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**Change in hair growth-related gene expression profile in human isolated hair follicles induced by 5-alpha reductase inhibitors – dutasteride and finasteride – in the presence of testosterone**

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## INTRODUCTION

Dihydrotestosterone (DHT) plays a key role in the pathogenesis of male androgenetic alopecia (AGA) and is converted from the androgen, testosterone, by 5-alpha reductase (5AR). DHT binds to androgen receptors in the hair follicles, causing shortening of the anagen or growing phase of the hair cycle and leading to hair follicle miniaturisation.<sup>(1)</sup> Over time, large terminal hairs are lost and progressively replaced by thin, short, villus-like hairs, resulting in a characteristic pattern of baldness.<sup>(1,2)</sup>

Oral treatment with 5AR inhibitors (5ARIs; e.g. dutasteride and finasteride) is used for men with AGA, and is recommended by Japanese guidelines as the first-line treatment for male AGA and female pattern hair loss.<sup>(3)</sup> Although these agents inhibit testosterone conversion to DHT, their mechanisms of action are different – finasteride specifically inhibits type II 5AR, but dutasteride inhibits both type I and type II 5AR.<sup>(4,5)</sup> Consequently, the superiority of dutasteride 0.5 mg to finasteride 1.0 mg, with respect to changes in hair count and width, has been demonstrated in a global phase III clinical trial<sup>(6)</sup> and documented in review articles.<sup>(7,8)</sup>

However, it is unclear whether type I 5AR is one of the enzymes driving the hair loss process. Moreover, downstream molecular events following 5AR inhibition are poorly understood. Therefore, in-depth molecular analyses are necessary to clarify the extent of the contribution of type I 5AR to hair growth.

This study investigated gene expression changes in growth factors and other related molecules responsible for hair growth using the bulbar portions of hair follicles (BPHF) isolated from plucked human hair and evaluated the involvement of type I 5AR in human hair growth.

## METHODS

This study was approved by the Manchester Consumer Healthcare Research Ethics Committee. In accordance with the Declaration of Helsinki, all volunteer hair donors provided written informed consent.

Anagen hair follicles were plucked from frontal, parietal and frontal/temporal areas of five healthy male donors, with varying degrees of AGA (Norwood-Hamilton classification ranging from score 2A–7). Plucked hair follicles were cultured in Dulbecco's Modified Eagle's Medium, containing proprietary growth media, L-glutamine 200 nM (Cat: G7513; Sigma Aldrich, Gillingham, UK) and penicillin-streptomycin (Cat: P0781; Sigma Aldrich), in the presence of DHT or testosterone in 0.1% (v/v) dimethyl sulfoxide (DMSO) or DMSO vehicle only. Hair follicles were incubated at 37°C in a 5% carbon dioxide atmosphere for 24 hours, with and without 5ARIs (dutasteride or finasteride), in the presence of testosterone, as previously described, with modification.<sup>(9)</sup> Post-culture, anagen hairs were visually assessed for dermal papilla cells (DPCs) and suitable candidates were chosen for lysis and RNA extraction. Total RNA was extracted from DPCs using the Invitrogen RNAqueous total RNA isolation kit (Cat: 10596935; Fisher Scientific, Loughborough, UK). Human fibroblasts derived from skin samples (human abdominal tissue sourced from Tissue Solutions, Glasgow, UK) were used as controls.

For quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analysis, RNA was reverse transcribed into complementary DNA (cDNA) as a template for qRT-PCR. PCR was conducted as follows: hot start at 95°C for 60 seconds (one cycle); denaturation at 96°C for 5 seconds and annealing and extension at 60°C (30 cycles); additional extension at 60°C for 3 minutes and from 60°C to 95°C for 3 seconds (one cycle). The genes assessed in this assay as well as the primers for assessed and housekeeping genes are shown in Supplementary Table I (Appendix).

The focus was on those genes reported to be involved in hair growth: fibroblast growth factor 7 (*FGF7*), secreted by cells in the dermal papilla, plays a role in stimulating hair matrix cell proliferation and hair growth;<sup>(10)</sup> insulin-like growth factor 1 (*IGF1*) promotes maintenance of anagen phase and the stimulation of hair growth;<sup>(11)</sup> and WNT family member 5A (*WNT5a*) mediates some of the effects of Sonic hedgehog in hair follicle morphogenesis and is capable of regulating proliferation.<sup>(12,13)</sup> Other related growth factors for hair growth were analysed, including alkaline phosphatase (*ALPL*), expressed in dermal papilla;<sup>(14)</sup> fibroblast growth factor 2 (*FGF2*), expressed in hair matrix cells;<sup>(10)</sup> vascular endothelial growth factor A (*VEGFA*), responsible for maintaining vasculature around the hair follicle during the anagen growth phase,<sup>(15)</sup> and platelet-derived growth factor A (*PDGFA*), stimulating morphogenesis of new capillaries in anagen.<sup>(16)</sup> Related surrogate markers were also analysed, including: lymphoid enhancer binding factor 1 (*Lef1*),<sup>(17)</sup> involved in hair follicle organogenesis; bone morphogenetic protein 4 (*BMP4*), found in the hair placode, i.e. in the sites of *Lef1/b-catenin* expression;<sup>(18)</sup> *Noggin*, driving hair follicle morphogenesis and an antagonist for *BMP4*;<sup>(19,20)</sup> and WNT inhibitory factor 1 (*Wif1*), an antagonist for WNT family.<sup>(21,22)</sup>

The study was conducted sequentially in three steps.

In Step 1, we assessed the assay system and confirmed type I and type II 5AR expression in BPHF. As DHT has been reported to change hair growth factor gene expression levels,<sup>(23)</sup> gene expression levels of *FGF7*, *IGF1*, *WNT5a* and relevant factors were evaluated using human BPHF in the presence of DHT 1.5 nM, 3 nM or 15 nM (or DMSO control). DHT concentrations used were based on a phase II clinical trial of dutasteride in patients with AGA,<sup>(24)</sup> during which baseline serum DHT concentrations were in the range 371–482 pg/mL (approximately 1.5 nM). Therefore, the starting DHT concentration, in our study, was 1.5 nM, with high concentrations of 3 nM and 15 nM also investigated.

In Step 2, we evaluated whether using testosterone in this assay system mimicked the conversion to DHT by 5AR *in vivo*. Changes in gene expression levels of the hair growth factors assessed in Step 1 (*FGF7*, *IGF1* and *WNT5a*) plus related factors (*VEGFA*, *Lef1*, *BMP4*, *PDGFA*, *Noggin* and *Wif1*) were investigated in isolated human BPHF following stimulation with testosterone 3 nM, 10 nM or 30 nM (or DMSO control). Testosterone concentrations were based on results of the phase II clinical trial<sup>(24)</sup> and Step 1. In the phase II study, baseline serum testosterone concentration was around 4.5 ng/mL (approximately 15 nM). In Step 1, DHT 3 nM was suggested as an optimal concentration and considering the tenfold higher potency of DHT than that of testosterone, the submaximal concentration of testosterone was speculated to be 30 nM.

In Step 3, we assessed the expected inhibitory effects of dutasteride and finasteride on gene expression of hair growth-related factors in human BPHF following testosterone stimulation (10 nM, as determined in Step 2). Concentrations of dutasteride were 0.3 nM, 0.03 nM and 0.003 nM, and concentrations of finasteride were 1.5 nM, 0.15 nM and 0.015 nM, based on phase II/III trial results.<sup>(6)</sup> In the previous study, the range of concentrations for dutasteride 0.5 mg and finasteride 1.0 mg in therapeutic plasma steady-state were around 40 ng/mL (76 nM) and 9 ng/mL (24 nM), respectively, which after correcting for protein binding equated to 0.2 ng/mL (0.38 nM) and 0.63 ng/mL (1.69 nM), respectively.

Data was analysed by normalising targets to the geomean of two housekeeping genes. Analysis was primarily conducted on the Partek Genomic Suite (Partek, St Louis, MO, USA) using contrasts of vehicle versus treatment samples for intra-patient and global cohort analyses. Statistical significance was calculated by analysis of variance.

## RESULTS

In Step 1, changes were observed in gene expression subsequent to DHT stimulation and optimal DHT concentration was determined. Following DHT stimulation, steroid 5AR 1 and 2 (*SRD5A1* and *SRD5A2*) were detected, indicating that both type I and type II 5AR were expressed in BPHF (Fig. 1a). Up to 3 nM, DHT stimulation showed a trend to decrease gene expression levels of *FGF7* ( $p = 0.52$ ), *IGF1* ( $p = 0.85$ ) and *WNT5a* ( $p = 0.08$ ) (Fig. 1b). However, 15 nM DHT stimulation did not show consistent changes in the gene expression levels of any assessed factors, except for *AR* and *VEGFA*. Therefore, 3 nM was the optimal concentration of DHT in this assay system and was used as the base concentration for Step 2.

In Step 2, there were changes in gene expression following testosterone stimulation. Testosterone stimulation of BPHF showed a trend to decrease the gene expression levels of three hair growth factors: *FGF7* ( $p = 0.53$ ); *IGF1* ( $p = 0.93$ ); and *WNT5a* ( $p = 0.51$ ) (Fig. 2a). Similar changes in the gene expression levels of *Lef1* and *BMP4* were observed, while *Noggin* and *Wif1* showed opposite changes (Fig. 2b). Although the *VEGFA* gene expression increased in a concentration-dependent manner alongside that of testosterone, this change was opposite to that observed in Step 1. As observed in Step 1, the maximal testosterone concentration (30 nM) was considered to cause saturation in *WNT5a*, *PDGFA* and *Noggin*; therefore, 10 nM was determined as the optimal concentration to use for Step 3.

In Step 3, there was a 5ARI effect on changes in gene expression levels in the presence of testosterone. The expected inhibitory effects of dutasteride and/or finasteride on the observed hair growth factor gene expression changes under testosterone stimulation were investigated, with testosterone at an optimal concentration (10 nM), as determined in Step 2. Gene expression levels of *FGF7*, *IGF1* and *WNT5a*, with dutasteride or finasteride, under testosterone stimulation showed a consistent trend of re-increasing (Fig. 3a). The fold change (mean  $\pm$  standard error of the mean) in *FGF7* expression, when compared with DMSO control,

ranged from  $1.00 \pm 0.25$  to  $1.53 \pm 10.04$  ( $p = 0.10$ ) for finasteride and from  $0.93 \pm 0.36$  to  $1.22 \pm 0.10$  ( $p = 0.23$ ) for dutasteride. Similarly, *IGF1* expression ranged from  $0.56 \pm 0.78$  to  $4.34 \pm 2.71$  ( $p = 0.72$ ) with finasteride treatment and from  $1.70 \pm 0.78$  to  $2.73 \pm 1.24$  ( $p = 0.61$ ) with dutasteride treatment. Meanwhile, *WNT5a* expression ranged from  $1.03 \pm 0.99$  to  $1.14 \pm 0.10$  ( $p = 0.26$ ) with finasteride and  $1.08 \pm 0.25$  to  $1.21 \pm 0.20$  ( $p = 0.65$ ) with dutasteride treatments.

In our study, the expression of *Noggin* and *Wif1* showed a trend of increasing in the presence of testosterone stimulation, whereas *WNT5a* expression showed a trend of decreasing with testosterone stimulation (Fig. 2b).

*PDGFA* gene expression showed a consistent trend towards decreasing with increasing 5ARI concentrations (Fig. 3b). Changes in *VEGFA* and *Lef1* gene expression were inconsistent (Fig. 3b).

Additionally, hair growth factor expression under testosterone stimulation was evaluated in isolated human fibroblasts to confirm whether observed changes in gene expression were induced specifically in BPHF. No genes were shown to decrease under testosterone stimulation in the human fibroblasts (data not shown), indicating that effects on gene expression were specific to BPHF.

## DISCUSSION

By utilising BPHF isolated from plucked human hair in this assay, we investigated why dutasteride was more effective at promoting human hair growth than finasteride, as demonstrated in a clinical trial.<sup>(6)</sup> We also evaluated the involvement of type I 5AR in human hair growth.

Our three-step assay system was considered to detect changes in gene expression of hair growth factors and other related molecules. By measuring changes in the expression levels of targeted genes, new 5ARI candidates and medicines may be evaluated at the gene level.

However, the assay system needs further development. In Step 1, stimulation of BPHF with 15 nM DHT resulted in saturation of the trend of decreasing gene expression for three factors – *FGF7*, *IGF1* and *WNT5a* – possibly due to the over-concentration of DHT and/or DHT-induced down-regulation of 5AR in the cells. Furthermore, changes in gene expression of other related molecules (*VEGFA*, *Lef1* and *PDGFA*) were shown to be inconsistent in all steps. This could be due to the use of BPHF, with other components, and not purely isolated DPCs.

Notably, dutasteride and finasteride showed the trend to cancel suppressive effects of testosterone on hair-related gene expression, presumably in DPCs in Step 3, as the expression levels of targeted genes (*FGF7*, *IGF1* and *WNT5a*) were increased. Considering the inhibitory potency of dutasteride and finasteride, there was a trend towards differing changes in expression of the investigated genes and factors between the two 5ARIs. The effect of finasteride on *FGF7*, *IGF1* and *WNT5a* expression plateaued at the middle concentration evaluated (i.e. 0.15 nM), but the effect of dutasteride was linear to the highest concentration tested (or 0.3 nM), although these findings were not statistically significant. This observation suggests that dutasteride may have a stronger inhibitory potency to increase growth factor expression than finasteride, possibly due to the inhibition of type I 5AR by dutasteride or greater inhibition of type II 5AR by dutasteride when compared to finasteride. Our results suggest that type I 5AR may play an important role in hair growth, as well as type II 5AR.<sup>(10,11)</sup> Furthermore, *WNT5a* expression in the developing hair follicles requires Sonic hedgehog; this suggests that *WNT5a* may mediate some effects of Sonic hedgehog in hair follicle morphogenesis, a hypothesis supported by the fact that both *WNT5a* and Sonic hedgehog are capable of regulating proliferation.<sup>(12)</sup> *Noggin* is a known inhibitor of *BMP4*;<sup>(19)</sup> therefore, results were consistent with *BMP4* expression decreasing in a concentration dependent manner. Similarly, results for *Wif1*, a WNT antagonist,<sup>(22,25)</sup> were consistent with those of *WNT5a*,

showing a trend for reduced expression. Thus, *FGF7*, *IGF1* and *WNT5a* were confirmed as key factors for hair growth in the study.

As with other *in vitro/ex vivo* investigations, this study had potential limitations. Changes in gene expression were not verified as leading to corresponding changes in protein levels. Although not the focus of this study, effects on protein expression should also be assessed. Furthermore, concentrations of DHT converted from testosterone in the culture medium were not assessed in Steps 1 and 2. Testosterone induced changes in expression of several genes and testosterone conversion to DHT was inhibited by 5AR in BPHF. However, more precise investigations, including the determination of DHT concentration in the medium, may be necessary. Another limitation was the small sample size; larger sample sizes, with over 35 participants, should be considered in the future. Therefore, interpretation of these results for testosterone stimulation, alone and in the presence of dutasteride/finasteride, should be carefully considered. In addition, hair growth *ex vivo* culture was not assessed directly, given the study design; 24-hour incubation time is too short to reach levels of gene expression changes that would lead to changes in efficacy endpoints (e.g. hair shaft lengths, hair width, hair count). Finally, data analyses and interpretation may be difficult due to our use of BPHF instead of pure DPCs; assays using pure DPCs are expected to show more clear-cut results and provide more in-depth interpretation.

Our study indicates that dutasteride and finasteride are potent modulators of the expression of genes encoding hair growth factors and other molecules potentially related to hair growth. Dutasteride may be more potent than finasteride in modulating the expression levels of key hair growth genes (e.g. *FGF7*, *IGF1* and *WNT5a*). This study provides supporting evidence that type I 5AR may be involved in hair growth in addition to type II 5AR. These findings indicate an in-depth downstream mechanism of action by 5AR and provide further clarification of the importance of type I 5AR function.

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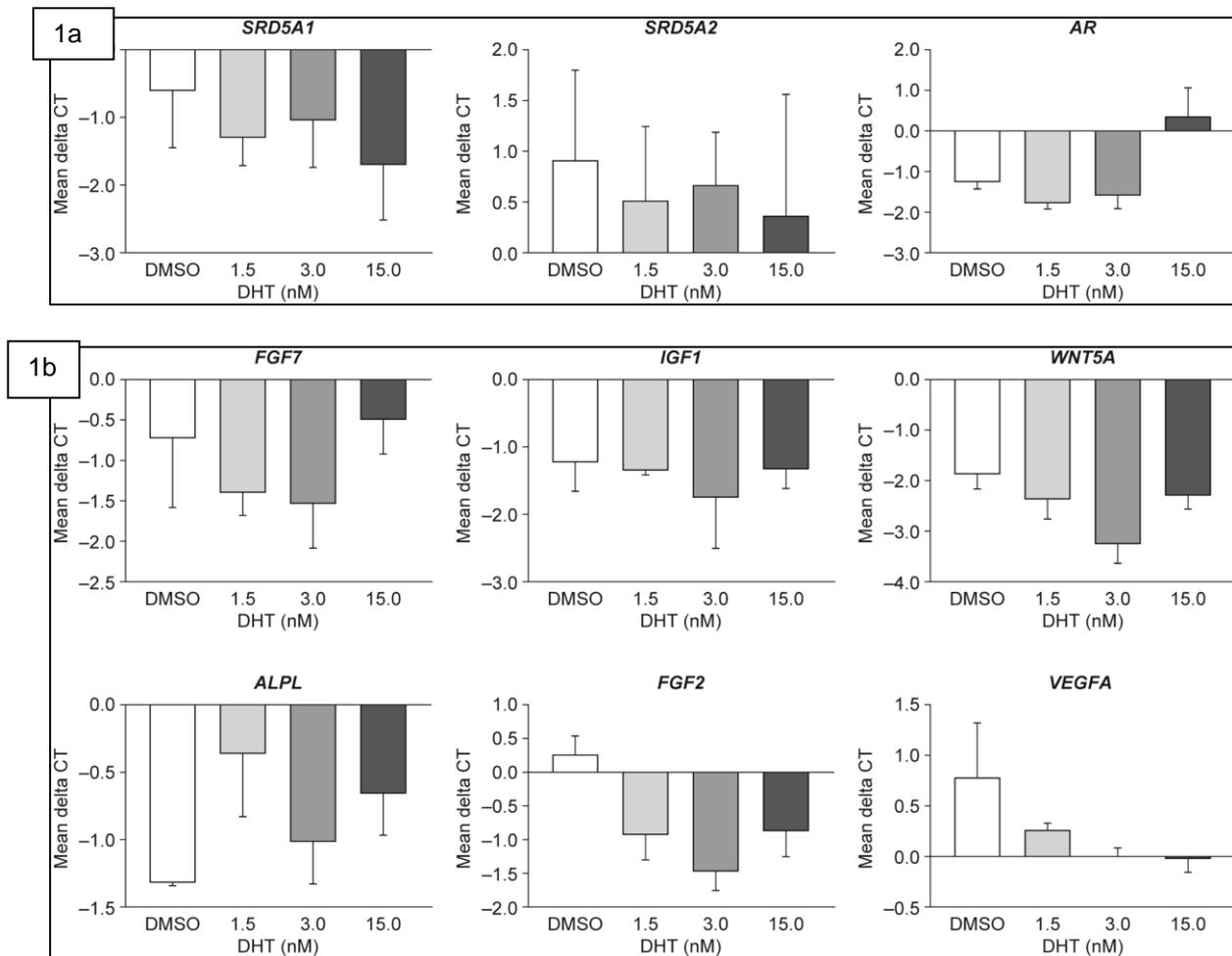
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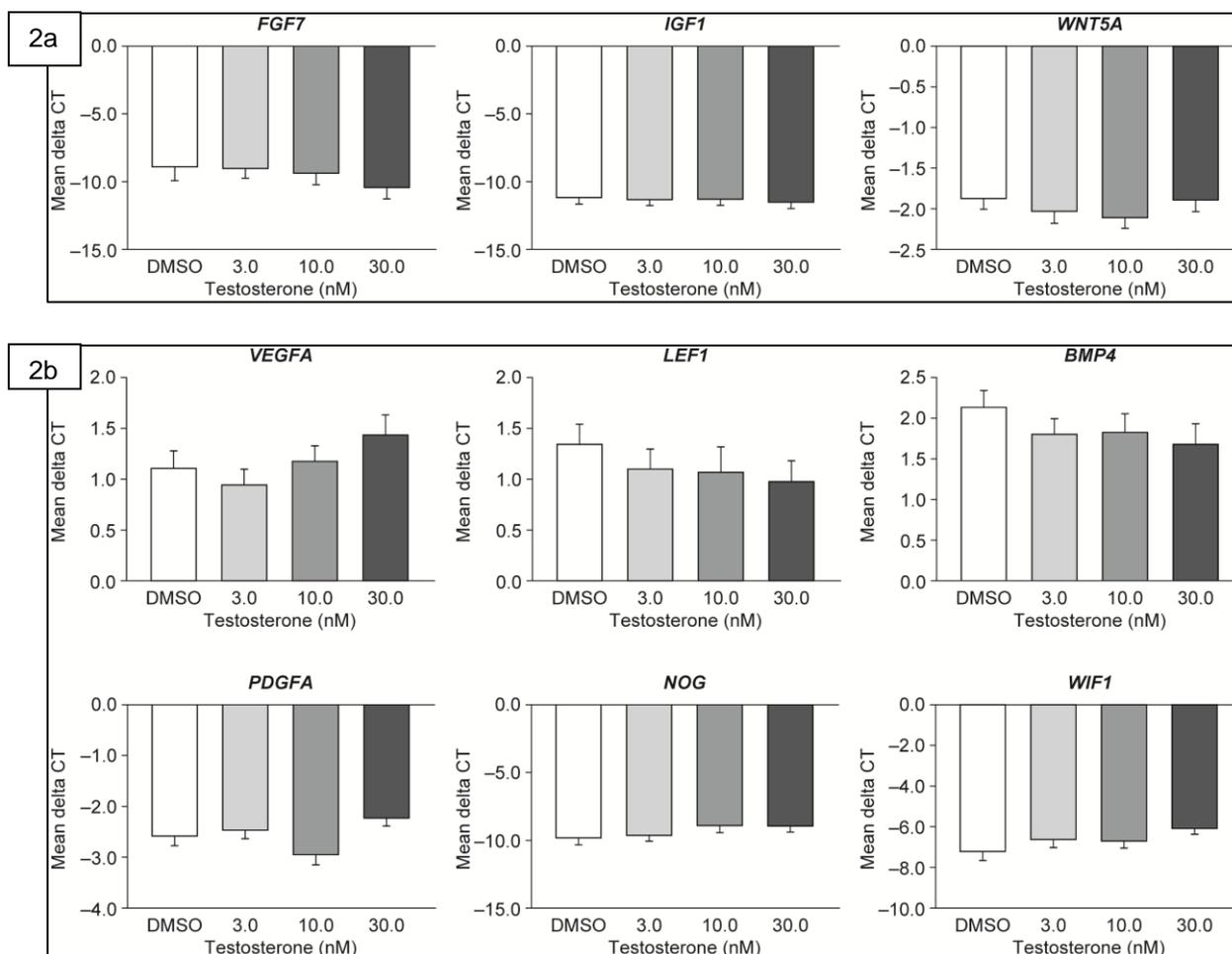
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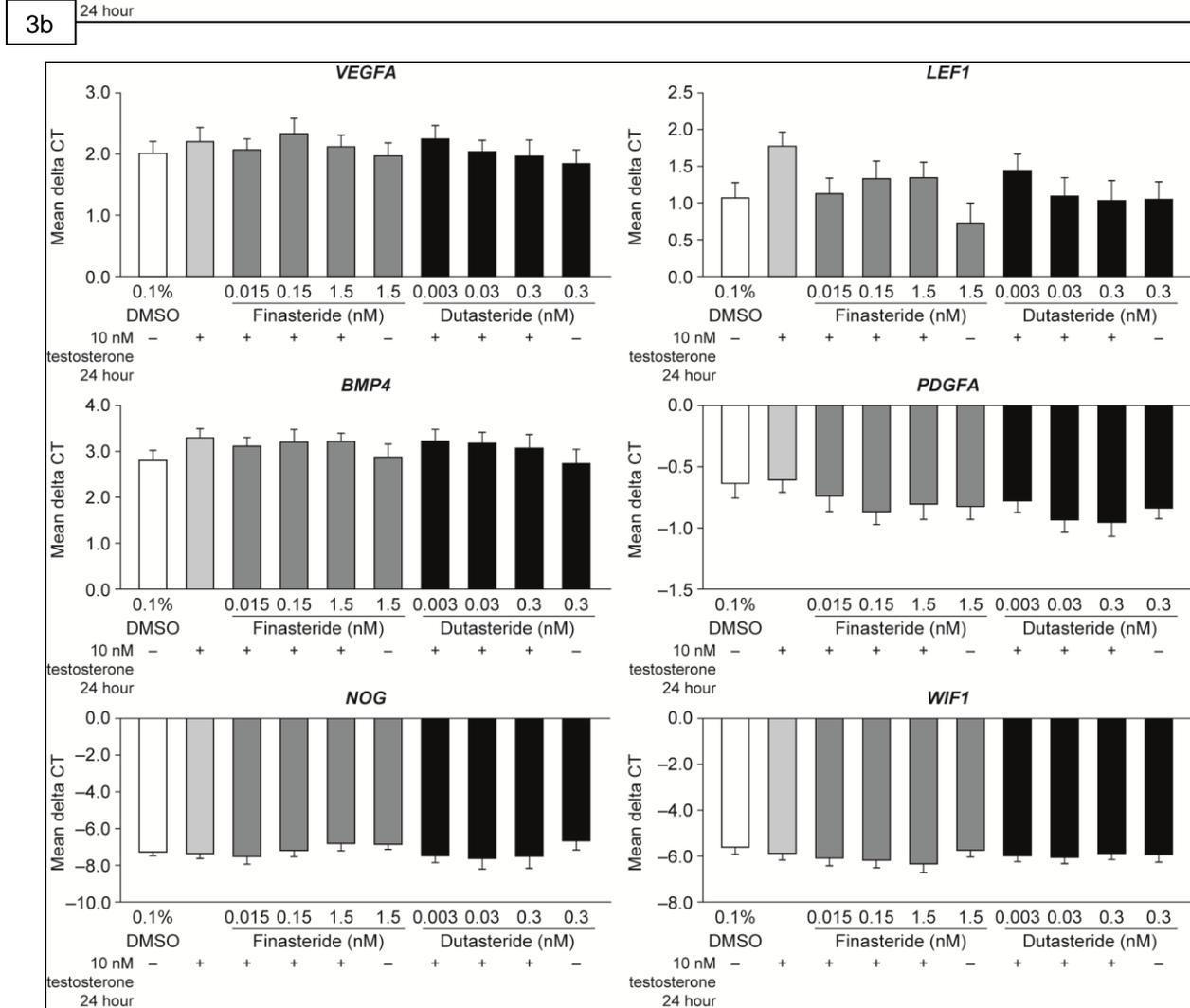
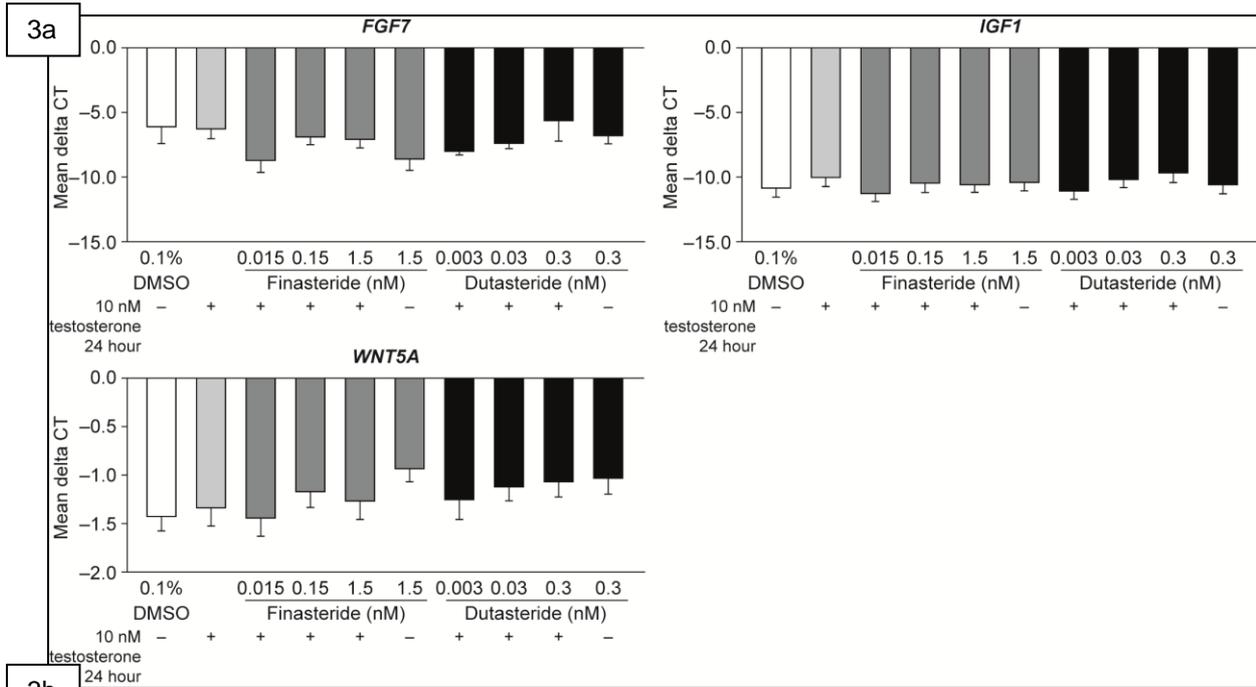
## FIGURES



**Fig. 1** Charts show changes in gene expression of (a) *SRD5A1*, *SRD5A2* and *AR*, and (b) other factors related to hair growth induced by DHT. *ALPL*: alkaline phosphatase; *AR*: androgen receptor; delta CT: difference of expression between gene of interest and reference sequence; DHT: dihydrotestosterone; DMSO: dimethyl sulfoxide; *FGF2*: fibroblast growth factor 2; *FGF7*: fibroblast growth factor 7; *IGF1*: insulin-like growth factor 1; *SRD5A1*: steroid 5 alpha-reductase 1; *SRD5A2*: steroid 5 alpha-reductase 2; *VEGFA*: vascular endothelial growth factor A; *WNT5a*: WNT family member 5A



**Fig. 2** Charts show changes in gene expression of (a) *FGF7*, *IGF1* and *WNT5a*, and (b) other factors related to hair growth induced by testosterone. *BMP4*: bone morphogenetic protein 4; delta CT: difference of expression between two genes; DMSO: dimethyl sulfoxide; *FGF7*: fibroblast growth factor 7; *IGF1*: insulin-like growth factor 1; *Lef1*: lymphoid enhancer binding factor 1; *NOG*: *Noggin*; *PDGFA*: platelet-derived growth factor A; *VEGFA*: vascular endothelial growth factor A; *Wif1*: WNT inhibitory factor 1; *WNT5a*: WNT family member 5A



**Fig. 3** Charts show changes in gene expression of (a) *FGF7*, *IGF1* and *WNT5a*, and (b) other factors related to hair growth under 10 nM testosterone stimulation in the presence of dutasteride or finasteride. *BMP4*: bone morphogenetic protein 4; delta CT: difference of expression between two genes; DMSO: dimethyl sulfoxide; *FGF7*: fibroblast growth factor 7; *IGF1*: insulin-like growth factor 1; *Lef1*: lymphoid enhancer binding factor 1; *NOG*: *Noggin*; *PDGFA*: platelet-derived growth factor A; *VEGFA*: vascular endothelial growth factor A; *Wif1*: WNT inhibitory factor 1; *WNT5a*: WNT family member 5A

## APPENDIX

Supplementary Table I. List of assessed genes and the primers for assessed and housekeeping genes.

Genes	Full name of gene	Gene accession no. (reference sequence)	Forward primer	Reverse primer	Step 1	Step 2	Step 3
<i>IGF1</i>	Insulin-like growth factor 1	NM_001111283.N	GAGCTGGTGGATGCTCTTCA	GACTGCTGGAGCCATACCC	X	X	X
<i>FGF2</i>	Fibroblast growth factor 2	NM_002006	TGCTAACCGTTACCTGGCTA	AAGAAACACTCATCCGTAACACA	X	-	-
<i>FGF7</i>	Fibroblast growth factor 7	NM_002009.N	AAAGGGACCCAAGAGATGAAGAA	CACCCCTTTGATTGCCACAA	X	X	X
<i>VEGFA</i>	Vascular endothelial growth factor A	NM_001171623.N	GAGGAGGGCAGAATCATCAC	GTCTCGATTGGATGGCAGTA	X	X	X
<i>WNT5a</i>	WNT family member 5A	NM_003392.N	TACGAGAGTGCTCGCATCC	CACATCAGCCAGGTTGTACAC	X	X	X
<i>ALPL</i>	Alkaline phosphatase	NM_000478	CCCTTCACTGCCATCCTGTA	GCAGACTGCGCCTGGTA	X	-	-
<i>AR</i>	Androgen receptor	NM_001011645.N	CGCACCTGATGTGTGGTAC	GCCCATTTGCTTTTGACAC	X	X	X
<i>SRD5A1</i>	Steroid 5 alpha-reductase 1	NM_001324323.N	TGGTTAACGGGCATGTTGATA	TGGCTGCAGTTACGTATTCA	X	X	X
<i>SRD5A2</i>	Steroid 5 alpha-reductase 2	NM_000348.N	TGATGGGTGGTACACAGACA	AGCTGGCGCAATATATAGTCAC	X	X	X
<i>Noggin</i>	Noggin	NM_005450.N	CCATGCCGAGCGAGATCAA	CCTCCGCAGCTTCTTGCTTA	-	X	X
<i>PDGF-A</i>	Platelet-derived growth factor subunit A	NM_002607.5	GCCCATTCGGAGGAAGAGAA	CTGACTCCGAGGAATCTCGTAAA	-	X	X
<i>Lef1</i>	Lymphoid enhancer binding factor 1	NM_016269.	AAGAAAGTGCAGCTATCAACCA	GCTGTCTTTCTTTCCGTGCTA	-	X	X

<i>Wif1</i>	WNT inhibitory factor 1	NM_007191.N	GATGCTCACCAGGCAAGAGTA	GTGCCATTTTCCCCTCTGAAAC	-	X	X
<i>BMP4</i>	Bone morphogenetic protein 4	NM_001347916.N	CCACAGCACTGGTCTTGAGTA	GGTCCCTGGGATGTTCTCC	-	X	X
<b>Housekeeping genes</b>							
<i>TTF1</i>	Transcription termination factor 1	NM_007344.N	GCTGAAAGCTGTCTATGTTCC	GCAAAGGTAGAGTCGTCTCA	-	-	X
<i>CCNK</i>	Cyclin K	NM_001099402.1	TTGGCCAGTTTGGAGATGAC	TCAAACCTGATGGTCTGCAGTAA	X	X	X
<i>POL2J</i>	RNA polymerase II subunit J	NM_006234.4	CCTGTCAGTCAAAGCAGGAAC	TGGACAAGAAGCCTGTGGAA	X	X	X

-: not assessed; X: assessed