2.046	2.402
ADRB2	2.106	2.590	HIF1A	2.557	2.670
AGT	2.008	1.881	HIF1A	2.806	2.850
AGTR1	2.788	2.643	HIF1A	2.822	2.546
AGTR1	2.803	2.621	HMOX1	1.895	1.934
AMOT	1.851	1.910	HTATIP2	6.764	6.038
ANGPTL4	2.054	2.220	ITGA1	2.008	1.956
ANXA2	2.500	2.211	ITGA4	1.784	1.748
APOLD1	2.371	2.661	JUNB	2.325	2.002
BGN	2.314	2.324	KLF5	1.738	1.802
CAV1	2.884	2.708	LEPR	2.042	1.987
CAV2	1.857	1.954	LOX	3.116	2.974
CAV2	1.819	1.775	MMP14	2.090	2.003
CAV2	1.827	1.741	NOS3	2.154	1.955
CCBE1	2.250	2.171	NOTCH1	1.713	1.813
CDH13	1.829	1.867	P2RX4	1.830	1.806
CDH2	1.734	1.782	PDE5A	2.809	2.697
CHD7	2.047	2.187	PDPN	1.931	1.957
CITED2	2.967	2.623	PLAU	1.788	1.882
COL1A1	5.069	4.926	PLCD3	1.750	1.770
COL1A2	1.808	1.757	PLXDC1	1.992	1.846
COL3A1	3.723	3.615	PPAP2B	1.879	1.870
COL18A1	16.750	15.675	PPAP2B	2.235	2.225
CTGF	3.546	3.587	RECK	2.463	2.351
CTGF	3.963	4.229	RECK	2.458	2.314
CXCL12	2.635	2.631	SCG2	5.365	5.938
CXCL12	2.391	2.151	SGPL1	1.910	1.828
CXCL12	2.518	2.643	SMAD7	1.841	1.729
CYR61	3.167	3.023	SMO	1.783	1.830
DHCR7	23.464	20.353	SOD2	-	1.752
DHCR7	17.994	14.520	SOD2	2.306	2.355
DICER1	1.860	1.800	SOD2	1.969	2.367
DICER1	1.896	1.898	TGFA	3.281	3.060
EDNRA	1.831	1.978	TGFA	2.146	2.328
EREG	1.986	2.303	THY1	2.871	2.781
FGF2	2.087	2.018	TNFAIP2	3.180	2.792
FGF2	1.971	1.954	ZC3H12A	1.890	2.237
GCH1	2.957	3.348	ZFP36L1	3.244	3.055
GNA13	1.784	1.755	ZMIZ1	1.835	1.746

FIGURE LEGENDS

Figure 1: Characterisation of immortalised balding DPC line (BAB) and non-balding DPC line (BAN). (a) BAB grows in a non-monolayer manner and does not cover entire culture area at 100% confluency, (b) BAN grows in a monolayer and displays whorl-like pattern. Bar = 1000 µm. Both (c) BAB and (d) BAN express dermal papilla marker protein, versican. Cell nuclei marked by DAPI staining (blue). Bar = 100 µm. (e) 83 out of 117 (70.9%, $p < 0.001$) DP signature genes were expressed in BAN (green square). 75 out of 117 (64.1%, $p < 0.001$) DP signature genes were expressed in BAB (blue square). 69 DP signature genes were expressed in both BAN and BAB. 28 DP signature genes not expressed in BAB and BAN are listed outside of blue and green squares.

Figure 2: Differential gene expression in non-balding (BAN) compared to balding DPC (BAB). (a) Gene expression in BAN and BAB are inherent and DHT-dependent. (b) Differential genes in BAB compared to BAN under 1 and 10 nM DHT treatment, and (c) commonly up- and (d) down-regulated genes under both concentrations of DHT stimulation. RT-qPCR validation of down-regulated genes (e) *CAV1*, (f) *EDNRA*, (g) *IGFBP5* and (h) *SCG2* in BAB as compared to BAN under 10 nM DHT treatment. Expression fold decrease in BAB compared to BAN from microarray analysis presented in upper plots. *GAPDH*-normalised relative gene expression in BAB compared to BAN from RT-qPCR analysis (ddCp) presented in the lower plots. Fold change threshold cut-off of 1.7 indicated in red. # indicate fold change ≤ 1.7 from microarray analysis.

Figure 3: Gene ontology (GO) clustering analysis of differentially-expressed genes in balding (BAB) compared to non-balding DPC (BAN). (a) Down-regulated genes in BAB compared to BAN were highly enriched in vasculature-related GO clusters (sub-terms: vasculature development, blood vessel development and blood vessel morphogenesis, **Table 2**), cell motion and cell migration, cell death, phosphate and phosphorous metabolism and protein kinase (**Table S4a**). (b) GO clustering of up-regulated genes in BAB compared to BAN (**Table S4b**). Maximum enrichment score of each GO cluster

is indicted below respective term. (c,d) Expression levels of vasculature-related proteins, CYR61, MMP14 ($p = 0.0004$) and CAV1 ($p = 0.0002$) were found to be decreased in BAB compared to BAN. ** indicate $p < 0.001$. Cell nuclei marked by DAPI staining (blue). Bar = 100 µm. HepG2 as negative control for CAV1.

SUPPLEMENTAL DATA

Supplementary Materials and Methods

Supplementary Results

Supplementary Discussion

Figure S1: Microarray data analysis pipeline for (a) balding DPC (BAB) and non-balding DPC (BAN) samples and (b) primary balding DPC and non-balding DPC

Figure S2: Validation of eight DP signature genes by RT-qPCR.

Figure S3: DP signature gene expression in DPC and fibroblast.

Figure S4: Vasculature-related gene expression in BAB, BAN and fibroblast.

Figure S5: Validation of vasculature-related differences in balding DPC (BAB) compared to non-balding DPC (BAN) with transwell endothelial cell migration assay.

Figure S6: Overlap of differential genes in BAB compared to BAN with and without DHT stimulation.

Figure S7: Overlap of down-regulated vasculature-related genes in BAB as compared to BAN with and without DHT treatment.

Figure S8: Trend analysis of significantly expressed genes in non-balding (BAN) and balding DPC (BAB) under DHT treatment.

Table S1: (a) Expression level of DP signature genes in non-balding (BAN) and balding (BAB) DPC. (b) Expression level of DP signature genes in primary balding DPC and primary non-balding DPC. (c) Expression level of DP signature genes in primary DPC (HFDPC) and primary fibroblast (NHDF and L5-F).

Table S2: (a) Commonly up-regulated genes in BAB compared to BAN under 1 nM and 10 nM DHT stimulation. (b) Commonly down-regulated genes in BAB compared to BAN under 1 nM and 10 nM DHT stimulation

Table S3: (a) Detailed list of vasculature-related genes that are down-regulated in balding DPC (BAB) compared to non-balding DPC (BAN) with DHT treatment. (b) List of vasculature-related genes that are down-regulated in balding DPC (BAB) compared to non-balding DPC (BAN) without DHT treatment. (c) List of vasculature-related genes that are down-regulated in primary balding DPC as compared to primary non-balding DPC. (d) Expression level of vasculature-related genes in primary fibroblast.

Table S4: (a) Top functional enrichment clusters of down-regulated genes in BAB compared to BAN under 1 nM and 10 nM DHT stimulation. (b) Top functional enrichment clusters of up-regulated genes in BAB compared to BAN under 1 nM and 10 nM DHT stimulation.

Table S5: qPCR validation primer sequences

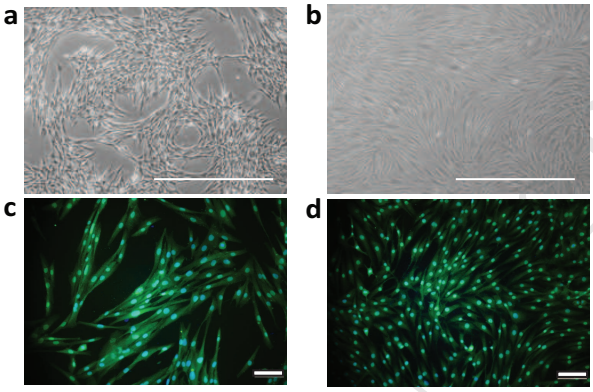
Table S6: (a) Up-regulated genes in BAB compared to BAN without DHT stimulation. (b) Down-regulated genes in BAB compared to BAN without DHT stimulation.

Table S7: (a) Top functional enrichment clusters of down-regulated genes in BAB compared to BAN without DHT stimulation. (b) Top functional enrichment clusters of up-regulated genes in BAB compared to BAN without DHT stimulation.

Table S8: Overlap of expressed genes in BAB and BAN and differentially-expressed genes identified in BAB compared to BAN with AGA risk loci of genome-wide significance

Table S9: (a) Motif analysis in promoter regions of down-regulated genes in BAB compared to BAN under DHT treatment (b) Motif analysis in promoter regions of up-regulated genes in BAB compared to BAN under DHT treatment. (c) Up-regulated genes in BAB compared to BAN with NFY and Sp1

binding motif in the promoter. (d) Up-regulated genes in BAB compared to BAN with E2F, NYF and CHR promoter motifs.



e

Expressed in BAN only

APOD
ARHGEF3
BMP4
DIO2
FOXO1
GAS7
GMFG
GUCY1A3
IFI27
LXN
LZTS1
NOG
PLCG2
RBP1

Total: 14

Expressed in both BAB and BAN

ACACB	H3F3B	PHF15	SPRY4
ALPL	HBEGF	PIK3R1	SVIL
BAMBI	HEY1	PLA2G4A	SYNM
BCL2	INHBB	PTGS2	TFAP2A
CASP1	JARID2	PTPRE	TFAP2C
CLCA2	LEF1	RABGAP1L	THBD
CPE	LRP4	RGS2	TMEM100
CREM	LYN	RNF144A	TNFAIP3
CSGALNACT1	METTL7A	RORA	TRIM2
CSTA	MIPEP	SAT1	TRPS1
CTSH	MITF	SDC1	TWIST1
CYP26B1	MLLT11	SEMA4C	VAV3
DYNC1I1	MMD	SERPINI1	VCAN
EDNRA	MTSS1	SETBP1	WNT5A
FABP5	MYLIP	SLC7A8	ZMYND8
FGF7	NDP	SLCO3A1	
FRZB	NET1	SNCAIP	
GPR125	NLRP1	SPRY1	

Total: 69

Expressed in BAB only

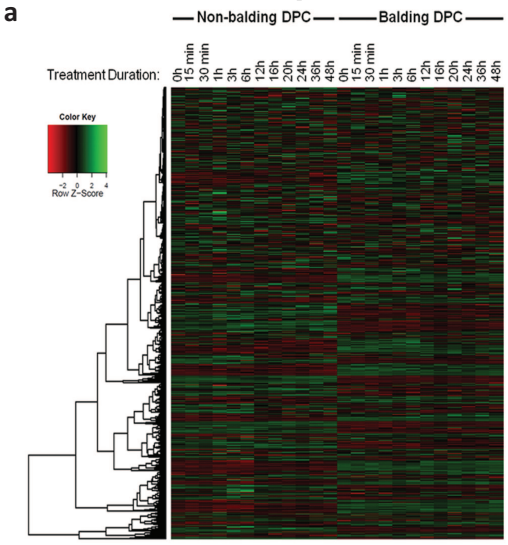
BMP2
CCND2
GPM6B
MEF2C
SOSTDC1
STON1

Total: 6

Not expressed in both BAB and BAN

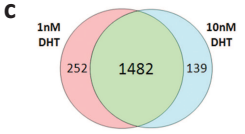
A2M	LPL
ALX4	MAF
APOE	MGP
AQP1	MREG
CRIP2	MTUS1
EDN3	NTRK2
EGR3	PEG3
ETV1	PIK3R3
GPRC5B	RASL11B
HEY2	SFN
HLA-DPB1	SNAI1
IGF1	SPARCL1
LAMC3	SPON1
LHCGR	WIF1

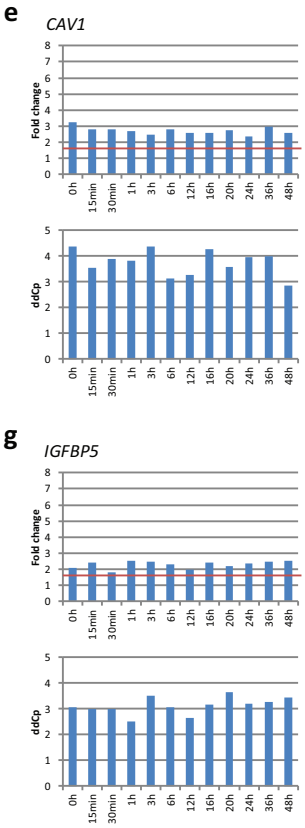
Total: 28

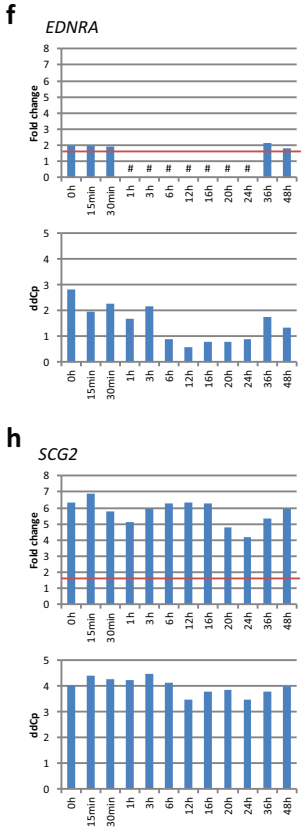


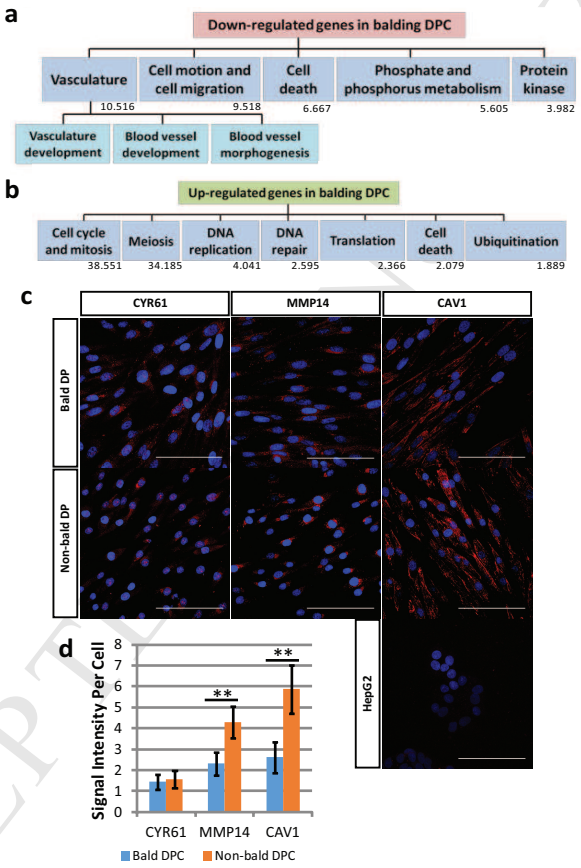
b

p ≤ 0.001, fold change ≥ 1.7	Balding DPC versus Non-balding DPC	
	1nM DHT	10nM DHT
Up-regulated genes	1734	1621
Down-regulated genes	1725	1654
Total:	3446 (+ 13 common genes)	3267 (+ 8 common genes)








a
Down-regulated genes in balding DPC
Vasculature
Cell motion and cell migration
Cell death
Phosphate and phosphorus metabolism
Protein kinase
10.516
9.518
6.667
5.605
3.982
Vasculature development
Blood vessel development
Blood vessel morphogenesis
b
Up-regulated genes in balding DPC
Cell cycle and mitosis
Meiosis
DNA replication
DNA repair
Translation
Cell death
Ubiquitination
38.551
34.185
4.041
2.595
2.366
2.079
1.889
c
CYR61
MMP14
CAV1
Bald DP
Non-bald DP
HepG2
d
Signal Intensity Per Cell
0
1
2
3
4
5
6
7
8
**
**
CYR61
MMP14
CAV1
Bald DPC
Non-bald DPC