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Title:

Androgens downregulate BMP2 impairing the inductive role of dermal papilla cells on hair follicle stem cells differentiation

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None

ABSTRACT

Hair follicle cyclical regeneration is regulated by epithelial-mesenchymal interactions. During androgenetic alopecia (AGA), hair follicle stem cells (HFSC) differentiation is impaired by deregulation of dermal papilla cells (DPC) secreted factors. We analyzed androgen influence on BMPs expression in DPC and their effect on HFSC differentiation to hair lineage.

Androgens downregulated BMP2 and BMP4 in DPC spheroids. Addition of BMP2 restored alkaline phosphatase activity, marker of hair-inductivity in DPC, and DPC-induced HFSC differentiation, both inhibited by androgens. Concomitantly, in differentiating HFSC, an upregulation of BMPRIa and BMPRII receptors and nuclear ß-catenin accumulation, indicative of Wnt/β-catenin pathway activation, were detected. Our results present BMP2 as an androgen-downregulated paracrine factor that contributes to DPC inductivity and favors DPC-induced HFSC differentiation to hair lineage, possibly through a crosstalk with Wnt/ß-catenin pathway.

A comprehensive understanding of androgen-deregulated DPC factors and their effects on differentiating HFSC would help to improve treatments for AGA.

KEYWORDS

epithelial-mesenchymal interactions; androgenetic alopecia; BMP; WNT; dermal papilla

1.INTRODUCTION

Hair follicle (HF) homeostasis and cyclical regeneration are regulated through balancing quiescence and activation of hair follicle stem cells (HFSCs) residing in the upper segment of the HF, known as bulge. HF regeneration begins with signal exchanges between quiescent HFSC and underlying mesenchymal dermal papilla cells (DPC).

The HF undergoes cycles of growth (anagen), regression (catagen) and rest (telogen). When the telogen to anagen transition occurs, the HFSC near the DPC, are activated to produce an entire new hair shaft during each anagen phase (Blanpain et al., 2004,Morris et al., 2004,Oshima et al., 2001). DPC play a pivotal role providing the molecular signals that activate HFSC to initiate the next cycle of hair growth and orchestrating the complex hair differentiation program (Cotsarelis, 2006,Tumbar et al., 2004). Many of the paracrine factors and the signalling pathways involved in this crosstalk, at different hair cycle stages, have been reported (Alonso et al., 2006). However, little is known about their specific roles.

Androgenetic alopecia (AGA), is the most common type of baldness, characterized by patterned hair loss from the scalp, caused by shortening of the anagen phase of hair cycling and progressive miniaturization of the hair follicle (HF). Although etiological studies have proved that this phenomenon is mediated mainly by androgens (Hamilton, 1942), most of the molecular mechanisms underlying androgen-related actions remain unknown. In the hair follicle, androgens target cells would be mainly the DPC, and the other components of the HF (ORS, hair bulb or bulge) would be indirectly controlled through paracrine factors.

Although the synthesis of steroid hormones takes place mainly in adrenal glands, ovaries, testis, placenta and brain, skin constitutes an important peripheral steroidogenic tissue. In the skin, androgens can originate from the main circulating endocrine precursor dehydroepiandrosterone (DHEA) sulfate or can be endogenously generated, since the cells present in the skin and its appendages express the enzymes and accessory proteins necessary for their synthesis *de novo* from cholesterol (Slominski et al., 2004, Slominski et al., 2015).

As the hair cycle is regulated by several growth factors from DPCs (Stenn et al., 2001), deregulation of these secreted factors could cause a shortening of the anagen period leading to AGA development.

It has been shown that Wnt/β-catenin, Sonic Hedgehog (SHH), Bone Morphogenetic Protein (BMP), Notch, Transforming Growth Factor β2 (TGF- β2), NF-κB, and Fibroblast Growth Factors (FGFs) signaling pathways are involved in the HF morphogenesis and regeneration during hair cycle (Rishikaysh et al., 2014).

Studies have shown that dihydrotestosterone (DHT) acts on DPCs of AGA phenotype and deregulates paracrine factors that would be responsible for catagen induction in HFs (Inui et al., 2002, Kitagawa et al., 2009,Kwack et al., 2012,Kwack et al., 2008).

We had shown that androgens regulate the expression of DPC secreted factors, which are involved in normal HFSC differentiation, via the inhibition of the canonical Wnt signaling pathway (Leiros et al., 2012). This androgen modulating effect on autocrine or paracrine DPC functions could play a major role avoiding normal HFSC differentiation during AGA development. The observation that stem cells number was maintained in hair follicles from bald scalp, whereas progenitor cells were markedly diminished supports this notion (Garza et al., 2011). We had also described that the WNT antagonist DKK1 and the WNT agonist WNT10b are DHT-regulated paracrine factors that modulate the HFSC differentiation inhibition involved in AGA (Leiros et al., 2017). The identification of new androgen-regulated autocrine or paracrine factors produced by scalp DPC would lead to a better understanding of AGA pathogenesis and could provide new therapeutic targets.

BMPs are members of the TGFβ family of signaling proteins and play an important role during development and tissue formation (Derynck et al., 2003,Massague et al., 2000,Miyazawa et al., 2002,ten Dijke et al., 2004). BMP signaling plays a key role in HFSC maintenance and progenitor cell differentiation (Kobielak et al., 2003). Moreover, BMPs present in HF bulb microenvironment, act on DPC to maintain key signature features *in vitro* and hair-inducing activity *in vivo*(Rendl et al., 2008). They are critical pieces of the complex epithelial–mesenchymal crosstalk necessary to make hair. A very important molecule belonging to this family, BMP2, acts through the binding to its receptors BMPR-IA and BMPR-IB with high affinity (Kotzsch et al., 2008). Studies in mice have found that BMP2, BMPR-IA, and Noggin are involved in HF cycle transition (Botchkarev et al., 2002,Guha et al., 2004,Kobielak et al., 2003,Kulessa et al., 2000). As BMPs have also been shown to inhibit the HF telogen-anagen transition (Botchkarev et al., 2001,Plikus et al., 2008), their function in hair cycle control remains controversial.

In this work, we show for the first time, a downregulation of BMP2 and BMP4 expression in androgen sensitive human DPC, after DHT treatment. Using spheroids in a cellular model to mimic epithelial-mesenchymal interactions, we found that BMP2 is involved in the HFSC differentiation induced by DPC. We also observed a crosstalk between BMP and Wnt/β catenin pathways that is involved in this process.

2. MATERIALS AND METHODS

2.1 Cell line cultures

Androgen-responsive human DP cell line (DPC) was obtained after stable transfection of a DPC immortalized cell line, kindly provided by Dr. Michael P. Philpott (Barts and The London School of Medicine and Dentistry, Queen Mary University of Lon- don) with pCI-neoAR (mammalian expression vector pCI-neo Promega, Fitchburg, WI, USA that express androgen receptor (AR)) (Leiros et al., 2017).

The immortalized stem cell line from human bulge, Tel-E6E7 (HFSC) (Roh et al., 2008), was kindly provided by Dr. Stephen Lyle (University of Massachusetts Medical School, MA, U.S.A.) and cultured on feeder layer (FL) of mytomicin C-inactivated 3T3-Swiss albino cells (ATCC) in cFAD medium (Roh et al., 2004).

Generation of DPC-spheroids and monolayer cultures:

For DPC-spheroids cultures, 2.5 $x10^4$ cells/well were seeded in 96-wells plate coated with 5% PVA (Polyvinyl alcohol). For monolayer cultures, $1.5x10^5$ cells/well were seeded in 6-wells plate. The cultures were left undisturbed for 3 days. When indicated, cultures were supplemented with 10^{-7}M DHT (Sigma-Aldrich, USA) and maintained during 3 days.

2.2 Generation of DPC conditioned media

For the different treatments, DPC-spheroids cultures were supplemented as follow: 10^{-7} M DHT (Cat. 31573 Sigma-Aldrich, St. Louis, MO, USA) or 300 ng/ml of BMP2 (Cat. AK8356, Akron Biotech, FL, USA) or BMP4 (Cat. AK8352, Akron Biotech, FL,USA). After 3 days, conditioned media were collected and used in HFSC differentiation assays. Conditioned medium without any supplement was used as control.

HFSC differentiation assays using DPC-conditioned media: 10^5 HFSC/well were seeded in 6-wells plate in cFAD medium and cultured during 3 days, whereupon the medium was replaced with media conditioned by DPC-spheroids with different treatments (in 1:1 rate with cFAD) and cultured another 3days.

2.3 Total RNA isolation, cDNA synthesis and real time PCR

Total RNAs from DPC or HFSC were isolated using Trizol protocol (Invitrogen-Thermofisher, Waltham, MA, USA) and cDNAs were obtained by M-MLV Reverse transcriptase (Promega, Madison, WI). Real time PCRs were carried out in Step-One Real time PCR device (Applied Biosystems Invitrogen, Carlsbad, CA) using SYBR Green MasterMix (Applied Biosystems Thermofisher, Waltham, MA, USA) and the following set of specific primers: BMP2 (Forward:5´-ATGGATTCGTGGTGGAAGTG -3´, Reverse: 5´-GTGGAG TTCAGATGATCAGC-3´), BMP4 (Forward:5´-AGCATGTCAGGATTAGCCGA -3´, Reverse: 5´-TGGAGATGGCACTCAGTTCA -3´), Dkk-1 (Forward: 5´-TCACGCTATGTGCTGCCCCG-3´, Reverse: 5´-TCTGGAATACCCATCCAAGGTGCT-3´), Wnt10b (Forward: 5´-CTCTGGGATGTGTAGCCTTC-3´, Reverse: 5´-GGCTCTGGAGTTGAGAAGTG-3´), K6hf (Forward: 5´-CTAGAGCCCCTCTTTGATTCCT-3´, Reverse: 5´-GCAGCATCTACGTCCTTTTTCA-3´), BMPRIa (Forward:5´-TCAGACTCCGACCAGAAAAAGT -3´, Reverse: 5´-TGGCAAAGCAATGTCCATTAGTT-3´), BMPRIb (Forward:5´-CCTCCCTCTGCTGGTCCAAAGGA -3´, Reverse: 5´-GCTACCTTTTCGCCACGCCACT-3´), BMPRII (Forward:5´- CGGCTGCTTCGCAGAATCA-3´, Reverse: 5´-AGGTGCTACCTTTCGAGCATA-3´). Hypoxanthine-guanine phosphoribosyltransferase (HPRT) (Forward: 5´-CTCAACTTTAACTGGAAAGAATGTC-3´, Reverse. 5´-TCCTTTTCACCAGCAAGCT-3´) was used as internal mRNA expression control.

2.4 Western blot assays

To evaluate BMP2 protein levels in DPC, whole cell extracts were obtained with RIPA lysis buffer [20mM Tris pH 7,5, 1 mM EDTA, 150mM NaC1, 10mM KC1, 1% NP-40, 0,1% deoxycholate, 0,1% sodium dodecyl sulphate (SDS)] with protease inhibitors. To evaluate K6hf protein expression in differentiating HFSC, whole cell extracts were obtained with urea 5M lysis buffer. After SDS polyacrylamide gel electrophoresis and electroblotting, membranes were incubated with the following primary antibodies for BMP2 (ab6285 Abcam), K6hf (Gp-K6hf, Progen), GAPDH (sc-32233, Santa Cruz Biotechnology, Inc.). Horseradish peroxidase-conjugated secondary antibodies (GE Healthcare, Amersham, U.K.) were used. Blots were developed using the chemiluminescence imaging system G: BOX Chemi XRQ (Syngene, Frederick MD, USA Inc.) and quantified with ImageJ software.

2.5 Immunohistochemical staining of DPC spheroids

Formalin fixed, paraffin-embedded DPC spheroids were sliced and stained with BMP2 and Vimentin antibodies (Santa Cruz Biotechnology, Inc.) and developed with HRP-DAB method (Bio Genex).

2.6 Alkaline phosphatase activity

After treatments, spheroids were collected in one tube, washed with PBS and sonicated in lysis buffer (NP40 0.4% in Cl2Mg 1mM). Alkaline phosphatase activity was measured in diethanolamide buffer with p-nitrophenyl phosphate (pNPP) as substrate (10mM). Color production was detected at 405 nm.

2.7 β-catenin immunofluorescence in HFSC

Media conditioned by DPC-spheroids were generated as described above. HFSC in cFAD medium (FL) were used as control. HFSC were cultured on glass coverslips with conditioned or control media during 20h and fixed in chilled MeOH. β-catenin was detected by immunofluorescence using anti-human β-catenin antibody (cat. 616153, BD Transduction Laboratories ™ USA) followed by an anti-mouse secondary antibody conjugated to fluorochrome Alexa fluor 594 (Thermo Fisher Scientific, USA) and analyzed by confocal microscopy (Olympus FV 1000).

3.RESULTS

3.1 Androgens downregulate BMP2 and BMP4 expression in DPC

Our previous results concerning the impairment of HFSC differentiation by androgens, suggested a deregulation of DPC secreted factors. We also identified DKK1 and WNT10b as paracrine factors which modulate the HFSC differentiation.

In this work, we studied the influence of androgens on BMPs expression and BMP pathway in DPC and their effect on HFSC differentiation to hair lineage. Balding DPC have higher AR expression than non-balding DPC, evidenced by an elevated androgen sensitivity in AGA (Hibberts et al., 1998). In DPC primary cultures, AR expression decreases through the successive passages (Inui et al., 2002,Kwack et al., 2008). Therefore, we used a human DP derived cell line that expresses AR, as model of AGA, to evaluate the effect of androgens on BMP2 and BMP4 expression. In this cell line, specific traits of dermal papilla, such as α-smooth muscle actin (α-SMA), alkaline phosphatase activity, and a functional androgen receptor (AR) had been previously confirmed (Leiros et al., 2017). In order to analyze the effect of culture condition on BMPs expression, DPC were grown both in 2D monolayer (ML) and 3D spheroids (Sph) cultures as done before (Leiros et al., 2017). In DPC Sph, dihydrotestosterone (DHT) significantly downregulates the expression of both BMP-2 and BMP-4 mRNA. However, when DPC are cultured in monolayer, BMPs expression is not affected by androgens (Fig1. A, B). Moreover, basal level of both genes is significantly higher in Sph compare to ML. It seems that culture condition (2D or 3D) affects BMPs expression. To confirm this hypothesis, we analyzed BMPs expression profile in a time course assay. BMP2 and BMP4 mRNAs augment as spheroids are forming up to 96h, with a peak at 72h (Fig1. C). DPC have an aggregative conformation *in vivo*. It was shown that spheroid culture model recapitulates the tissue microenvironment that promotes an appropriate cellular phenotype (Schmeichel et al., 2003). Therefore, we continued working with DPC spheroids. Then, we evaluated the effect of androgens on BMP2 protein level. BMP2 expression diminished about 23% after treatment with DHT, as shown by Western blot and immunohistochemistry on DPC Sph (Fig 1. D, E).

3.2 BMP-2 restores inductivity marker in androgen treated human DPC spheroids

Alkaline phosphatase activity is considered a DP marker that correlates with hair-inducing capacity (Ohyama et al., 2010). DPC Sph cultured in presence of DHT for three days, diminished 25% their alkaline phosphatase activity. Addition of hrBMP2 to the culture medium, restored this activity to basal level, nevertheless, we did not observe this effect after addition of hrBMP4 (Fig.2). Therefore, the autocrine action of BMP2 on DPC could restore their inductive properties.

3.3BMP2 restores the DPC mediated HFSC differentiation inhibited by androgens

Given that BMP2 and BMP4 expression is regulated by DHT, we studied the effect of BMPs on the ability of DPC-conditioned culture media to induce human HFSC cell line differentiation, in presence or not of androgens. We cultured DPC as spheroids, as they show a relative higher basal expression level of BMPs that can be regulated by DHT and higher hair-inductive properties than monolayers.

Conditioned culture media from DPC spheroids induced HFSC differentiation into hair lineage. When HFSC were cultured in DPC Sph-conditioned media (Control), it was observed that K6hf (K75) mRNA expression, a keratin used as hair follicle lineage committed marker (Roh et al., 2004,Wang et al., 2003), was upregulated 5-fold compared to HFSC cultured in non-conditioned medium (FL) (Fig. 3A). Nevertheless,

when HFSC were cultured in medium conditioned by DPC spheroids in presence of DHT 10^{-7}M (DHT), a significant reduction in differentiation was observed respect to HFSC cultured with control medium (3.2 vs 5 fold) (Fig. 3A). We evaluated if BMP2 and BMP4 are DHT-downregulated factors involved in HFSC differentiation to hair lineage. In order to study if they can restore DPC- inductive action, 300 ng/ml of BMP2 orBMP4 were added to collected conditioned media from DHT-treated DPC immediately before being used. We observed that the addition of BMP2, significantly upregulated K6hf mRNA expression, overcoming the inhibitory DHT effect (DHT+BMP2, 6.9 fold vs DHT, 3.2 fold) (Fig. 3A). This result indicates that BMP2 secreted by DPC could have positive paracrine effects on human HFSC differentiation induced by DPC. Lastly, HFSC differentiation in DHT- conditioned medium supplemented with BMP4, is higher but not statistical different from DHT alone (DHT+BMP4, 4.85 fold vs DHT, 3.2 fold).

In an attempt to mimic the epithelial-mesenchymal interactions that occur in the hair follicle *in vivo*, we performed a differentiation assay with heterotypic spheroids composed of HFSC and DPC, or HFSC and 3T3 Swiss Albino murine fibroblasts cells used as FL, that do not induce differentiation. Briefly, in a 96 well plate coated with PVA 5%, we plated 3T3 Swiss Albino cells (FL) or DPC in control medium (C), DPC with medium supplemented with DHT (DHT), DPC with medium supplemented with BMP2 (BMP2), or DPC with medium supplemented with both DHT and BMP2 (DHT+BMP2), and let spheroids form for 24h. Then we added HFSC to form the heterotypic spheroids, and let them differentiate for another 96h. We observed an inductive effect of BMP2 on DPC mediated-HFSC differentiation to hair lineage (4,88 BMP2vs 3.56 Control). BMP2 also prevented DHT inhibition on HFSC differentiation (4,2 DHT+BMP2 vs 2.16 DHT) (Fig. 3B).

We also analyzed K6hf protein expression by Western blot in HFSC following differentiation induced by DPC conditioned media. BMP2 treatment was able to prevent DHT inhibition and augmented K6hf expression when HFSC were cultured in medium conditioned by DPC treated with DHT 10^{-7}M and supplemented with 300 ng/ml BMP-2 immediately before being use (DHT+BMP2) or medium conditioned by DPC in presence of DHT and BMP-2 simultaneously (DHT/BMP2*) (Fig. 3C).

These results would indicate that BMP-2 exerts paracrine and autocrine significant actions on HFSC and DPC respectively, modulating DPC induced-HFSC differentiation.

3.4BMP2 modulates BMPRIa and BMPRII expression in differentiating HFSC

We showed that BMP2 is able to overcome the inhibitory effect of androgens in the HFSC differentiation process to hair lineage. However, the role of BMPs secreted from DPC in this process is not clear yet. In order to identify the molecular mechanisms modulated by BMPs, that may influence the HFSC differentiation, we first explored possible autocrine effects of BMP2 in DPC spheroids.

It is known that WNT signaling regulators, Wnt10b and Dkk-1 are downregulated and upregulated respectively by androgens in DPC, resulting in an inhibition of HFSC differentiation to hair lineage. We studied a possible effect of BMP2 on the regulation of these ligands expression in DPC spheroids that could compensate the inhibitory action of androgens. However, neither Wnt10b nor Dkk-1 expression was affected by treating DPC with BMP2, in presence or absence of DHT (Fig. 4).

In order to study the paracrine action of DPC secreted BMPs, we first analyzed expression of major BMP receptors in HFSC, in a differentiation context. We observed that BMPRIa, BMPRIb and BMPRII are downregulated in HFSC after 72h of DPC-induced differentiation. However, when DHT-treated DPC conditioned medium is supplemented with BMP2, expression of BMPRIa and BMPRII is significantly upregulated or kept elevated in differentiating HFSC. This fact, may allow for BMP pathway to be responsive for a longer time, affecting expression of keratins, which would be a reflection of the differentiation process (Fig. 5; and see Discussion).

We did not observe a similar effect with DPC conditioned medium supplemented with BMP4.

3.5 BMP2 favors nuclear ß-catenin accumulation in differentiating HFSC

Active Wnt/ßcatenin pathway is required for normal HFSCs differentiation to hair lineage. There are various examples of crosstalk between WNT and BMP signaling pathways, that can be either synergistic or antagonistic, depending on the cellular context or the biological process (Itasaki et al., 2010). We evaluated if BMP2 can activate or potentiate Wnt/ß-catenin pathway in HFSC induced to differentiate with DPC conditioned media. We analyzed presence of nuclear ß-catenin by immunofluorescence. When HFSC were grown in FL (basal condition), ß-catenin was associated to plasmatic membrane. In a little percentage of cells (9.3%) it was in the nucleus, particularly in those cells that were in mitosis (Fig. 6). When HFSC were stimulated with DPC-conditioned medium from spheroids, 17.44 % of cells (Control) showed nuclear accumulation of ß-catenin. Relatively, it is 88% more than FL, indicating pathway activation. When DPC-spheroids were cultured in presence of DHT, conditioned medium (DHT) lost the ability to induce nuclear ß-catenin accumulation in HFSC (6.06% DHT vs 17.4% Control). Notably, HFSC cultured in DHT treated DPC-conditioned medium and supplemented with BMP2 immediately before use, showed the highest percentage of cells with nuclear ß-catenin (23,3% DHT+BMP2 vs 6.06% DHT) (Fig. 6). These results unveiled a synergistic crosstalk between BMP and Wnt/ßcatenin pathways, that may be involved in DPC induced-HFSC differentiation.

4. DISCUSSION

Androgens are essential in the process of the HF miniaturization that occurs during the development of androgenetic alopecia.

Androgens deregulate the production of DPC secreted factors, that can act in an autocrine way or have influence over the follicular epithelial cells. Thus, research have been focused on identifying these androgen-regulated factors (reviewed in Ceruti et al., 2018).

Bone morphogenetic proteins (BMPs), belong to the TGF superfamily and several reports have assigned them a role in the hair cycle or in hair follicle formation. Even if many of them indicate an inhibitory effect of BMPs in hair cycle control (Botchkarev et al., 2001,Jamora et al., 2003), other investigations, in noggin transgenic mice or Msx2 deficient mice, demonstrated that disruption of BMP signaling affects growth and differentiation of the anagen hair follicle. This evidence suggested that BMPs positively contribute to hair cycle control, especially to hair keratin formation (Kulessa et al., 2000,Ma et al., 2003). Moreover, using conditional gene targeting in mice keratinocytes, it was shown that BMP receptor IA is essential for the differentiation of progenitor cells of the inner root sheath and hair shaft (Kobielak et al., 2003). Other important work demonstrated that BMP signaling is required to maintain DP cell key phenotype*in vitro* and is crucial for stimulation of HFSC and HF growing *in vivo*(Rendl et al., 2008). Nevertheless, the precise role of BMPs in human hair cycle control remains controversial.

We report for the first time to our knowledge, a DHT driven downregulation of both BMP2 and BMP4 mRNAs in androgen sensitive DPC cultured as spheroids. In light of the above, we hypothesized that these factors have positive effects on the control of the human hair cycle. In accordance with our results, reduced expression of BMP2 was observed in cultured AGA dermal papillae (Midorikawa et al., 2004). Likewise, in a study aiming to identify molecular biomarkers associated with premature AGA, authors observed the same pattern for BMP2 and BMP4 in a gene expression analysis from bald men and healthy volunteers (Michel et al., 2017).

We alsoreport, that culture condition (2D or 3D) affects BMPs expression in DPC. BMP2 and BMP4 mRNAs level increases in a time dependent way during cell aggregation until spheroids are shaped. This point is relevant, as DP is a natural spheroidal tissue and DPC cultured as spheroids recover their inductivity (Higgins et al., 2013). Moreover, we have previously reported changes in gene expression of various Wnt pathway regulatory factors (DKK1, Wnt10b, Wnt5a), that depend on DPC culture condition. In the same

way, it was shown that addition of BMPs (BMP2, BMP4 or BMP6) to cultured DPC can restore certain features of their molecular identity that were loosen in the monolayer, without changing proliferation or cell morphology (Rendl et al., 2008 Supplementary material).

In addition, we show that BMP2 restored the alkaline phosphatase (ALP) activity of androgen treated human DPC spheroids, indicating that BMP2 could reestablish DPC inductive properties. Moreover, it was recently reported that overexpression of ALP in human dermal papilla spheroids increased the levels of phosphorylated AKT (p-AKT) and phosphorylated GSK3β (p-GSK3β), which in turn resulted in increased β-catenin levels in the nucleus. This was translated in an improved hair-inductive capacity(Kwack et al., 2019).

Our analyses have shown a downregulation of BMPs in androgen sensitive DPCs, but the relationship between these differences and the defined molecular mechanism underlying androgen-related actions remains unknown. After the catagen phase, some HFSCs residing in the bulge area migrate to the bulb to regenerate a new hair. A flow cytometry analysis of epithelial follicular cells from balding and non-balding areas of AGA patients, revealed that stem cells number was maintained in bald scalp whereas progenitor cells were markedly diminished (Garza et al., 2011). This observation suggests that the ability of HFSC to convert into progenitor cells is perturbed, either due to an autonomous defect or an inability to respond to signaling initiating the conversion. Alternatively, the secreted factors from DPC that initiate HFSC differentiation may be deregulated. Our previous work support this second proposal. We reported that androgens inhibit the Wnt signaling in androgen sensitive DPC and these cells were unable to induce the differentiation of HFSC, revealed by the diminished expression of follicular keratin K6hf in these cells (Leiros et al., 2012). This suggests that DPC-secreted factors involved in the molecular crosstalk with the precursor bulge cells are disturbed by the presence of androgens. In fact, our results showed a downregulation of the Wnt agonist WNT10b and an upregulation of the antagonist DKK1 after DHT treatment in DPC, and these regulations were involved in androgen inhibition of DPC-induced HFSC differentiation (Leiros et al., 2017).

In order to enlarge the list of factors which may play a role as mediators of DPC inductive action, we evaluated if BMP2 and BMP4 are DHT-downregulated factors involved in HFSC differentiation to hair lineage. We observed that the addition of BMP2 to media conditioned by DPC spheroids, significantly upregulated the expression of the keratin K6hf in HFSC, overcoming the inhibitory androgen effect. Our results indicate that only BMP2 but not BMP4, could have positive paracrine effects on HFSC differentiation induced by DPC.

So as to elucidate the mechanism of action of BMP2 in differentiating HFSC, we studied the expression of its major receptors. We found a downregulation of BMPRIa, BMPRIb and BMPRII during DPC-induced differentiation in HFSC. However, concomitant BMP2 treatment, increases BMPRIa and BMPRII expression levels.If the abundance of BMPRIa or BMPRII is a limiting component of the differentiation process in HFSC, additon of BMP2 could be favouring the recycling or the synthesis of them. It was reported that receptor activity is important for receptor endocytosis, receptor recycling and *de novo* synthesis. Besides, receptor recycling and *de novo* synthesis, are required for efficient BMP2 endocytosis at later time points. Moreover, the importance of specific cell surface binding sites was confirmed by the overexpression of BMPRIa, which led to a clear increase in BMP2 internalization(Alborzinia et al., 2013).BMP2 itself seems to be a limiting factor, at least in our model, as its addition induces expression of K6hf up to a level even higher than that of the control sample.In view of these results, it appears that BMP signaling could be counteracting the known inhibitory effects of DHT on Wnt/β-catenin pathway in HFSC, but also supporting differentiation via other mechanisms, bypassing the DHT restrain.

It is known that BMP2 is not significantly degraded upon internalization, and sorting to late endosomal particles could provide a reservoir for later use or may be associated with other cellular functions (Alborzinia

et al., 2013). BMP ligand/receptor unlimited availability, would allow for BMP pathway to be responsive for longer time, inducing expression of keratins, which is a reflection of the hair differentiation process (Fig. 4). BMPRIa is expressed in all different hair bulb populations. Thus, BMPs have both autocrine and paracrine potential regulatory actions within the microenvironment of the bulb (Botchkarev et al., 2004,Kobielak et al., 2003,Rendl et al., 2005,Rendl et al., 2008).

It had been reported that factors secreted by DPC triggerWnt pathway activationevidenced by the accumulation of β-catenin in the cytoplasm and nucleus of differentiating HFSC (Roh et al., 2004). When DPC are cultured in presence of androgens, their ability to induce Wnt/β-catenin pathway activation in HFSC is lost, as we demonstrated by culturing HFSC with the conditioned medium of DPC treated with DHT (Leiros et al., 2017). In this work, we show Wnt/β-catenin pathway reactivation after supplementing this conditioned medium with BMP2, evidenced by the highest percentage of cells with nuclear β-catenin. This result brings evidence that BMPs underregulation by androgens in DPC converges to inhibit Wnt/β-catenin signaling in differentiating HFSC. We suggest that a crosstalk between BMP and Wnt/ß-catenin pathways may be involved in DPC induced-HFSC differentiation. However, the molecular mechanism underlying this process is not elucidated.

Crosstalk of BMP and Wnt/β-catenin signaling may occur at various levels in the cascade. One possible spot of interaction between them in the cytoplasm may involve the PI3K/PKB pathway. PI3K, is recruited to membrane tyrosine kinases receptors upon their stimulation (e.g. by BMP2). Then, activated PKB can phosphorylate the Wnt pathway inhibitor GSK3β, inhibiting its activity (Haq et al., 2003). Hence, GSK3 inhibition would result in stabilizing β-catenin.

Several studies have reported an interrelation between the action of BMPs and Wnt/β-catenin signaling pathways during osteoblast differentiation (reviewed in Lin et al., 2011). In one of them, the positive effects of BMP2 on β-catenin expression level and on its translocation to nucleus, were diminished by blocking the PI3K pathway, giving a role to PI3K on the regulation of this crosstalk (Lee et al., 2010). Other mechanism that links GSK3 function with the BMP pathway, involves Smad1 phosphorylation by GSK3. GSK3 phosphorylates Smad1 at specific sites that cause ubiquitination and degradation of Smad1.When GSK3 is inhibited, Smad1 activation by BMPR1 is maintained at least a few hours longer compared with the situation where GSK3 is active (Fuentealba et al., 2007). It also could exist an interaction between Wnt and BMP signaling at gene expression level. In a murine model of renal dysplasia, authors found that mice with high BMP signaling show elevated activity of the Wnt/β-catenin pathway and discovered a Smad1/Tcf4/β-catenin complex, which drives expression of *c-myc* in excess amounts (Hu et al., 2003,Hu et al., 2005). In this case, Wnt and BMP pathways seem to work on a common target, and two signals show additive or synergistic effects. Indeed, it seems that BMP2 exerts that synergistic effect on K6hf expression in HFSC. We must elucidate if this is a direct or indirect action at the promoter or enhancer level.

It must be highlighted that, the outcome of cross-interactions between BMP and Wnt/β-catenin signaling, can vary depending on cell types, developmental stage, or even the particular target gene. In the hair follicle, the effect of this crosstalk can differ depending on the hair cycle phase and the balance of positive and negative signals in the follicle microenvironment.

In summary, our report suggests that androgen-driven BMP2 downregulation in DPC, perturbs HFSC differentiation into hair lineage, thus contributing to AGA development. In turn, addition of BMP2 can reestablish DPC inductive properties diminished by androgens. Moreover, when BMP2 is not a limiting factor, expression of its receptors in HFSC is higher, positively affecting hair lineage differentiation through a crosstalk with the Wnt/β-catenin pathway.

Based on DPC and HFSC cell lines that maintain both the characteristic fates from their origin tissue and their capabilities *in vitro*, this cellular model contributes to identify factors secreted by dermal papilla and involved in HFSC differentiation. Further studies, involving hair follicle cultured *ex vivo* or animal models, are needed in order to conclude about their physiological significance. A better understanding of androgen

altered epithelial-mesenchymal interactions that occur during AGA, would help to the development of improved treatments.

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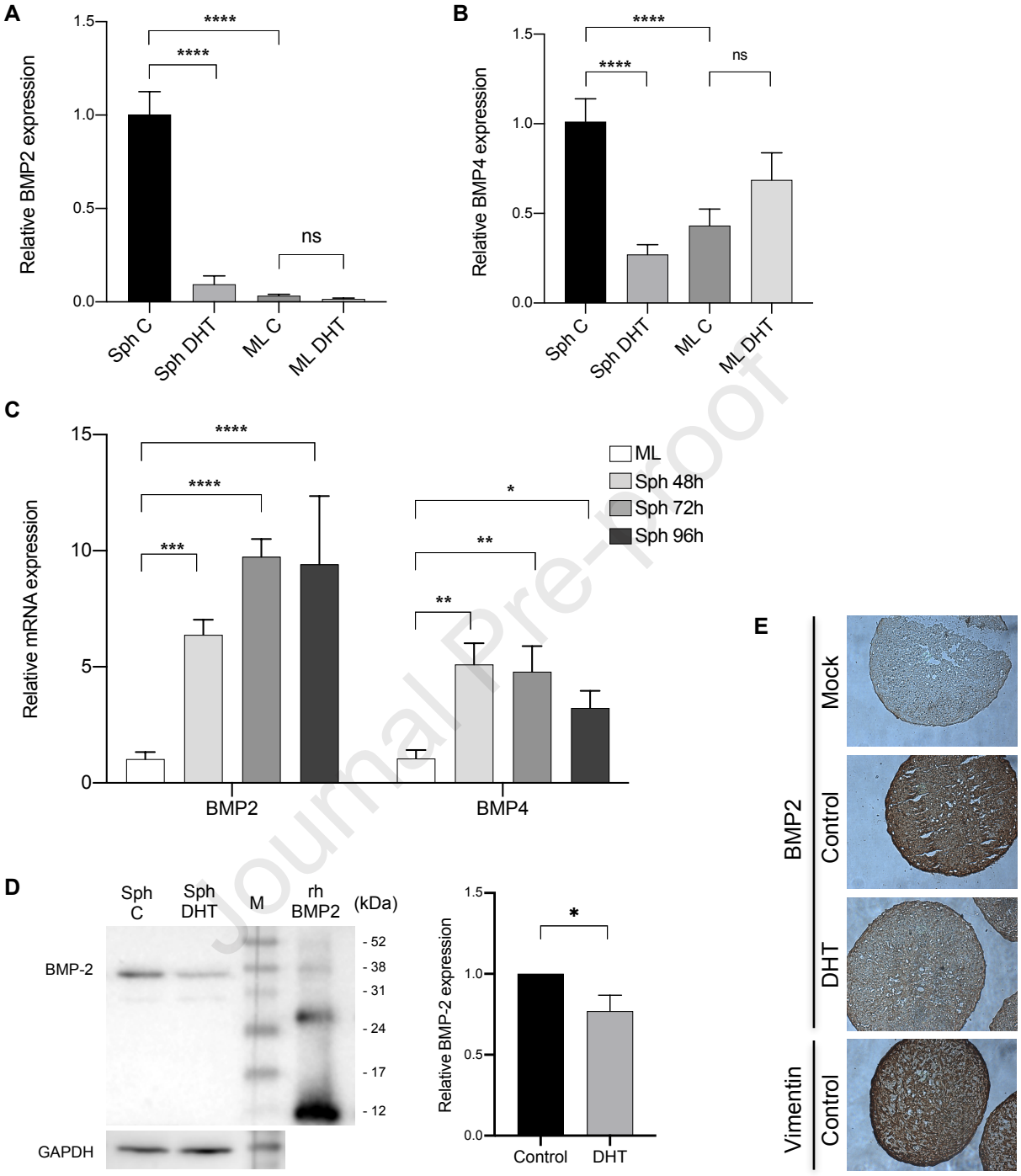


Fig. 1. BMP2 and BMP4 expression is regulated by androgens in DPC spheroids. Androgen sensible-DPC were cultured as spheroids (Sph) or monolayer (ML). BMPs expression was measured by: A,B) real time qPCR after 72h of DHT 10^{-7}M treatment. One way ANOVA test, alpha 0.05; C) real time qPCR in ML and during Sph formation at indicated time points. Two way ANOVA test, alpha 0.05; D) Western blot after 72h of DHT 10^{-7}M treatment. Unpaired t-test with Welch´s correction $P<0.05$; E) immunohistochemical staining in DPC Sph after 72h of DHT 10^{-7}M treatment. Vimentin was used as DP marker.

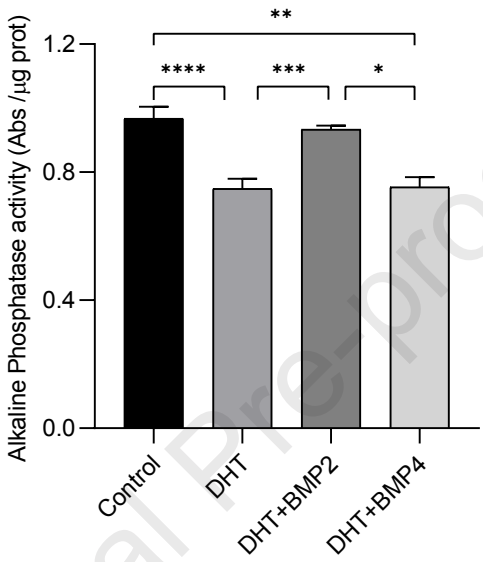


Fig. 2. BMP-2 restores inductivity marker in androgen treated human DPC spheroids. DPC spheroids expressing AR were treated or not with DHT 10^{-7}M alone or with 300 ng/ml BMP2 or BMP4 addition. Alkaline phosphatase activity was measured after 72h in cellular extracts using p-nitrophenyl phosphate (pNPP) as substrate (10mM) and expressed as the ratio between absorbance at 405 nm and µg of protein. Brown-Forsthy and Welch´s ANOVA test, (*) $P<0.05$.

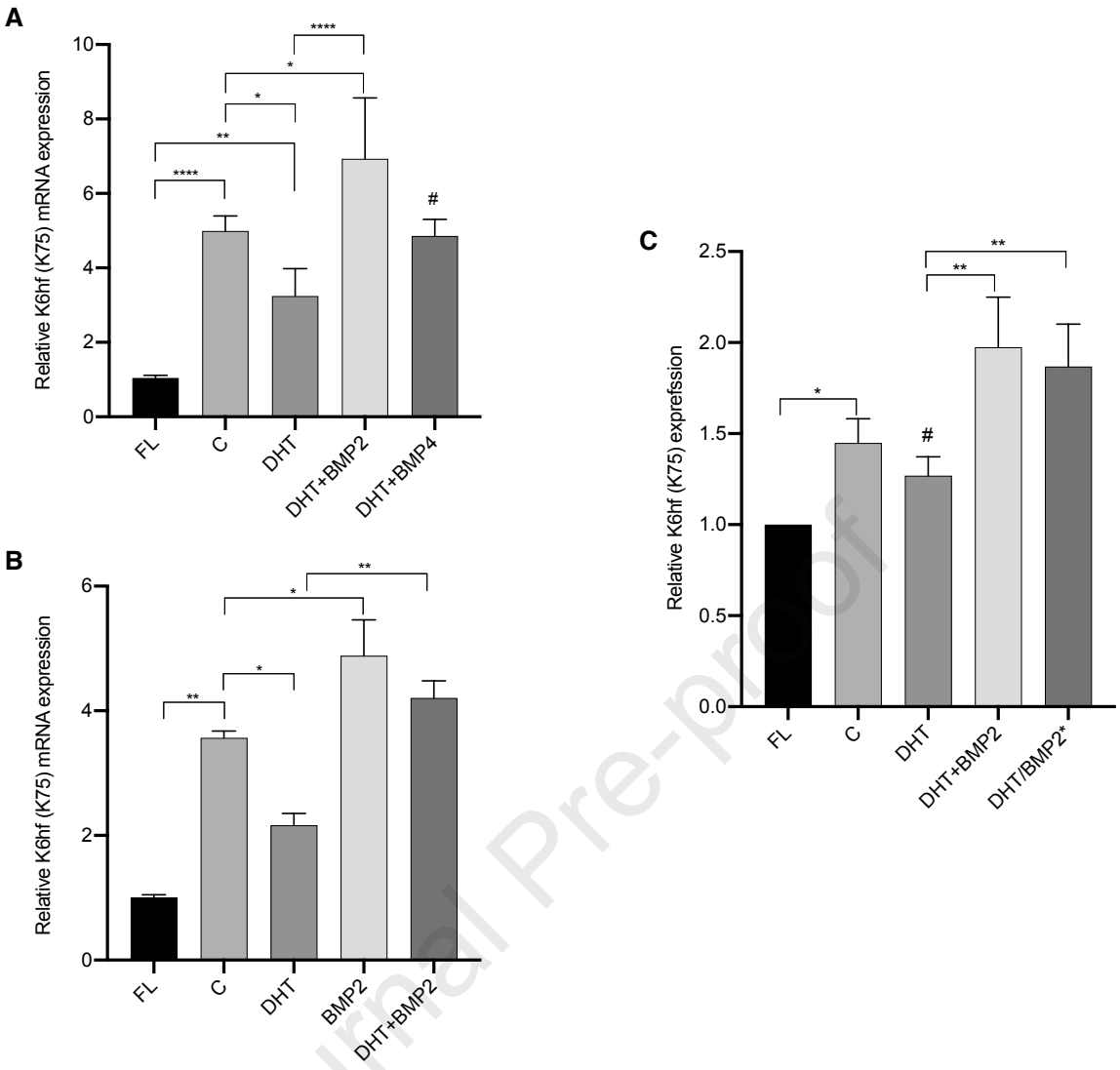


Fig. 3. Androgen and BMPs effects on HFSC differentiation. A) Real time qPCR analysis of K6hf expression in untreated HFSC (FL) or cultured with differentiating medium conditioned by: DPC-Sph (C), DPC-Sph treated with 10^{-7}M DHT (DHT), DPC-Sph treated with 10^{-7}M DHT and supplemented with 300 ng/ml BMP2 immediately before use (DHT+BMP2), DPC-Sph treated with 10^{-7}M DHT and supplemented with 300 ng/ml BMP4 immediately before use (DHT+BMP4). **B)** Real time qPCR analysis of K6hf expression in a differentiation assay with heterotypic spheroids. In a 96 well plate coated with PVA 5%, we plated 3T3 Swiss Albino cells (FL) or DPC, in control medium (C); DPC with medium supplemented with DHT (DHT); DPC with medium supplemented with BMP2 (BMP2); or DPC with medium supplemented with both DHT and BMP2 (DHT+BMP2), and let spheroids form for 24h. Then we added HFSC to form heterotypic spheroids, and let them differentiate for another 96h. HPRT mRNA was used as the internal control. **C)** Western blot analysis of K6hf expression in HFSC treated as in A. GAPDH was used as loading control. Results are expressed relative to HFSC in FL. Statistical analysis was performed using One way Anova-Tukey-Kramer test. *$p < 0.05$,**$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, # ns.

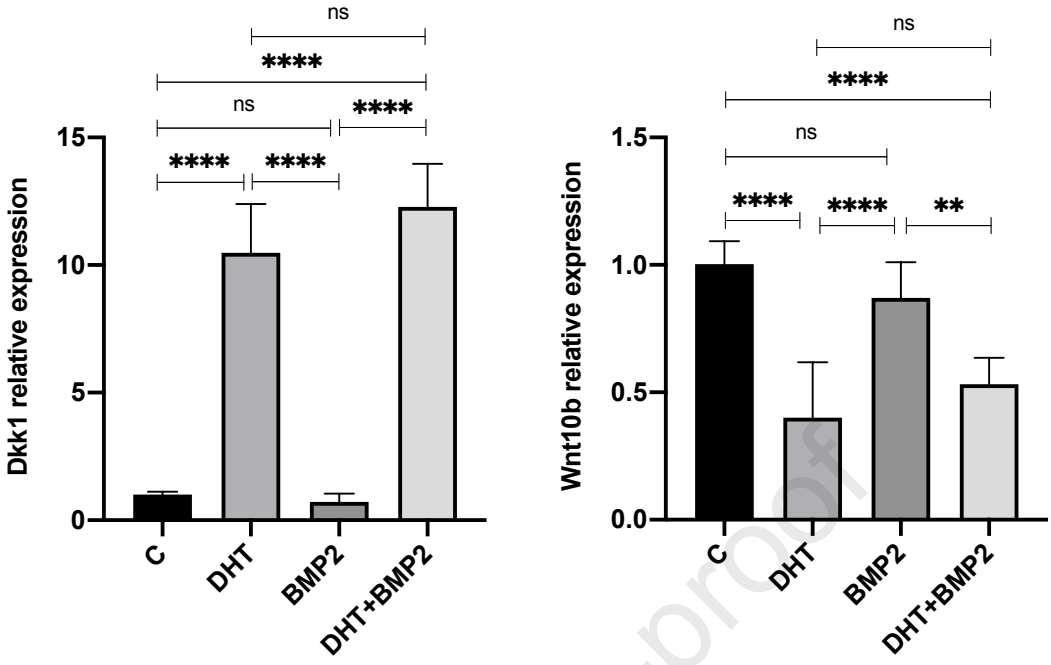


Fig. 4. **Androgens and BMP2 effect on Wnt pathway ligands expression in DPC spheroids.** Real time qPCR of WNT inhibitor Dkk-1 (left palnel) or WNT activator Wnt10b (right panel) in DPC spheroids treated or not with DHT and/or BMP2 for 72h. HPRT mRNA was used as the internal control. Statistical analysis was performed using One way Anova-Tukey-Kramer test. *$p < 0.05$, ns: non significant.

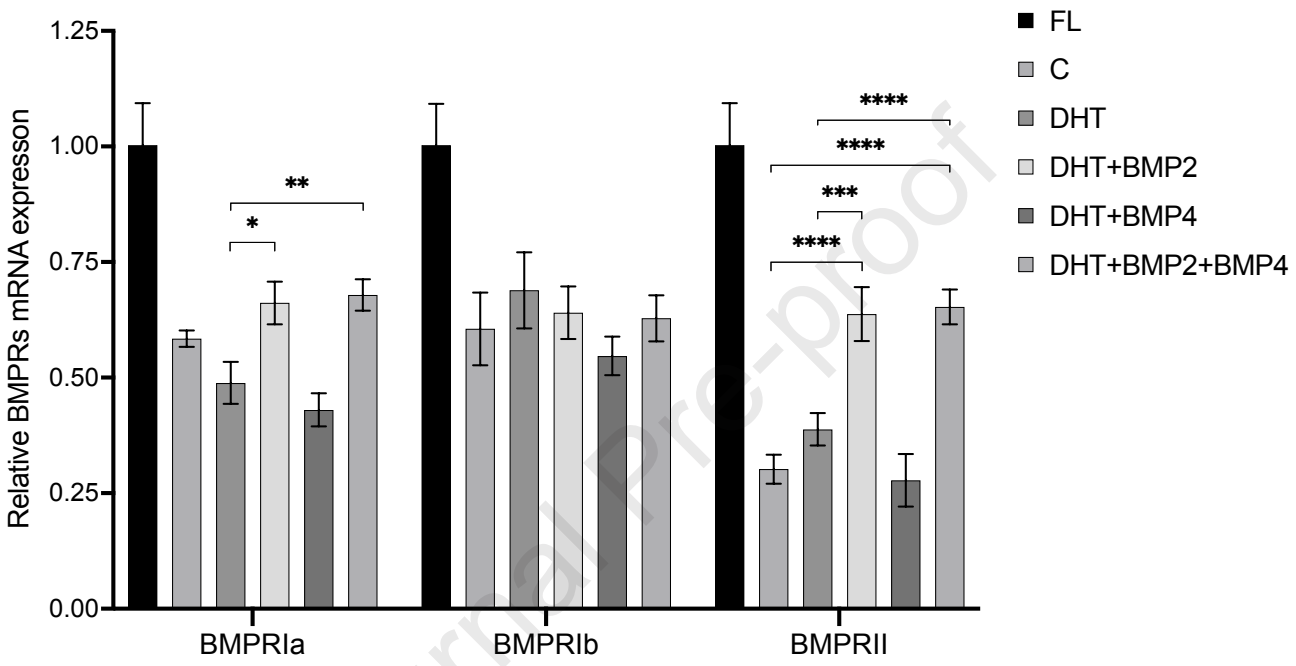
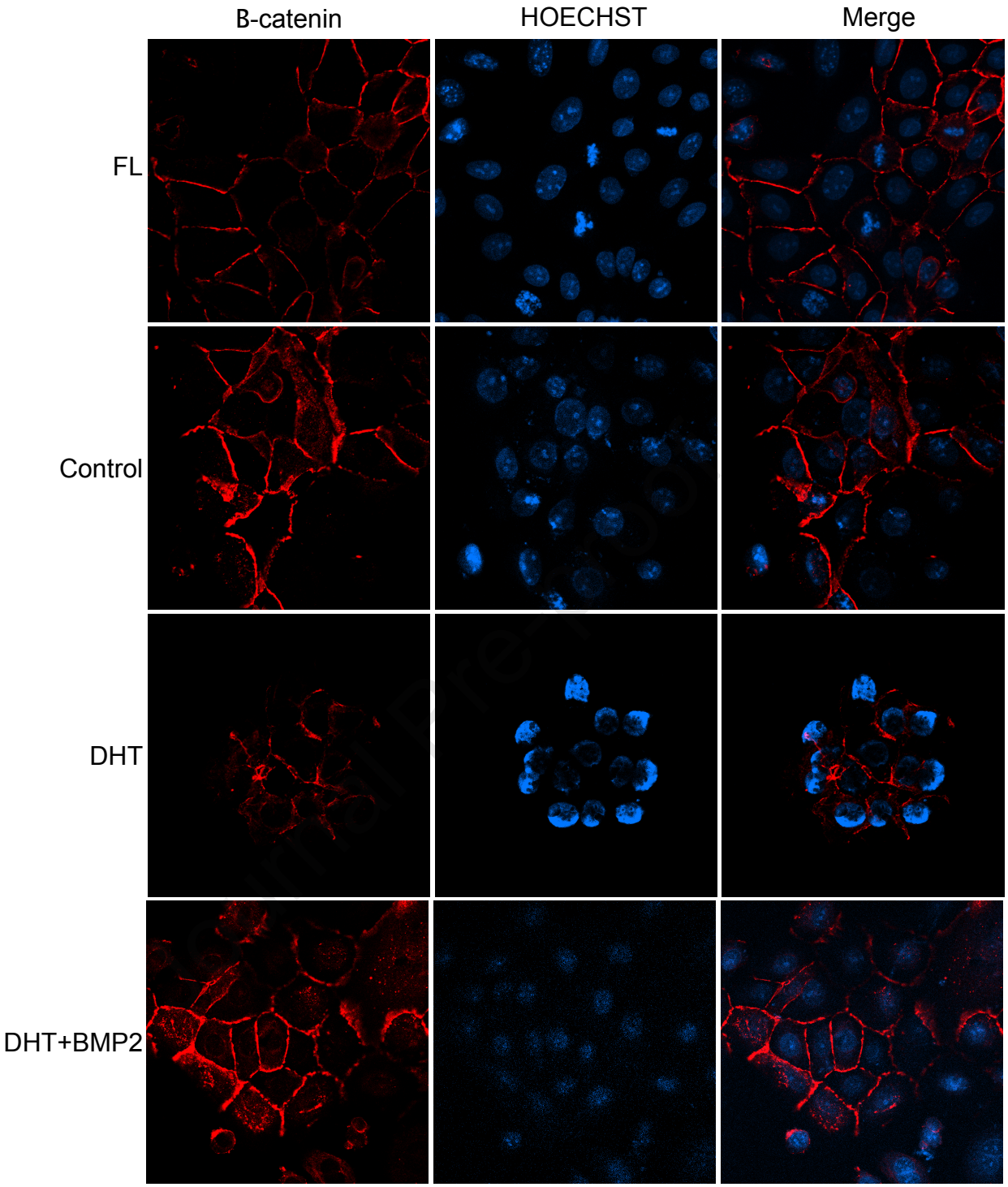


Fig. 5. BMPs effect on BMPRs expression in differentiating HFSC. Real time qPCR analysis of BMPRIa, BMPRIb and BMPRII expression in untreated HFSC (FL) or cultured with medium conditioned by: DPC-Sph (C), DPC-Sph treated with 10^{-7}M DHT (DHT), DPC-Sph treated with DHT and supplemented with 300ng/ml BMP2 immediately before use (DHT+BMP2), DPC-Sph treated with DHT and supplemented with 300ng/ml BMP4 immediately before use (DHT+BMP4), DPC-Sph treated with DHT and supplemented with BMP2 and BMP4 before use (DHT+BMP2+BMP4). HPRT mRNA was used as the internal control. Results are expressed relative to HFSC in FL. Statistical analysis was performed using Two way Anova-Tukey multiple comparisons test. *$p < 0.05$,**$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.



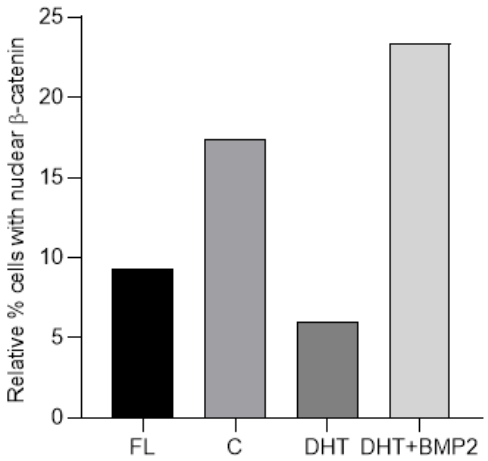


Fig. 6. **β-catenin nuclear translocation in differentiating HFSC.** Confocal immunofluorescence images (400x magnification) of β-catenin (red) in HFSC cultured in different DPC-conditioned or supplemented media for 20h as indicated: FL (non conditioned medium), Control (medium conditioned by DPC Sph), DHT (medium conditioned by DPC Sph treated with 10^{-7}M DHT), DHT+BMP2 (medium conditioned by DPC Sph and supplemented with 300ng/ml BMP2). Nuclear staining with Hoechst dye (blue). At least 100 cells of different fields were analyzed for each treatment.

HIGHLIGHTS.

Androgens down regulate BMP2 and BMP4 in dermal papilla cells (DPC).

BMP2 contributes to human DPC spheroids inductivity.

BMP2 restores hair follicle stem cell (HFSC) differentiation inhibited by androgens.

BMPRIa and BMPRII are upregulated by BMP2 paracrine action on differentiating HFSC.

A crosstalk between BMPs and Wnt pathways is observed in differentiating HFSC.