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Androgens modify Wnt agonists/antagonists expression balance in dermal papilla cells preventing hair follicle stem cell differentiation in androgenetic alopecia

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ABSTRACT

In androgenetic alopecia, androgens impair dermal papilla-induced hair follicle stem cell (HFSC) differentiation inhibiting Wnt signaling. Wnt agonists/antagonists balance was analyzed after dihydrotestosterone (DHT) stimulation in androgen-sensitive dermal papilla cells (DPC) cultured as spheroids or monolayer. In both culture conditions, DHT stimulation downregulated Wnt5a and Wnt10b mRNA while the Wnt antagonist Dkk-1 was upregulated. Notably, tissue architecture of DPC-spheroids lowers Dkk-1 and enhances Wnt agonists' basal expression; probably contributing to DPC inductivity. The role of Wnt agonists/antagonists as mediators of androgen inhibition of DPC-induced HFSC differentiation was evaluated. Inductive DPC-conditioned medium supplemented with DKK-1 impaired HFSC differentiation mimicking androgens' action. This effect was associated with inactivation of Wnt/β-catenin pathway in differentiating HFSC by both DPC-conditioned media. Moreover, addition of WNT10b to DPC-medium conditioned with DHT, overcame androgen inhibition of HFSC differentiation. Our results identify DKK1 and WNT10b as paracrine factors which modulate the HFSC differentiation inhibition involved in androgen-driven balding.

1. Introduction

The hair follicle (HF) is a regenerating system, which physiologically undergoes cycles of growth (anagen), regression (catagen), and rest (telogen). The epithelial stem cells reservoir located at the hair follicle bulge (Blanpain et al., 2004; Morris et al., 2004; Oshima et al., 2001) is responsible for the regeneration of the HF during cycling. This process implicates a crosstalk between epithelial precursor cells and the underlying mesenchymal dermal papilla (DP), encapsulated by the overlying epithelial matrix cells. Growth factors from the DP cause epithelial cells to proliferate and differentiate into the hair follicle cell lineages to produce the hair shaft (Blanpain et al., 2004; Botchkarev and Kishimoto, 2003). Many paracrine factors and signaling pathways involved in this crosstalk at different hair cycle stages have been reported (Botchkarev and Kishimoto, 2003; Roh et al., 2004).

Androgenetic alopecia (AGA) (Randall et al., 2000) is the most common type of hair loss in men. During AGA, large and terminal scalp hairs are replaced by smaller hairs in precise patterns. Although most molecular mechanisms of AGA are not clear, the process is attributed to the effect of dihydrotestosterone (DHT) on hair follicles of the scalp. The skin and its appendages are important sites for estrogen and androgen production, activation or metabolism. Indeed, the androgenic effects on hair follicle could not be an isolated phenomenon but a complex one including systemic circulating androgens as well as the locally produced. These steroids act in auto or paracrine fashions to regulate local homeostasis (Slominski et al., 2012, 2013).

The DP is hypothesized as the androgen's target in hair follicles suffering AGA that sends signals to follicular epithelial cells (Randall et al., 2001). Androgen-driven alteration of the autocrine and paracrine factors produced by scalp DP may be a key to AGA

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development. The screening of DHT-regulated genes in balding DP showed that Wnt inhibitory molecule dickkopf 1 (DKK-1) is one of the most upregulated genes (Kwack et al., 2008). Activation of the Wnt/β-catenin signaling pathway is important for hair morphogenesis (Millar, 2002) and for the maintenance of DP inductive properties required for HF regeneration and growth of the hair shaft (Shimizu and Morgan, 2004). Wnt/β-catenin signaling leads to cytosol accumulation of β-catenin, which then translocates to the nucleus to act as a coactivator of the transcription factor TCF/Lef (T cell-factor proteins) regulating the expression of specific genes.

We had shown that androgens regulate the expression of factors secreted by androgen-sensitive DP cells which are involved in normal hair follicle stem cells differentiation via the inhibition of the canonical Wnt signaling system pathway (Leirós et al., 2012). This androgen modulating effects on autocrine or paracrine DP cell functions could play a major role in differentiation defects of hair follicle stem cells during AGA development. The observation that stem cells number was maintained in bald scalp whereas progenitor cells were markedly diminished supports this notion (Garza et al., 2011). Understanding the signals responsible for stem cells differentiation will be the next step in developing new treatments for AGA.

Wnt agonists (e.g., Wnt3a, 5a, 10b) are involved in HF induction and hair growth promotion abilities of the DP, and their expression have been reported in HFs of postnatal skin (Reddy et al., 2001).

Wnt10b mediates canonical signaling, promotes the proliferation of DP cell primary cultures, and maintains their ability for HF induction and hair growth (Ouji et al., 2012). Wnt5a has been found in DP and root sheath cells (Xing et al., 2011) of mature anagen follicles, and Wnt10b in IRS cells within the matrix, implying that they are involved in follicle morphogenesis. However, their specific roles remain unknown.

DP cells are distinguishable from dermal fibroblasts by their aggregative behavior and their ability to induce new hair follicle formation. This aggregative conformation *in vivo*, suggests that growth in monolayer culture (2D) is particularly 'unnatural' for DP cells. Key differences between 2D cultured DP cells and intact DP have been reported, including molecular signature changes probably involved in loss of their hair inductive capacity (Higgins et al., 2013). Spheroids (3D) culture models recapitulate the *in vivo* tissue counterparts by re-establishing the tissue microenvironment that promotes an appropriate cellular phenotype (Schmeichel and Bissell, 2003).

To assess the effect that culture conditions could have on androgen-driven signals, we set out this work establishing not only 2D but also 3D DP cells cultures.

In this work, we evaluate the impact of DP cells culture conditions and the role of DHT-mediated Wnt agonists/antagonists imbalance in HFSC differentiation induced by DP cells.

2. Materials and methods

2.1. Cell line cultures

The androgen-responsive human DP cell line (DPC) was obtained after stable transfection of a DPC immortalised cell line, kindly provided by Dr. Michael P. Philpott (Barts and The London School of Medicine and Dentistry, Queen Mary University of London) by electroporation with pCI-neoAR (mammalian expression vector pCI-neo Promega, Fitchburg, WI, USA that express androgen receptor (AR)). Cells stably expressing the AR gene (DPC-AR) were selected in DMEM culture medium (GIBCO-Invitrogen, CA) containing penicillin (100 U/ml), streptomycin (100 mg/ml), glutamine (4 mM), 10% fetal bovine serum (FBS) (GIBCO-Invitrogen, CA) and 400 μg/ml of antibiotic G418 (Geneticin, Termofisher, Waltham, MA, USA). DPC-AR selected clone was maintained in the same culture medium reducing G418 concentration to 100 μg/ml.

The immortalised 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) (mytomicin C-inactivated 3T3-Swiss cells) using cFAD medium (Roh et al., 2004).

Generation of DPC-spheroids and monolayer cultures:

For DPC-spheroids cultures, 5×10^4 cells/well were seeded in 96-wells plate coated with 5% PVA (Polyvinyl alcohol). In case of monolayer cultures, 1.5×10^5 cells/well were seeded in 6-wells plate. The cultures were left undisturbed for 4 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 (Sigma-Aldrich, St. Louis, MO, USA) or 25 ng/ml Dkk-1 (Acrobiosystems, Newark, DE, USA), 200 ng/ml WNT5a (R&D Systems) or 400 ng/ml WNT10b (R&D Systems). 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 4 days, whereupon the medium was replaced with media conditioned by DPC-spheroids with different treatments (in 1:1 rate with cFAD) and cultured during 72hs.

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 the Step-One Real time PCR machine (Applied-Biosystems-Invitrogen, Carlsbad, CA) using SYBR Green MasterMix (Applied-Biosystems-Thermofisher, Waltham, MA, USA) and the following set of specific primers: Dkk-1 (Forward: 5′-TCACGCTATGTGCTGCCCCG-3′, Reverse: 5′-TCTGGAATACCC ATCCAAGGTGCT-3′), Wnt10b (Forward: 5′-CTCTGGGATGTG-TAGCCTTC-3′, Reverse: 5′-GGCTCTGGAGTTGAGAAGTG-3′), Wnt5a (Forward: 5′-TTGAAGCCAATTCTTGGTGGTCGC-3′, Reverse: 5′-TGGTCCTGATACAAGTGGCACAGT-3′) and K6hf (Forward: 5′-CTA-GAGCCCCTCTTTGATTCCT-3′, Reverse: 5′-GCAGCATCTACGTC CTTTTTCA-3′). Hypoxanthine-guanine phosphoribosyltransferase (HPRT) specific set of primers (Forward: 5′-CTCAACTTTAACTG-GAAAGAATGTC-3′, Reverse. 5′-TCCTTTTCACCAGCAAGCT-3′) was used as internal mRNA expression control.

2.4. 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 with conditioned or control media during 3hr and fixed in chilled MeOH. β-catenin was detected by immunofluorescence using anti-human β- catenin antibody (B&D, USA) followed by an anti-murine antibody conjugated to fluorochrome Alexa fluor 555 (Thermo Fisher Scientific, USA) and analyzed by confocal microscopy (Olympus FV 1000).

2.5. Western blot assays

To evaluate Dkk-1, WNT5a and WNT10b protein levels in DPC with the different treatments, whole cell extracts were obtained by

RIPA lysis buffer [20 mmol L^{-1} Tris, pH 7,5, 1 mmol L^{-1} EDTA, 0,15 mol L^{-1} NaC1, 10 mmol L^{-1} KC1, 1% NP-40, 0,1% deoxycholate, 0,1% sodium dodecyl sulphate (SDS)] with protease inhibitors. After SDS–polyacrylamide gel electrophoresis and electroblotting, membranes were incubated with the following primary antibodies for DKK-1 (anti human DKK-1 polyclonal antibody-ACRO Biosystems, Newark, DE, USA), WNT5a (Human/Mouse Wnt5a Monoclonal Antibody, Catalog # MAB64, R&D Systems) and WNT10b (Human Wnt10b Monoclonal Antibody, Catalog # MAB7196, R&D Systems). To evaluate β-catenin nuclear translocation in DPC, nuclear extracts were obtained as described before (Mendez and Stillman, 2000). Briefly, cellular pellets were resuspended in Buffer A (10 mM HEPES (pH 7,9), 10 mM KCl, 1,5 mM $MgCl_2$, 0,34M Sucrosa, 10% Glycerol, 1 mM DTT, 0,1 mM PMSF, protease inhibitors and 0,1% Triton X-100 added prior to use) and incubated for 3 min on ice. Then they were centrifuged at 1300×g, 4 min at 4 °C. Supernatant corresponds to cytoplasmic fraction. Nuclear pellet was washed once with buffer A and centrifuged at 1500 RPM 4 min at 4 °C. Pellet was resuspended in buffer B (3 mM EDTA, 0,2 mM EGTA, 1 mM DTT, proteases inhibitors) and incubated 30 min on ice. Then it was centrifuged at 12000g, to obtain the nuclear extract in supernatant. The pellet contains the chromatinic fraction. Proteins were quantified with bicinchoninic acid assay (Thermo Scientific™ Pierce™ BCA Protein Assay). Western blots were done as previously described using a primary antibody specific for β-Catenin (cat. 616153, BD Transduction Laboratories ™). Anti PCNA antibody (sc-7907, Santa Cruz Biotechnology Inc., Santa Cruz, CA, U.S.A) was used as nuclear loading control. Horseradish peroxidase-conjugated secondary antibodies specific for primary antibody isotype (GE Healthcare, Amersham, U.K.) were used. Blots were developed using the chemiluminescence imaging system G: BOX Chemi XRQ (Syngene, Frederick MD, USA Inc.).

3. Results

3.1. Androgens modulate expression of Wnt ligands in DPC cultures

We have previously observed that androgens abrogate the ability of DP cells to induce HF stem cells differentiation into hair follicle lineage via the inhibition of Wnt signaling (Leirós et al., 2012). However, the autocrine and/or paracrine factors modulated by androgens involved in DPC induced HFSC differentiation remain uncertain. This differentiation process requires activation of Wnt signaling pathway both in DP cells and HF stem cells (Roh et al., 2004).

Therefore, androgens regulation of the Wnt-ligand antagonist Dkk-1, and the agonists Wnt10b and Wnt5a expression was studied in DPC. Moreover, as it was observed that DP cells increase their inductive ability when they are cultured as spheroids (Higgins et al., 2013), we also evaluated the effect that culture conditions could have on selected Wnt agonists/antagonists expression.

The AR expression in balding DP cells is significantly higher than in non-balding DP cells, resulting in elevated androgen sensitivity in AGA (Hibberts et al., 1998). In DP cells primary cultures, AR expression level gradually decreases through the successive passages (Kwack et al., 2008; Inui et al., 2002). We therefore used an androgen- responsive human DP cell line (DPC) as model for AGA in order to study the effect of androgen on Dkk-1 gene expression. In this cell line, obtained as described in Materials and Methods, specific traits of dermal papilla, such as α-smooth muscle actin (α-SMA), alkaline phosphatase activity, and a functional androgen receptor (AR) (Supplementary Fig. 1) were confirmed.

DPC cultured as monolayer (Fig. 1A) or spheroids (Fig. 1B) in presence of DHT 10^{-7} M during 72 h showed 50 and 43 fold increase in Dkk-1 mRNA expression, respectively, compared to Control (Fig. 1C and D). Western blot analysis performed in DPC-spheroids cultures also showed upregulation of DKK-1 protein after 48 h treatment with DHT, which was blocked by the androgen antagonist flutamide (Fig. 2A).

We then evaluated Wnt10b and Wnt5a mRNA expression. Interestingly, the regulation of the expression by DHT of both Wnt mRNA was opposite to that observed for Dkk-1. DHT downregulated Wnt10b expression 30% and 60% in monolayer and spheroids DPC cultures respectively (Fig. 1E, F). Similarly, Wnt5a expression was reduced 40% and 70% in monolayer and spheroids DPC cultures respectively (Fig. 1G, H). Western blot analysis performed in DPC-spheroids cultures also showed downregulation of WNT10b protein after 48 h treatment with DHT (Fig. 2B). However, WNT5a was barely detected and no regulation could be observed (Fig. 2C).

Although both culture conditions showed similar upregulation in Dkk-1 gene expression after DHT treatment, the basal expression was 12-fold higher in monolayer than in spheroids culture condition (Fig. 3A). Similarly, significant differences in Wnt10b and Wnt5a mRNA expression were observed between 2D and 3D DPC culture conditions. Nevertheless, in this case, the basal mRNA expression of Wnt10b and Wnt5a was approximately 10-fold lower in monolayer than in spheroids culture condition (Fig. 3B, C).

Spheroid culture condition dramatically reduces Dkk-1 and increases Wnt10b and Wnt5a mRNA expression in DPC cultures indicating that 3D-tissue architecture tips the balance of Wnt agonists/antagonists expression in favor of the first ones.

DKK-1 abrogates DPC-mediated HFSC differentiation, mimicking androgen effect.

Since the next step was to study the effect of DHT-inducible DKK-1 on the ability of DPC-conditioned culture media to induce HFSC differentiation, we thereafter cultured DPC as spheroids that showed the lowest Dkk-1 basal expression level.

In the present study, we observed that conditioned culture media from DPC-spheroids induced HFSC differentiation into hair lineage. When HFSC were cultured in DPC-conditioned media (DPC-Sph Control), it was observed that K6hf mRNA expression, a keratin expressed in the hair follicle companion layer and used as hair follicle lineage committed marker (Wang et al., 2003), was upregulated approximately 5-fold compared to HFSC cultured in non-conditioned medium (FL) (Fig. 4A). Nevertheless, when HFSC were cultured in presence of media conditioned by DPC-spheroids cultured with DHT 10^{-7} M (DPC-Sph DHT), a significant reduction in K6hf mRNA expression was observed with respect to HFSC cultured with DPC-Sph Control (Fig. 4A). In order to evaluate if DKK-1 is a DHT-regulated factor that can block DPC-inductive action, 25 ng/ml DKK-1 were added to DPC-spheroids and conditioned media were collected. When HFSC were cultured using this conditioned media (DPC-Sph/DKK-1), the K6hf mRNA expression was significantly reduced mimicking DHT effect (Fig. 4A). This result indicates that DKK-1 could have autocrine effects on DPC.

Given that Dkk1 is a DHT inducible gene, we evaluated if its paracrine action mediates androgen effect on the inhibition of HFSC differentiation induced by DP cells (Leirós et al., 2012). For that purpose, 25 ng/ml DKK-1 were added to the inductive DPC-conditioned medium before been used for HFSC culture (DPC-Sph Control + DKK-1). HFSC cultured with this medium showed a significant reduction in K6hf mRNA expression similarly to that observed with the DPC-conditioned media obtained in presence of DHT (DPC-Sph DHT) or DKK-1 (DPC-Sph/DKK-1), indicating that a DKK-1 paracrine action would be enough to abrogate HFSC differentiation induced by DPC (Fig. 4A). Moreover, we had observed that this paracrine inhibitory effect is directly correlated with the dose used (Supplementary Fig. 2).

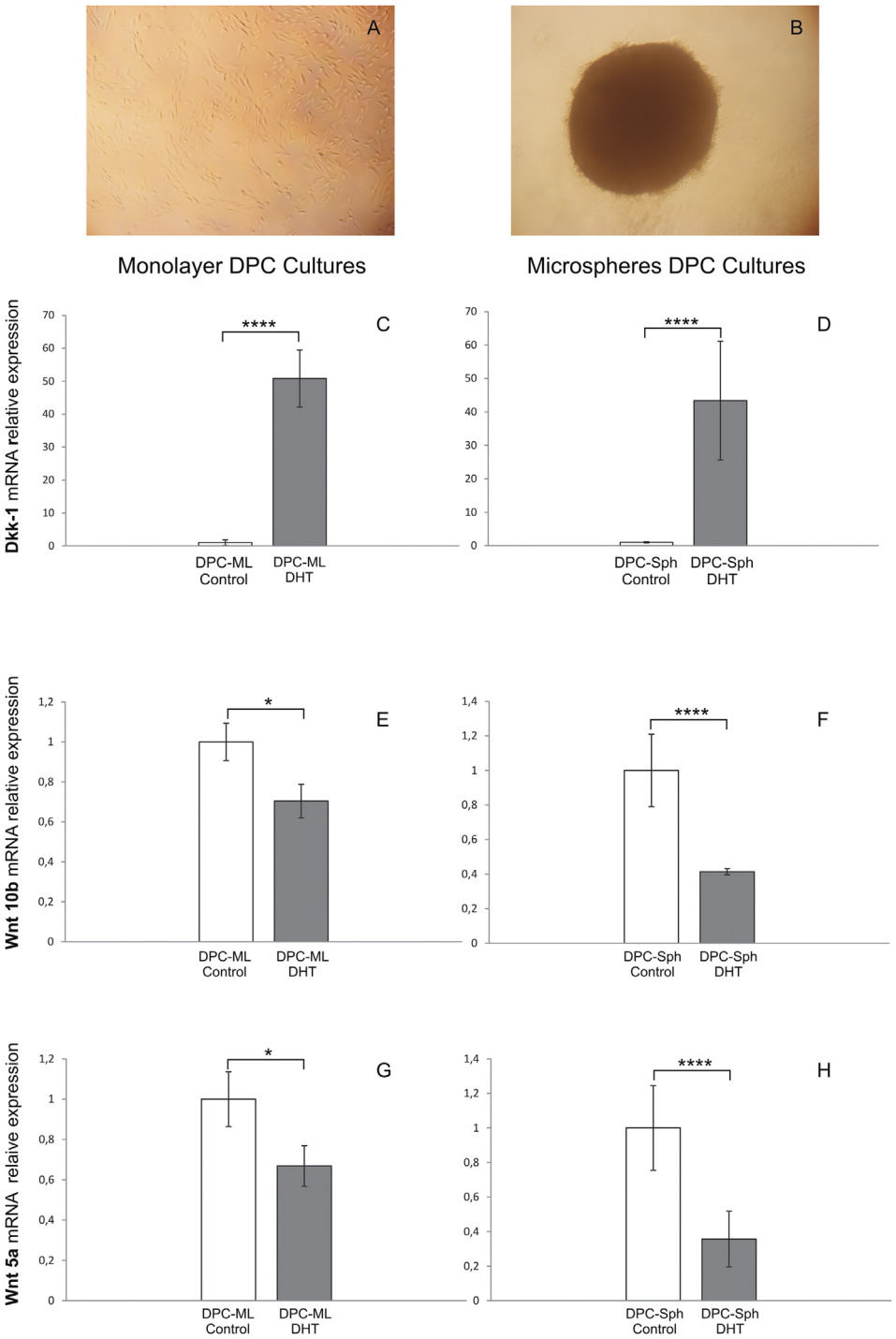

Fig. 1. Androgen effect on Wnt ligands mRNA expression in human DPC. DPC cultures as Monolayer (ML) (A) or Spheroids(Sph) (B) (40× magnification) were established. mRNA expression analysis, by Real-time PCR, of Dkk-1, Wnt10b and ligand Wnt5a in DPC-ML (C, E and G) or DPC-Sph (D, F and H) conditions in absence (DPC Control) or presence of 10^{-7}M DHT (DPC DHT) for 72 h. Values are means of triplicates ± SD using HPRT mRNA as the internal control. Results are expressed relative to DPC Control. Statistical analysis was performed using Unpaired T-Test. *p < 0.05; ****p < 0.0001.

3.2. Wnt 10b overcomes androgen inhibition in DPC-mediated HFSC differentiation

Given that Wnt 10b and Wnt 5a are DHT downregulated genes, we evaluated if they are paracrine factors that contribute to HFSC differentiation induced by DP cells and if their downregulation is involved in the impairment of DPC-mediated HFSC differentiation by androgens. In order to evaluate if the mentioned Wnt ligands can overcome androgen effect, 400 ng/ml of WNT10b or 200 ng/ml of WNT5a were added to the DPC-conditioned media obtained in presence of DHT (DPC-Sph DHT).

HFSC cultured with the conditioned medium supplemented with WNT10b (DPC-Sph DHT + WNT10b) showed a significant increase in K6hf mRNA expression respect to that observed with the DPC-conditioned media obtained in presence of DHT (DPC-Sph DHT) (Fig. 4B), indicating that Wnt 10b can overcome androgen

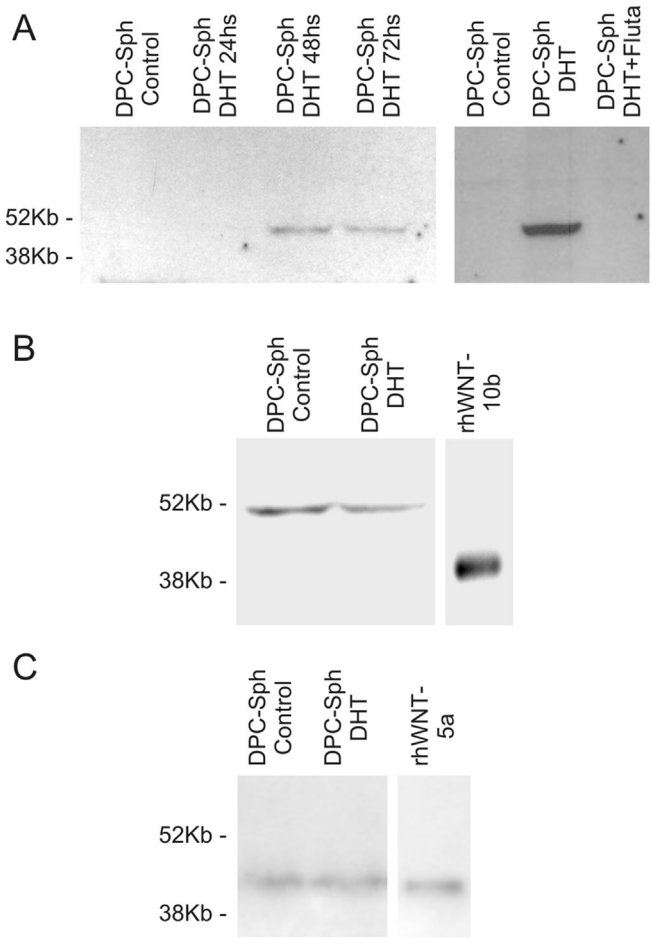

Fig. 2. Androgen effect on DKK1, WNT10b and WNT5a protein expression in human DPC. (A) Western blot for DKK-1 expression through culture time (left side): Androgen-responsive DPC cell line was cultured as microspheres without treatment (DPC-Sph control) or in presence of 10^{-7} M DHT during 24 h (DPC-Sph DHT 24 h), 48 h (DPC-Sph DHT 48 h) and 72 h (DPC-Sph DHT 72 h). Androgen effect on DKK-1 expression is mediated by androgen receptor (Right side): Androgen-responsive DPC cell line cultured as microspheres was treated with 10^{-7} M DHT (DPC-Sph DHT) or 10^{-7} M DHT and 100 μM Flutamide (DPC-Sph DHT + Fluta) during 72 h or not treated (DPC-Sph Control). (B) Western blot of androgen effect on WNT10b expression: Androgen-responsive DPC cell line cultured as microspheres was treated with 10^{-7} M DHT during 72 h (DPC-Sph DHT) or not treated (DPC-Sph Control). The last lane corresponds to recombinant human WNT10b protein. (C) Western blot to evaluate androgen effect on WNT5a expression: This western blot was done as in (B). In this case the last lane corresponds to recombinant human WNT5a. Numbers at the left side indicate molecular weight markers.

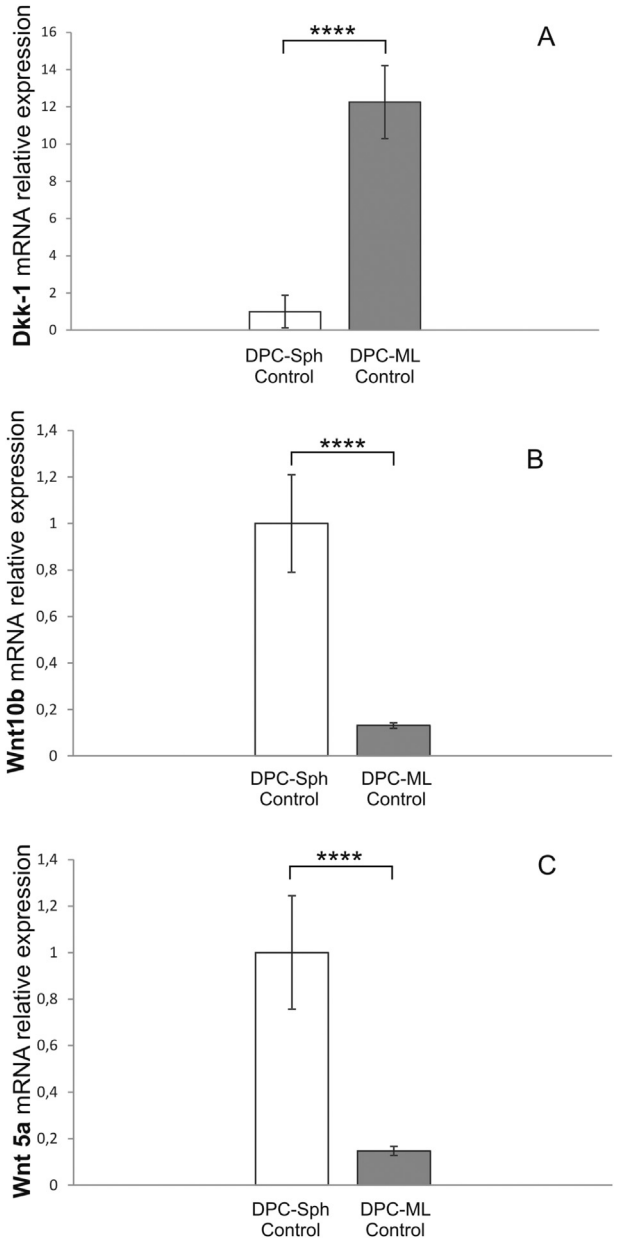

Fig. 3. Effect of DPC culture conditions on Wnt ligands basal expression in human DPC. mRNA basal expression analysis, by Real-time PCR, of Dkk-1 (A), Wnt10b (B) and Wnt5a (C) in spheroids (DPC-Sph Control) or Monolayer (DPC-ML Control) conditions. Values are means of triplicates ± SD using HPRT mRNA as the internal control. Results are expressed relative to DPC-Sph Control. Statistical analysis was performed using Unpaired T-Test. ****$p < 0.0001$.

effect in DPC-mediated HFSC differentiation.

However, no significant differences were observed in K6hf mRNA expression respect to that observed with the DPC-conditioned media obtained in presence of DHT (DPC-Sph DHT), when this medium was supplemented with WNT5a (Fig. 4B).

3.3. DKK-1 abrogates cytoplasmic β-catenin accumulation in HFSC treated with DPC-conditioned medium

As factors secreted by dermal papilla cause β catenin stabilization in differentiating HFSC (Roh et al., 2004), we evaluated the activation of Wnt/β-catenin pathway during HFSC-differentiation induced by DPC conditioned media after 3 days. In basal conditions HFSC showed β-catenin expression exclusively associated to plasmatic membrane (FL) (Fig. 5A). When HFSC were stimulated with DPC-conditioned medium from spheroids (DPC-Sph Control), we observed the accumulation of β-catenin in the cytoplasm and eventually in some nucleus (Fig. 5B), indicating pathway activation.

When DPC-spheroids were cultured in presence of DHT, its conditioned medium (DPC SPh/DHT) lost the ability to induce Wnt/β-catenin pathway activation in HFSC (Fig. 5C). Similar results were observed for DPC-conditioned medium supplemented with DKK-1 (DPC-Sph Control + DKK-1) (Fig. 5D).

We have also studied Wnt/β-Catenin signaling pathway activation during HFSC differentiation assays evaluating β-Catenin nuclear levels by western blot (Fig. 5E). In agreement with the previous experiment, when HFSC were stimulated with DPC-conditioned medium from spheroids (DPC-Sph Control) the β-Catenin nuclear level is increased relative to basal condition,

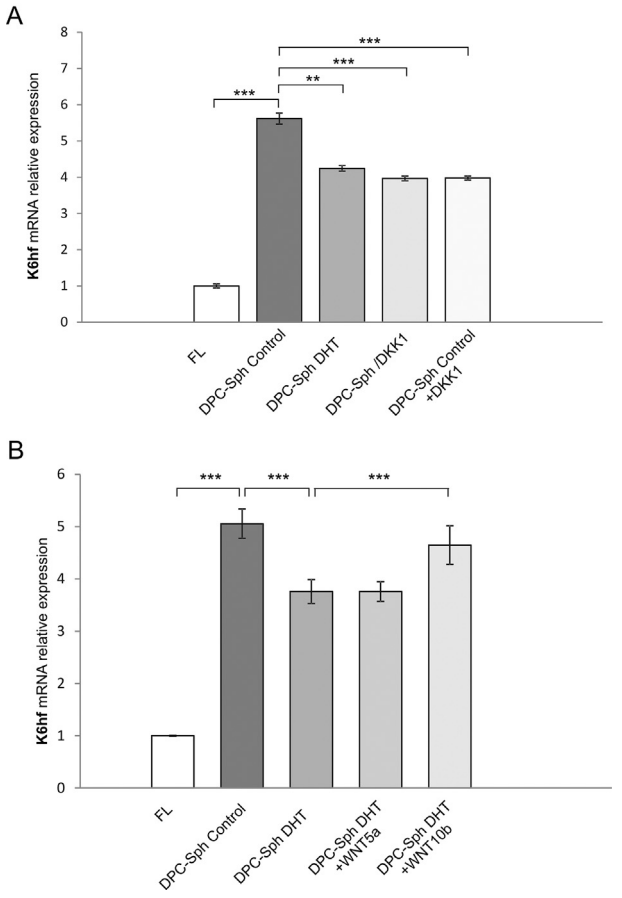

Fig. 4. A) Androgen and DKK-1 effects on HFSC differentiation. Real-time PCR analysis of K6hf mRNA expression in untreated Tel-E6E7 (FL) and cultured with: medium conditioned by DPC-Sph (DPC-Sph Control), medium conditioned by DPC treated with 10^{-7} M DHT (DPC-Sph DHT), medium conditioned by DPC treated with 25 ng/ml of Dkk-1 (DPC-Sph DKK-1), medium conditioned by DPC and supplemented with 25 ng/ml Dkk-1 immediately before use (DPC-Sph Control + DKK-1). B) Androgen and WNT10b and WNT5a effects on HFSC differentiation. Real-time PCR analysis of K6hf mRNA expression in untreated Tel-E6E7 (FL) and cultured with: medium conditioned by DPC-Sph (DPC-Sph Control), medium conditioned by DPC treated with 10^{-7} M DHT (DPC-Sph DHT), medium conditioned by DPC and supplemented with 200 ng/ml WNT5a immediately before use (DPC-Sph Control + WNT5a) and medium conditioned by DPC and supplemented with 400 ng/ml WNT10b immediately before use (DPC-Sph Control + WNT10b). Values are means of triplicates ± SD using HPRT mRNA as the internal control. Results are expressed relative to HFSCFL. Statistical analysis was performed using One way Anova-Tukey-Kramer test. $^{**}p < 0.01$, $^{***}p < 0.001$.

indicating pathway activation. Conversely, the conditioned media obtained from DPC cultured both in presence of DHT (DPC-SPh DHT) or DPC-conditioned medium from spheroids supplemented with DKK-1 (DPC-Sph Control + DKK-1) did not show such increase.

Both experiments confirm that Wnt signaling is activated in DPC-induced HFSC differentiation and the addition of DKK-1 is sufficient to disturb the process.

4. Discussion

In AGA, androgens are believed to play an important role in the gradual transformation of scalp hair follicles to smaller vellus hair through the dermal papilla.

DP from HF on a balding scalp contain higher levels of ARs than those from a non-balding scalp (Hibberts et al., 1998) and this has been proposed as a reason for the regional specificity of the androgen effect and a crucial factor for DP androgen sensitivity. Even if the molecular action of androgens on the DP remains unclear, it has been accepted that androgens alter the production of secreted factors in the DP which act in an autocrine way or have influence over the follicular epithelial cells (Slominski et al., 2012; Mendez and Stillman, 2000).

Wnt/β-catenin signaling pathway is important for the initiation and maintenance of hair morphogenesis (Millar, 2002) and the DP inductive properties required for HF regeneration and growth of the hair shaft (Shimizu and Morgan, 2004). We have hypothesized that the miniaturization of the hair follicle seen in AGA may result, at least partially, from deficient HFSC differentiation. Previously, we have shown that in DP cells from AGA patients, the Wnt-β-catenin signaling pathway is negatively influenced by ligand-activated androgen receptor, inhibiting HFSC differentiation (Leirós et al., 2012). In balding scalp samples, transient amplifying progenitor cell population was markedly diminished, supporting the notion that a deficient conversion of HFSC into progenitor cells plays a role in the pathogenesis of AGA (Garza et al., 2011).

In the present study, we focused on the expression of the Wnt ligand antagonist DKK-1, upregulated in response to DHT and reported to cause apoptosis in follicular keratinocytes co-cultured with DP cells (Kwack et al., 2008).

Since it was observed that the inductive ability of DPC and its intact transcriptional signature, can be partially restored when they are cultured as spheroids (Higgins et al., 2013), we compared the effect that culture conditions could have on androgen induction of Wnt agonists/antagonists. We observed that Dkk-1 mRNA expression level was induced by DHT both in monolayer (as previously described by Kwack et al. (2008)) or DPC-spheroids cultures. However, the basal expression of Dkk-1 was 12-fold higher in DPC cultured as monolayer than in spheroids. When we evaluated the androgen effect on the mRNA expression level of Wnt agonists, we found that Wnt10b and Wnt5a mRNA expression was downregulated by DHT. The culture conditions of DPC also influenced the basal expression of Wnt10b and Wnt5a mRNA, but in opposition to that observed for Dkk-1, their expression was approximately 10-fold lower in monolayer than in spheroids cultures. These observations support the idea that the 3D-tissue architecture of DPC cultures contributes to their inductivity.

We have also observed a significant attenuation of DPC-induced HFSC differentiation when DPC conditioned medium was supplemented with recombinant DKK-1, mimicking the blockade of differentiation observed with DHT. The data suggests that DKK-1 is indeed involved in the inhibition of the inductive ability of DPC and would be one of the paracrine factors upregulated by androgen action that contribute to abrogate HFSC differentiation. Besides, in HFSC incubated with DPC-conditioned medium, we observed Wnt/β-catenin canonical pathway activation. Medium conditioned by DPC in presence of DHT prevented β-catenin accumulation in cytosol and its translocation to nucleus. The addition of DKK-1 to DPC-conditioned medium mimicked the effect of DHT on β-catenin localization. These results are consistent with a previous report indicating that HFSC differentiation to hair lineages mediated by DP cells involved Wnt/β-catenin canonical pathway activation (Roh et al., 2004) and also indicate that androgens would block this pathway increasing DKK-1 secretion by DP cells.

It was reported that DKK-1 is involved in anagen to catagen transition in the hair cycle by regulating the activity of follicular keratinocytes (Kwack et al., 2008, 2012). Moreover, DKK-1 expression level is elevated in the bald scalp of patients with AGA. Anyway, beyond the role that DKK-1 could play, a decreased mRNA expression of the Wnt agonists, Wnt10b and Wnt5a, was observed after DHT treatment. These data suggest that not only Dkk-1 upregulation but rather a global imbalance of agonist/antagonist

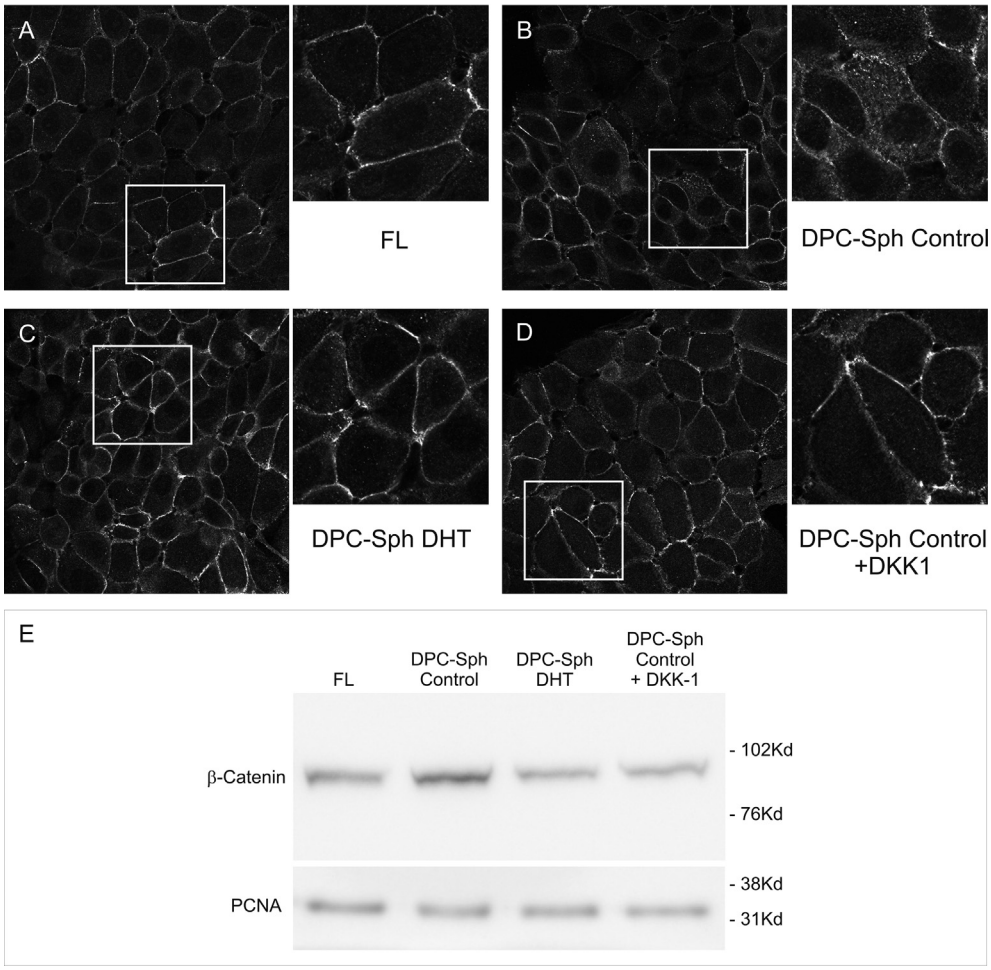

Fig. 5. Wnt/β-catenin pathway activation during HFSC differentiation process induced by DPC-conditioned media. Confocal immunofluorescence images (400× magnification) of β-catenin in HFSC cultured in different DPC-conditioned or supplemented media for 3 h as indicated: A) FL (cFAD medium), B) DPC-Sph Control (medium conditioned by DPC), C) DHT-Sph DHT (medium conditioned by DPC treated with 10^{-7} M DHT), D) DPC-Sph Control + Dkk-1 (medium conditioned by DPC and supplemented with 25 ng/ml Dkk-1). Insert pictures correspond to 1000× magnification. E) β-Catenin nuclear expression level HFSC differentiation process induced by DPC-conditioned media. Western blot for β-catenin in HFSC cultured in different DPC-conditioned or supplemented media for 9 h as indicated: A) FL (cFAD medium), B) DPC-Sph Control (medium conditioned by DPC), C) DHT-Sph DHT (medium conditioned by DPC treated with 10^{-7} M DHT), D) DPC-Sph Control + Dkk-1 (medium conditioned by DPC and supplemented with 25 ng/ml Dkk-1). Numbers at the right side indicate molecular weight markers.

Wnt ligands would be a key factor involved in the mesenchymal-epithelial crosstalk during AGA development. However only WNT10b showed to overcome HFSC differentiation blockade by DPC conditioned medium cultured with DHT.

Wnt10b mediates the canonical signaling that promotes hair shaft growth (Li et al., 2011). It is a potent sustainer of the HF-inducing ability of DP cells, and plays an important role in the maintenance of DP cells and trichogenesis (Ouji et al., 2013). It promotes differentiation of skin epithelial cells in vitro, which showed characteristics of the hair shaft and inner root sheath of the HF after Wnt10b treatment (Ouji et al., 2006).

Moreover, it was observed that Wnt10b treatment, via β-catenin activation, resulted in regenerating anagen hair follicles larger in size and, the subsequent co-treatment with DKK1, reduced hair follicle enlargement (Lei et al., 2014). These data identify potential mechanisms controlling hair follicle miniaturization and suggest that a balance of Wnt10b/DKK1 governs reciprocal signaling between cutaneous epithelium and mesenchyme to regulate proper hair follicle size and in agreement with our data, could regulate HFSC differentiation as well.

Furthermore, Wnt10b expression is significantly upregulated by 1,25-dihydroxyvitamin D3 (VD3) in human DP cells. In a rat model of *de novo* hair regeneration by murine DP cells transplantation, pre-treatment with VD3 significantly enhanced hair folliculogenesis (Aoi et al., 2012). It could be inferred that the upregulation of Wnt10b would contribute to preserve the inductive capacity of cultured DP cells. These results are in accordance with ours, in which the downregulation of Wnt10b by DHT would contribute to decrease DPC inductivity and thus, probably, lead to HF miniaturization during AGA.

Wnt5a has been generally accepted as a Wnt inhibitor (Kwack et al., 2013); however, in some contexts, it can also activate the canonical pathway (Mikels and Nusse, 2006). Wnt5a is a specific DP signature gene (Rendl et al., 2005). Hu et al. (2009) have reported it as a paracrine factor of DP cells highly expressed in the anagen phase, having a unique function in control of hair follicle differentiation (Xing et al., 2011). Nevertheless, WNT5a was not able to overcome androgen effect on our HFSC differentiation model.

As shown by our previous results (Leirós et al., 2012), androgens target on sensitive DP cells impairing their inductive ability to differentiate HFSC into hair lineage. Nevertheless, up to now the molecules modulated by androgens involved in HFSC differentiation were not yet found. In the present study we have observed that DHT disturbs the balance of Wnt agonist/antagonist in DPC,

upregulating Dkk-1 mRNA and downregulating Wnt10b and Wnt5a mRNA. However these effects were also confirmed at level protein only for DKK-1 and WNT10b. We also show that the mere addition of DKK-1 was sufficient to abrogate the inductive ability of conditioned DPC medium and the addition of WNT10b overcame HFSC differentiation blockade by DPC conditioned medium cultured with DHT.

It was previously reported that androgens upregulate Dkk-1 gene expression in dermal papilla cells (Kwack et al., 2008), but it wasn't explained which is the particular pathway involved in such regulation. Until now it doesn't exist, at least in our knowledge, bibliography that has elucidated this issue. Dkk-1 gene expression upregulation could result from a direct androgen action on this gene itself or an indirect mechanism mediated by blocking Wnt-signaling pathway by GSK-3β activation (Leirós et al., 2012). Instead, the Wnt10b and Wnt5a gene expression downregulation could be an androgen indirect effect such as an autocrine regulation mediated by DKK-1 overexpression. A previous study has shown that adding hrDKK-1 to preadipocyte cultures induced adipogenesis by upregulation of adipogenic marker genes such as PPAR-γ and C/EBP-α and downregulation of Wnt3a, Wnt10b and β-catenin, whereas had no significant effect on non-canonical Wnt agonist Wnt5a (Lu et al., 2016). In this sense it was also reported that glucocorticoids at doses greater than 10 nM exert inhibitory effects on mature osteoblastic cells Wnt signaling, whereby Wnt7b and Wnt10b mRNA expression was downregulated and expression of the Wnt inhibitors sFRP1 and DKK-1 was increased ((Mak et al., 2009). These results agree with our own presented in the current manuscript indicating that Wnt agonist downregulation by androgens could be a secondary effect driven by an early androgen-mediated DKK-1 over expression in DPC.

Our results also showed that cell culture conditions can also impact in the equilibrium of Wnt agonists/antagonists secreted by DP cells. This fact provides evidence that the loss of 3D architecture impairs this balance reducing Wnt agonists secretion whereas increasing Wnt antagonists. This effect could explain why DP cells cultured in monolayer lose hair inductive ability through successive passages (Jahoda et al., 1984).

Altogether, our data strongly suggest that the WNT agonist/antagonist expression imbalance in DPC driven by androgens would be responsible for the failure of HFSC to differentiate into hair lineage, thus contributing to AGA development.

The identification of more factors secreted by DP cells responsible for differentiation of HF stem cells would contribute to elucidating the regulation of epithelial-mesenchymal interactions that occur at the onset of hair regeneration and will lead to further understanding of the mechanisms involved in AGA development.

Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.mce.2016.10.018.

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