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Evidence for altered Wnt signaling in psoriatic skin

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Abstract

The Wnt gene family encodes a set of highly conserved secreted signaling proteins that have major roles in embryogenesis and tissue homeostasis. Yet the expression of this family of important mediators in psoriasis, a disease characterized by marked changes in keratinocyte growth and differentiation, is incompletely understood. We subjected 58 paired biopsies from lesional and uninvolved psoriatic skin and 64 biopsies from normal skin to global gene expression profiling. *WNT5A* transcripts were up-regulated 5-fold in lesional skin, accompanied by increased Wnt-5a protein levels. Notably, *WNT5A* mRNA was markedly induced by IL-1α, TNF-α, IFN-γ and TGF-α in cultured keratinocytes. *FZD2* and *FZD5*, which encode receptors for Wnt5A, were also increased in lesional psoriatic skin. In contrast, expression of *WIF1* mRNA, encoding a secreted antagonist of the Wnt proteins, was down-regulated >10-fold in lesional skin, along with decreased WIF-1 immunostaining. Interestingly, pathway analysis along with reduced *AXIN2* expression and lack of nuclear translocation of beta-catenin indicated a suppression of canonical Wnt signaling in lesional skin.

Our results suggest a shift away from canonical Wnt signaling towards non-canonical pathways driven by interactions between Wnt-5a and its cognate receptors in psoriasis, accompanied by impaired homeostatic inhibition of Wnt signaling by WIF-1 and Dkk.

Keywords

Wnt-signaling; psoriasis; WIF-1; Wnt proteins; keratinocytes

Introduction

The Wnt family of signaling proteins are highly conserved, lipid-modified, secreted molecules that participate in multiple developmental events during embryogenesis (Logan and Nusse, 2004). The Wnt proteins have also been shown to have fundamental roles in controlling cell proliferation, cell-fate determination, and differentiation during adult homeostasis (van Amerongen *et al.*, 2008). Classically, Wnt signaling has been divided into two major pathways. First, the canonical signaling pathway involves the use of Frizzled (Fz) receptors paired with low-density lipoprotein receptor-related proteins (LRP) 5 and 6 as co-receptors (Wehrli *et al.*, 2000). This leads to the activation and nuclear translocation of β-catenin and is typically linked to cell fate determination and stem cell maintenance. Alternatively, Wnt signaling occurs

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via the non-canonical pathway involving Fz receptors, independent of the β-catenin activation cascade (Kuhl *et al.*, 2000). In mammals 19 Wnt proteins and 10 Fz transmembrane receptors are known (van Amerongen *et al.*, 2008). To date, several non-canonical pathways have been described, involving the receptor tyrosine kinase Ror2 (Oishi *et al.*, 2003), the atypical tyrosine kinase Ryk (Lu *et al.*, 2004), and Wnt-Ca^{2+} signaling pathways (Kohn and Moon, 2005), all signaling modes associated with controlling cell adhesion and movement. Based on this concept, Wnt proteins have been divided into two main categories depending on which pathway they activate (Sen and Ghosh, 2008). Wnt-1, Wnt-3A and Wnt-8 have been classified as canonical Wnts whereas others such as Wnt-5a and Wnt-11 have been classified as non-canonical Wnts (van Amerongen *et al.*, 2008). However, it has recently become evident that this is probably an over-simplification, as typical non-canonical Wnt ligands such as Wnt-5a (Liu *et al.*, 2005) and Wnt-11 (Tao *et al.*, 2005) have been shown to be able to activate β-catenin signaling. Thus, it is likely that individual Wnt proteins may activate multiple pathways, depending upon which Fz receptors are expressed on the cell surface (van Amerongen *et al.*, 2008).

Three families of secreted proteins are known to inhibit Wnt signaling activity. These are the secreted Frizzled-related protein family (sFRPs) which bind Wnt proteins and prevent them from binding to the Fz receptors (Kawano and Kypta, 2003); the Dickkopf (Dkk) protein family that promote the internalization of LRP making it unavailable for Wnt binding (Logan and Nusse, 2004); and finally WIF-1, a secreted protein that binds to Wnt proteins and inhibits their activity (Hsieh *et al.*, 1999). As is evident from the description above, this combination of multiple ligands along with multiple receptors and soluble inhibitors creates an extremely complex system.

Psoriasis is a disease characterized by chronic inflammation and altered differentiation and hyperproliferation of keratinocytes. In normal skin the fraction of proliferating keratinocytes is probably around 20% (Wright and Camplejohn, 1983), whereas in psoriasis it is almost 100%, and the mean cell cycle time is reduced from 13 days to 36 h (Weinstein *et al.*, 1985). Moreover, it has been suggested that this hyperproliferation is not restricted to the basal epidermal layer containing keratinocyte stem cells, but may also involve suprabasal cells (Leigh *et al.*, 1985). Despite the fundamental roles played by Wnt proteins in controlling cell proliferation and differentiation, surprisingly little is known about the state of Wnt signaling in psoriasis. Of the Wnt proteins, only Wnt-5a has been described to be up-regulated in lesional psoriatic skin as determined by gene expression (Reischl *et al.*, 2007) and was recently shown to synergize with type 1 interferons (Romanowska *et al.*, 2009). However, the pathogenic role of this molecule in psoriasis is presently unknown. The aims of this study were to determine the cellular source of the increased expression of Wnt-5a in psoriasis and to characterize the expression of other mediators of canonical vs. non-canonical Wnt signaling.

Materials and Methods

Study Subjects

58 psoriatic cases and 64 normal healthy controls were enrolled for the study. The criteria for entry as case was the manifestation of one or more well demarcated, erythematous, scaly psoriatic plaques that were not limited to the scalp. In instances of only a single psoriatic plaque, a plaque size of at least 1% of total body surface area was required. Study subjects did not use any systemic anti-psoriatic treatments for 2 weeks prior to biopsy. Informed consent was obtained from all subjects, under protocols approved by the Institutional Review Board of the University of Michigan. This study was conducted in compliance with good clinical practice and according to the Declaration of Helsinki Principles. Local anesthesia was obtained with lidocaine HCl 1% with epinephrine 1:100,000 (Hospira, Inc, Lake Forest, IL). Two biopsies were taken from each patient; one 6mm punch biopsy was obtained from lesional skin of

patients and the other from uninvolved skin, taken at least 10cm away from any active plaque. One to two biopsies were obtained from healthy controls. Immediately upon removal, biopsies were snap frozen in liquid nitrogen and stored at -80°C.

Microarrays

The biopsy samples were prepared as previously prescribed (Gudjonsson *et al.*, 2009). Data from the most up- or down-regulated probes were used for analysis.

Quantitation of mRNA Levels

Quantitative reverse transcription-polymerase chain reaction (QRT)-PCR was performed on paired lesional and nonlesional samples from 10 psoriatic patients and 10 normal controls. The RNA used was from the same samples used for the gene microarrays. The reverse transcription reaction was performed on 0.5 μg of RNA template and cDNA was synthesized using anchored-oligo(dT)18 primers as instructed by the manufacturer (Roche Diagnostics, Mannheim, Germany). QRT-PCR was carried out using a LightCycler2.0 system (Roche Diagnostics). The reaction profile consisted of an initial denaturation at 95°C for 15 minutes followed by 40 cycles of PCR at 95°C for 10 seconds (denaturation), 58°C for 10 seconds (annealing) and 72° C for 10 seconds (extension). The fluorescence emitted was captured at the end of the extension step of each cycle at 530nm. Primers for the genes *WIF1*, *WNT5A*, *WNT3A*, *FZD1*, *FZD10*, *DKK2*, *KRT16*, *LRP6*, *RAB5A*, *RAB27A*, *GNA15*, *CCND1* were obtained from Superarray Biosciences (Frederick, MD). Results were normalized to the expression of the housekeeping gene ribosomal protein, large, P0 (*RPLP0/36B4*) (Laborda, 1991). QRT-PCR of cultured normal human keratinocytes (NHK) was carried out using cDNA prepared as above and primer sets for *DEFB4*, *CXCL1*, *WNT5A* and *WIF1* were obtained from Applied Biosystems and run on an Applied Biosystems 7900HT Fast Real Time PCR System with results normalized to *RPLP0* expression.

Immunohistochemistry

Immunohistochemistry was performed on 5μm fresh frozen tissue sections from uninvolved, lesional psoriatic and control skin using goat anti-Wnt5a (1 μg/ml, Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-WIF-1 (5 μg/ml, R&D Systems, Minneapolis, MN) and goat anti-Fzd5 (2 μg/ml, R&D Systems) antibodies overnight at 4°C, followed by the appropriate biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA), streptavidin-horseradish peroxidase conjugate (Vector) and visualized with 3-amino-9-ethyl-carbazole (BioGenex, San Ramon, CA), followed by hematoxylin counterstaining (Biocare Medical, Concord, CA). Stained sections were examined by light microscopy, and each stained tissue section was subjected to image capture in its entirety via five digital images taken with a 20X objective. All images were subsequently analyzed using Image Pro software (Image-Pro Plus, Media Cybernetics, Silver Spring, MD), to quantify the area stained in epidermis vs. dermis (calculated as density per area).

Paraffin embedded slides from normal controls (n=3), uninvolved psoriatic (n=3) and lesional psoriatic (n=3) were used for β-catenin immunofluorescence staining. Antigen retrieval was performed for 20 minutes (Trizma base/EDTA) and the slides were then incubated with mouse anti-human β-catenin (Sigma, St. Louis, MO, USA,) overnight at 4°C. The slides were then washed three times and incubated with secondary antibody (AF594 chicken anti-mouse (Invitrogen, Carlsbad, CA). For Wnt5a CD31/CD34 counterstaining, paraffin embedded slides were processed as described above and stained with goat anti-mouse Wnt5a (R&D Systems, 1:200 dilution) overnight followed by incubation with biotinylated anti-goat antibodies for 30 minutes and avidin-488 (Vector Laboratories, Burlingame, CA, 1:200 dilution) for 10 minutes. The slides were then washed and counterstained with either mouse anti-human CD34 (Dako North America, Carpinteria, CA, ready for use) for 1 hour, or CD31 (Dako, 1:20) for 1 hour,

followed by AF594 chicken-anti-mouse antibodies (Invitrogen, 1:300) for 30 minutes. The slides were washed three times and mounted with Vectashield fluorescent medium containing DAPI (Vector Laboratories, Burlingame, CA).

Keratinocyte Cultures

Normal human keratinocytes (NHK) were obtained from sun-protected adult skin by trypsin flotation and propagated in modified MCDB 153 medium (M154, Cascade Biologics, Portland, OR) as described previously (Stoll *et al.*, 2001), with the calcium concentration set at 0.1mM. To determine the proliferative effects of exogenous Wnt-5a and WIF-1, NHK were seeded at a density of 2000 cells/cm^2 on Costar® 12 well polystyrene plates (Corning Life Sciences, Lowell, MA) and allowed to attach for 48 hours. Cells were treated with either M154, Wnt5a-conditioned medium (from L Wnt-5A cell line), or medium conditioned by the parental untransfected cell line (L-M (TK-1) line). The L-M(TK-1) (CCL-1.3) and L Wnt-5A cell lines (CRL-2814) were obtained from American Type Culture Collection (ATCC) (Manassas, VA).

To condition medium for use in experiments with NHK, the L Wnt5a cell line was grown as recommended, then passaged 1:10 and grown in M154 without selection agents for 3 days. After 3 days culture medium was collected and stored at 4°C while a second batch of conditioned medium was obtained from the same cells. On day 6, cells were discarded and the 2 batches of media were combined and stored at -20°C until required,

Before use, conditioned media were diluted 1:4 in fresh M154and subsequently added to the NHK cultures, in the presence or absence of recombinant human WIF-1 (300 ng/ml) (R&D Systems) or Wnt5a-antibody (5ìg/ml; R&D Systems) for variable time periods (24-96h). Cells were trypsinized and counted with a hemacytometer at the times indicated. All experiments were performed in triplicates. Evaluation of growth was also measured by flow cytometry after 96 hours using CFSE-labeled NHK (CellTrace CFSE Detection kit, Invitrogen, Carlsbad, CA). NHK were in a similar manner exposed to recombinant Wnt-3a (200ng/ml) and recombinant Wnt-5a (200ng/ml) (R&D Systems) for variable time periods (24-96h) and manually counted.

To examine the induction of Wnt proteins by pro-inflammatory cytokines, NHK cultures were grown to 40% confluency or maintained to 4 days post-confluence. Cultures were then starved of growth factors in unsupplemented M154 for 24 hours before use. Cultures were stimulated with recombinant human TNF-α (10ng/ml), IL-17A (20ng/ml), IFN-γ (20ng/ml), IL-22 (20ng/ml), IL-1α (10 ng/ml) (R&D Systems) or TGF-α (24ng/ml, R&D Systems) for 24 hours and processed for RNA isolation as described above.

Western Blots

One 6-mm punch biopsy was obtained from normal skin from healthy individuals (n=3) and paired lesional and non-lesional biopsies were obtained from psoriatic patients (n=3) as described above. Biopsies were snap frozen in liquid nitrogen and stored at -80°C. Protein lysates were obtained by pulverizing the biopsies while still frozen and the pulverized tissue was transferred to a 2ml glass tissue grinder with 500ìl of RIPA lysis buffer. The samples were centrifuged at 4,500 rpm for 4 min and supernatants collected. Protein concentrations were measured and all samples diluted to 1mg/ml. Samples were separated on 10% SDS polyacrylamide gels and transferred to Immobilon-P membranes (Millipore, Billerica, MA). Western blots were blocked with TBS/0.1% Tween-20 (TBS-T) containing 5% nonfat dry milk for 1 hour at room temperature and incubated with Wnt-5a antibody (Cell Signaling Technology, Danvers, MA, 1:1000) overnight at 4°C. The blots were washed 3 times with TBS-T and then incubated with HRP-conjugated rabbit secondary antibody (GE, 1:2500) for 1.5 hours at room temperature. Blots were washed again and detected by chemiluminescence

using ECL (GE) and Kodak X-Omat fil (Kodak). Anti-β-actin (Sigma, 1:2500 dilution) was used as a loading control.

Statistical analyses

Student's *t*-test was used to analyze differences. A paired *t*-test was used when uninvolved and lesional psoriatic datasets were compared, unpaired t-test was used for other comparisons. Quantitative immunohistochemistry data were tested for significance using Student's *t*-test assuming equal variances and p-values = 0.05 were considered significant.

Ingenuity Pathway Analyses

Microarray data were analyzed using Ingenuity Pathway Analysis software (Ingenuity Systems, www.ingenuity.com). For network generation, a data set containing gene identifiers and corresponding expression values for WNT pathway genes was uploaded into the application. Each gene identifier was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base. These genes, called focus genes, were overlaid onto a global molecular network developed from information contained in the Ingenuity Pathways Knowledge Base.

Results

Differential expression of Wnt pathway genes in psoriasis lesions

We have previously used a microarray dataset to explore the differences between normal skin and uninvolved psoriatic (Gudjonsson *et al.*, 2009b) skin, to assess the expression of candidate risk genes in psoriasis (Nair *et al.*, 2009) and the activity of the sonic hedgehog pathway in psoriasis (Gudjonsson *et al.*, 2009a). Here we show that this dataset contains a differential expression of Wnt pathway genes in psoriatic lesions. Global gene expression analysis revealed significant changes in several members of the Wnt ligand family and several of the Fz receptors in lesional psoriatic skin compared with both normal and uninvolved psoriatic skin (Figure 1). Of the Wnt ligands, *WNT5A* showed a 5.0-fold up-regulation ($p<0.0001$) and *WNT10A* had a 1.3-fold up-regulation ($p<0.0001$). In contrast, *WNT2*, *WNT2B*, *WNT5B*, *WNT7B* were all down-regulated (1.3-, 1.3-, 1.2-, 1.3-fold respectively, all $p<0.0001$) (Table 1). Of the Fz receptor genes, *FZD2* and *FZD5*, which encode receptors for Wnt-5a, had a 1.2 and 1.3-fold up-regulation, respectively ($p<0.0001$), whereas *FZD1*, *FZD4*, *FZD7*, *FZD8* and *FZD10* were decreased (1.3-, 1.5-, 1.7-, 1.4- and 1.5-fold, respectively, $p<0.0001$, table 1). The Fz homologs act in concert with the low-density lipoprotein receptor-related proteins LRP5 or LRP6. *LRP6* demonstrated a 1.6-fold down-regulation and data for *LRP5* was inconclusive due to a limited probe set.

Among the soluble inhibitors and modulators of Wnt signaling, *WIF-1* was most strongly decreased in psoriatic skin, being expressed at 14-fold less than in uninvolved skin ($p<0.0001$). The secreted frizzled-related protein transcripts *SFRP1*, *SFRP2*, *SFRP4* and *SFRP5* were all down-regulated (1.3-, 1.3-, 1.3- and 1.1-fold, respectively, $p<0.001$). Additionally, the Dickkopf homolog genes *DKK1*, *DKK2* and *DKK3* were down-regulated by 1.6-, 2.2- and 1.3 fold, respectively ($p<0.0001$), whereas *DKK4* showed a modest 1.2 fold up-regulation ($p<0.0001$).

Downstream members of the Wnt-canonical pathway, such as β-catenin 1 (*CTNNB1*) and β-catenin interacting protein 1 (*CTNNB1*) were down-regulated by 1.5 and 1.9 fold, respectively, ($p<0.0001$). Consistent with previously published studies (Belso *et al.*, 2008), cyclin D1 (*CCND1)*, which is downstream of β-catenin (Prasad *et al.*, 2007), was down-regulated (2.0-fold, $p<0.0001$), whereas *CCND2* was up-regulated (1.5-fold, $p<0.0001$).

Confirmation of microarray data by QRT-PCR

To validate the microarray results, we performed QRT-PCR for several Wnt-related genes including *WIF1*, *WNT5A*, *DKK2*, and *CCND1*, along with keratin 16 (*KRT16)* as a positive control for up-regulation in lesional psoriatic skin (Leigh *et al.*, 1995). This analysis confirmed the up-regulation of *WNT5A* (2.9 fold, $p<0.001$) and down-regulation of *WIF1* (9.3-fold, $p<0.05$), *DKK2* (8.4-fold, $p<0.001$), and *CCND1* (4.3-fold, $p<0.001$). As anticipated, *KRT16* demonstrated more than 40-fold up-regulation ($p<0.001$) (Figure 2).

The Wnt canonical pathway is suppressed in lesional skin

To determine the effect of gene expression changes in lesional psoriatic skin on the Wnt-pathway, we used the Ingenuity Pathway Analysis software tool (www.ingenuity.com). Gene expression differences between normal control skin and lesional psoriatic skin were overlaid onto a global molecular network within the Ingenuity Pathway Knowledge Base. This revealed global down-regulation of nearly all members of the canonical signaling pathway in psoriasis (Figure 3A). Decreased activity of the canonical Wnt-pathway was confirmed by QRT-PCR for *AXIN2 ($p<0.05$)*, a marker of canonical Wnt signaling (Jho *et al.*, 2002) (Figure 3B). Consistent with these results, we found β-catenin to be decreased in lesional psoriatic skin (Figure 3C) and there was decreased nuclear localization of β-catenin in psoriatic skin compared to normal or uninvolved skin (insets in Figure 3C).

Wnt-5a is upregulated in lesional skin whereas WIF-1 is down-regulated

We examined the protein levels of Wnt-5a and WIF-1 proteins in normal, psoriatic, and symptom-free skin from psoriatic patients by semi-quantitative immunohistochemistry. Lesional psoriatic skin demonstrated increased Wnt-5a staining in both the epidermis and dermis compared to control and uninvolved skin (Figure 4A). These findings were confirmed using computer-assisted image quantification (Figure 4C). There were strong foci of Wnt-5a staining in the papillary dermis of lesional skin. Counterstaining with CD34 and CD31 did not show any co-localization (data not shown) indicating that the source of this staining is not from vascular structures. In addition, tissue lysates from normal, uninvolved and lesional psoriatic skin demonstrated increased levels of Wnt-5a protein in lesional psoriatic skin (Figure 4B). In agreement with our gene chip and QRT-PCR data we observed decreased WIF-1 staining in the epidermis of lesional psoriatic skin, although, interestingly, there was a slight increase in the dermis (Figure 4A and C). There was slight nuclear staining of WIF-1 in epidermis of lesional skin (Figure 4A) but similar nuclear staining has been seen in bladder cancer (Urakami *et al.*, 2006) but not in renal cell carcinoma (Kawakami *et al.*, 2009). The significance of this nuclear staining in the psoriatic epidermis is at this time not clear.

WNT5A expression by keratinocytes is induced by several pro-inflammatory cytokines

Psoriatic skin is replete with pro-inflammatory cytokines and growth factors. To examine the effects of such a cytokine environment on Wnt expression by keratinocytes, NHK were stimulated with TNF-α, IL-17A, IFN-γ, IL-22, TGF-α and IL-1α or a combination of TGF-α and IL-1α for 24 hours. *WIF1* expression was undetectable in both proliferating and differentiated NHK (data not shown), whereas *WNT5A* mRNA was readily detectable in both (Figure 5). We observed an approximately 1.5 to 2-fold increase in *WNT5A* expression treated with TGF-α, TNF-α, IFN-γ or IL-1α. The combination of both TGF-α and IL-1α had an additive effect resulting in a 2 to 3-fold increase in *WNT5A* expression (Figure 5). There was no change in *WNT5A* expression with IL-22 and IL-17A stimulation. Interestingly, the effects of these cytokines were observed only in the more differentiated keratinocytes, suggestive of a role for differentiation in the development of cytokine responsiveness. Baseline expression of *WNT5A* in both proliferating and differentiated NHKs was comparable to that of control and uninvolved skin. However, the maximum induction of *WNT5A* expression observed after

cytokine or TGF-α stimulation was only about half the level of that observed in lesional psoriatic skin (data not shown), indicating that other additional mediators, or cell types, play a role in *WNT5A* mRNA induction in lesional psoriatic skin *in vivo*.

Effect of exogenous Wnt-5a and WIF-1 on keratinocytes

We were unable to observe any effect of recombinant Wnt-5a and Wnt-3a on NHK growth or migration (data not shown). As recombinant Wnt proteins lack post-translational modifications that are essential for their full activity we used secreted Wnt-5a from a tranfected cell line to assess the effects of Wnt agonists and antagonists on keratinocyte growth and function, NHK were cultured in the presence of diluted (20% v/v) conditioned medium from Wnt-5a transfected cell line and a control cell line in the presence of WIF-1 and anti-Wnt-5a antibodies for 24, 48, 72 and 96 hours and counted at each time point. Exogenous Wnt-5a had an suppressive effect on keratinocyte growth ($p<0.01$, Figure 6A), which was prevented by anti-Wnt5a antibodies. There was a minimal to no effect of WIF-1 on the anti-proliferative effect of Wnt5a (Figure 6A and B). These data were confirmed by flow cytometry using CSFE-labeled NHK examined after 96 hours of culture (Figure 6B).

Discussion

Psoriasis is a common chronic inflammatory skin disease characterized by marked changes in keratinocytes growth and differentiation. The basis of this alteration in epidermal growth and differentiation is incompletely understood but has been shown to be dependent on the activity of the immune infiltrate within the psoriatic lesions (Valdimarsson *et al.*, 1995). Several cytokines and growth factors have been implicated in this process based on mouse models (Gudjonsson *et al.*, 2007) and *in vitro/ex vivo* studies. These include cytokines and growth factors such as interleukin (IL)-1α (Lee *et al.*, 1997), vascular endothelial growth factor (VEGF) (Detmar *et al.*, 1994), the epidermal growth factors TGF-α (Elder *et al.*, 1989), amphiregulin (Cook *et al.*, 1992), HB-EGF (Stoll and Elder, 1998), and several cytokines secreted by the Th17 subset of T lymphocytes, most prominently IL-17 and IL-22 (Zaba *et al.*, 2007). As outlined earlier, the Wnt family of signaling proteins are a set of highly conserved molecules that participate and control processes such as cell proliferation, cell-fate determination and differentiation during adult homeostasis (van Amerongen *et al.*, 2008). The changes that are observed in lesional psoriatic skin have been noted to have many similarities to wound healing (Hertle *et al.*, 1992; Mansbridge *et al.*, 1984), which is of interest as wounding has been shown to activate the Wnt-mediated signaling pathway (Ito *et al.*, 2007). Thus, based on the functions of these signaling proteins and the marked changes that occur within psoriatic lesions it would not be unanticipated that these proteins may play a major role in the pathogenesis of psoriasis.

To date, very few studies have been performed to determine whether and how the Wnt pathway is activated in psoriasis. One of the reasons for this is the complexity of this signaling pathway. To date, 19 Wnt proteins and 10 Fz transmembrane receptors have been described (van Amerongen *et al.*, 2008) which require the LRP5 and LRP6 co-receptors for effective signaling (Wehrli *et al.*, 2000). Classically Wnt signaling has been divided into the canonical pathway which leads to activation and nuclear translocation of membrane-bound β-catenin and the non-canonical pathway which is independent of β-catenin (Logan and Nusse, 2004) but can be mediated by several different signaling cascades (Katoh, 2007; Logan and Nusse, 2004). To complicate matters, several of the Wnt proteins, including Wnt-5a, can activate either pathway depending on receptor context on the surface of the responding cells (van Amerongen *et al.*, 2008). Wnt-5a is typically classified as a non-canonical Wnt as its transcriptional activation has been reported to be β-catenin independent (Sen and Ghosh, 2008). However, Wnt-5a has been shown to be able to either activate or inhibit β-catenin signaling depending on receptor

context (Mikels and Nusse, 2006). The activity of the Wnt canonical pathway in psoriasis, as determined by β-catenin activation, has been controversial. A recently published study demonstrated increased nuclear β-catenin staining high in the suprabasal layer in lesional psoriatic skin (Hampton *et al.*, 2007) whereas another study demonstrated only membranous staining in lesional psoriatic skin (Yamazaki *et al.*, 2001), indicating lack of β-catenin activation in lesional psoriatic skin. Thus, based on these two reports it is not clear whether or not β-catenin activity is increased in lesional psoriatic skin.

To date only two studies have tried to directly address whether the Wnt pathway was involved in the pathogenesis of psoriasis (Reischl *et al.*, 2007; Romanowska *et al.*, 2009). One of these studies was based on microarray gene expression in 16 patients screening for 22,283 oligonucleotide probes (Reischl *et al.*, 2007). The authors determined that 10% of the differentially expressed genes in their study were directly or indirectly related to the canonical Wnt/β-catenin or to the non-canonical Wnt/Ca2+ pathways. Of these genes, *WNT5A* was the one most markedly changed, being 5-fold up-regulated compared to uninvolved psoriatic skin whereas *DKK2*, an inhibitor of Wnt signaling, was found to be down-regulated (Reischl *et al.*, 2007). Based on decreased expression of cyclin D1 (*CCND1*) the authors argued that this indicated decreased activity of the canonical Wnt/β-catenin pathway (Reischl *et al.*, 2007). Our study, which is based on a microarray interrogating a much larger probe set (>55,000) and on a larger cohort (64 cases and 58 controls), has extended our knowledge on the status of the Wnt family in psoriasis. We have been able to confirm the findings of Reischl *et al.* (Reischl *et al.*, 2007) on the up-regulation of *WNT5A* and down-regulation of *DKK2* and several of the Fz receptors. Furthermore, we have been able to extend these findings and show that several members of the β-catenin pathway, including β-catenin itself, are down-regulated in lesional skin. Thus, there was both decreased expression of the beta-catenin gene and protein in lesional skin (Figure 3). In addition there was decrease in nuclear translocation of beta-catenin in psoriatic skin compared to normal and uninvolved skin (Figure 3C). These findings are consistent with the decreased *AXIN2* expression observed in lesional psoriatic skin (Figure 3B), but *AXIN2* is a reliable marker for canonical pathway activation (Jho *et al.*, 2002), and demonstrate that the activity of the canonical Wnt pathway is suppressed in lesional skin. As most if not all of the Wnt inhibitory genes are down-regulated in lesional psoriatic skin, including *WIF1*, an interesting question is why the canonical Wnt pathway is still depressed. Of all the Wnt family members, *WNT5A* was highly upregulated, *WNT10A* and *WNT7A* expression were moderately increased, and other Wnt members were either unchanged or showed decreased expression (Table 1). Importantly all the Fz receptors were down-regulated except *FZD5*, and *FZD2*, both of which have been demonstrated to be involved in non-canonical Wnt signaling (Ahumada *et al.*, 2002; Slusarski *et al.*, 1997) and both of which can interact with Wnt-5a (He *et al.*, 1997; Slusarski *et al.*, 1997). Thus, this pattern of gene expression in lesional psoriatic skin is consistent with a shift away from the canonical pathway towards the non-canonical signaling mediated by Wnt-5a and its cognate receptors.

The pathogenic role of Wnt-5a in psoriasis, if any, is still unclear. Our data suggests that the main source of Wnt-5a in psoriatic lesions is the epidermis (Figure 4). Wnt-5a has been implicated both in inflammatory responses of human mononuclear cells (Blumenthal *et al.*, 2006) and vascular proliferation (Masckauchan *et al.*, 2006), processes that have been implicated in psoriasis pathogenesis (Lowes *et al.*, 2007). Vascular changes in psoriasis are characterized by capillary elongation, widening and tortuosity predominantly in the dermal papillae (Hern and Mortimer, 2007). *WNT5A* has been described to be expressed by human endothelial cells and inhibit the canonical Wnt signaling (Masckauchan *et al.*, 2006). Additionally Wnt-5a has been shown to promote angiogenesis (Masckauchan *et al.*, 2006) and proliferation of endothelial cells in a dose dependent manner (Cheng *et al.*, 2008). Wnt-5a can induce expression of a number of genes including Tie-2 (Masckauchan *et al.*, 2006), which is a receptor tyrosine kinase and acts as the receptor for angiopoietins 1 and 2 (Kuroda *et al.*,

2001). Tie-2 has been demonstrated to be upregulated in psoriasis (Kuroda *et al.*, 2001) and transgenic mouse model over-expressing Tie-2 in the skin results in a psoriasis-like phenotype (Voskas *et al.*, 2005). It is well known that vascular changes reflected by redness of lesions take a longer time to resolve than the thickness and scaling. Given these data, it is enticing to speculate given the lack of normalization of Wnt-5a during anti-TNF treatment (unpublished observation) that Wnt-5a may have a role in promoting and/or maintaining vascular changes in lesional skin.

WNT5A has also been shown to be expressed by human antigen-presenting cells through stimulation through Toll-like receptors and directly by TNF-α (Blumenthal *et al.*, 2006). In this context, it is of interest that we observed increased expression of Wnt-5a in lesional dermis although less than that seen for the epidermis (Figure 4). However, we did not determine whether it was the inflammatory infiltrate or the vascular cells that were the main source of dermal Wnt-5a in psoriasis. TNF-α is a pro-inflammatory cytokine that has been shown to have a central role in psoriasis (Gottlieb *et al.*, 2005) and treatments directed against TNF-α are highly effective (Chew *et al.*, 2004; Leonardi *et al.*, 2003). As mentioned above and confirmed in our study, TNF-α has been shown to be able to directly induce the expression of Wnt-5a (Blumenthal *et al.*, 2006). In contrast, *WNT5A* expression in lesional skin does not decrease during anti-TNF-α treatment (unpublished observation), suggesting, that in psoriasis lesions, Wnt-5a is not acting down-stream of TNF-α. Wnt-5a has also been shown to be induced through stimulation of Toll-like receptor signaling, in which case, neutralizing antibodies against TNF-α did not have any suppressive effect (Blumenthal *et al.*, 2006) indicating that *WNT5A* expression can also occur independently of TNF-α, which is consistent with the findings presented in our study. Interestingly, neutralization of Wnt-5a led to a decrease in the production of the cytokines IFN-γ and IL-12p40 (the common subunit of IL-12 and IL-23) (Blumenthal *et al.*, 2006). IFN-γ and IL-23 have been shown to have crucial roles in the pathogenesis of psoriasis through the maintenance and effector functions of Th1 and Th17 cells (Cargill *et al.*, 2007; Krueger *et al.*, 2007; Uyemura *et al.*, 1993; Zaba *et al.*, 2007). Recently it was reported that Wnt-5a may synergize with type 1 interferons such as IFN-α and IFN-β (Romanowska *et al.*, 2009), and type I interferons have been implicated in the onset of psoriasis (Nestle *et al.*, 2005). These data support the notion that Wnt-5a has a role in amplifying and/or maintaining the inflammatory processes present in lesional skin.

Interestingly, IL-1α, TNF-α and IFN-γ and TGF-α, a member of the epidermal growth factor ligand family, were able to induce expression of WNT5A by keratinocytes. Although we were not able to detect any activity on keratinocytes with recombinant Wnt-5a or Wnt-3a *in vitro* as determined by both growth assays and migration assays (data not shown) we observed growth suppressive effect of secreted Wnt-5a on human keratinocytes *in vitro* (Figure 6). It should be noted that *in vivo* several of the Wnt proteins including Wnt-1, Wnt-3a and Wnt-5a have significant post-translational modifications (Kurayoshi *et al.*, 2007) that the commercially available recombinant proteins lack. The Wnts are heavily glycosylated, which is essential for folding and secretion (Kurayoshi *et al.*, 2007) and several, including Wnt-3a and Wnt-5a, are additionally conjugated to palmitate. This palmitate conjugation is essential for their biological activity and the lipid-unmodified form of Wnt-5a has been shown to be unable to bind to Fz5 and therefore unable to activate intracellular signal cascades and stimulate cell migration (Kurayoshi *et al.*, 2007). This might be one of the reasons for the observed lack of biological activity *in vitro* with either recombinant Wnt-3a and Wnt-5a. Taken together our data indicates that Wnt-5a may have a growth suppressive effect on the psoriatic epidermis, a finding that has been observed in other settings (Olson *et al.*, 1998), and may therefore be a part of a regulatory mechanism keeping proliferation under control.

In conclusion, our data indicates that there is a shift in lesional psoriatic skin away from canonical Wnt signaling towards non-canonical pathways likely mediated by the increased

expression and production of Wnt-5a and its cognate receptors along with impaired homeostatic inhibition of Wnt signaling by WIF-1 and the Dkk proteins. Our results and previously published studies indicate that Wnt-5a may have a role in inducing the marked vascular changes in lesional skin, influencing epidermal proliferation, and play a role in the amplification of inflammatory responses. Further studies are warranted to elucidate the exact role of the Wnt pathway in psoriasis pathogenesis.

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Abbreviations

Fzd	Frizzled
IL	interleukin
DKK	dickkopf
LRP	lipoprotein receptor-related proteins
KRT	Keratin
NHK	normal human keratinocyte
PCR	Polymerase chain reaction
PGA	Physician global assessment
RMA	robust multi-array average
SFRP	secreted frizzled-related protein
TGF	Transforming growth factor
TNF	Tumor necrosis factor
QRT	Quantitative real time PCR
WIF	WNT inhibitory factor

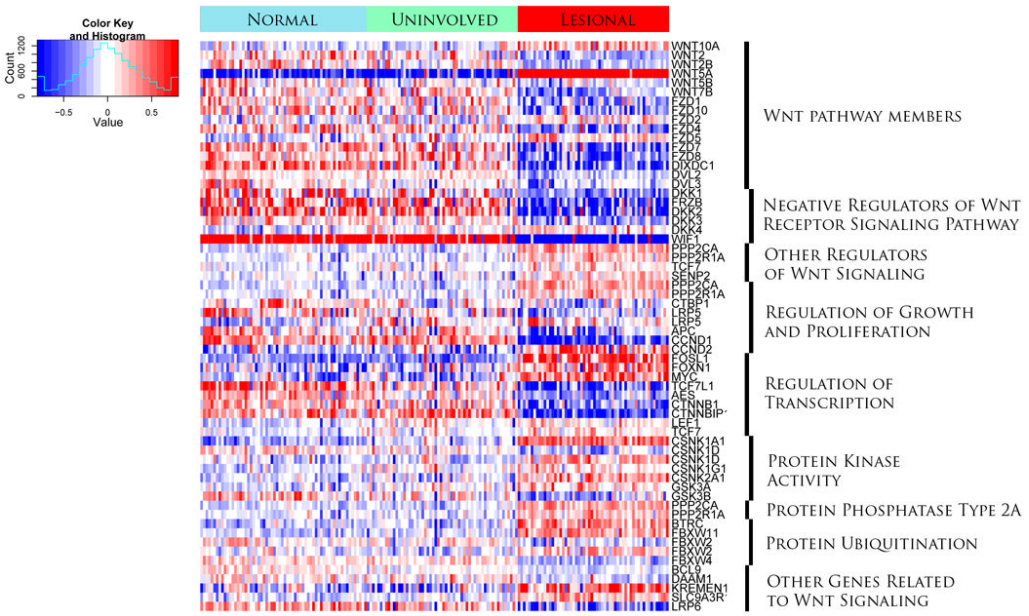

Figure 1. Microarray analysis reveals that WNT5A is strikingly upregulated, and WIF1 down-regulated in the vast majority of lesional skin samples

This gene expression heat map image used transcripts from 58 paired lesional and uninvolved psoriasis and 64 normal skin samples and displays Wnt pathway members, regulators of Wnt signaling, and regulators of growth, proliferation and transcription. In addition genes involved in protein kinase activity, protein phosphatases, protein ubiquitination and other genes related to Wnt signaling are shown. Color key: red, increased expression, blue, decreased expression, as indicated in the color key and histogram (inset).

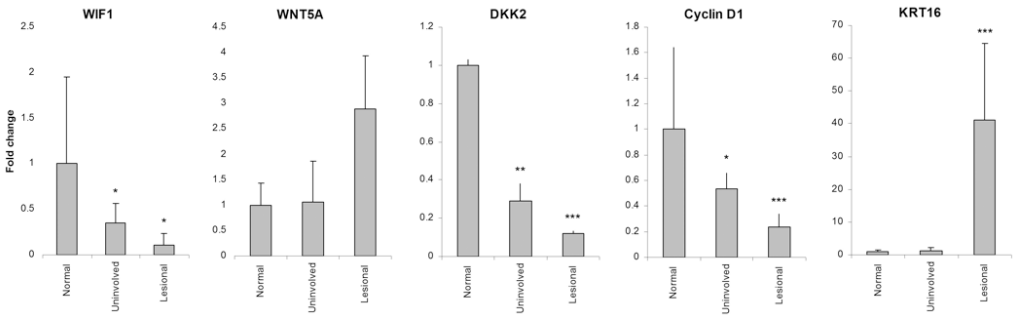

Figure 2. QRT-PCR confirmed the altered expression of several components of the Wnt signaling pathways in psoriasis

Keratin 16 (*KRT16*) expression was used as a positive control for lesional psoriasis skin. Data are expressed as fold-change relative to normal skin. Bars indicate mean ± S.D (n=10). Statistical significance denoted * $p < 0.05$, ** $p<0.01$, *** $p<0.001$.

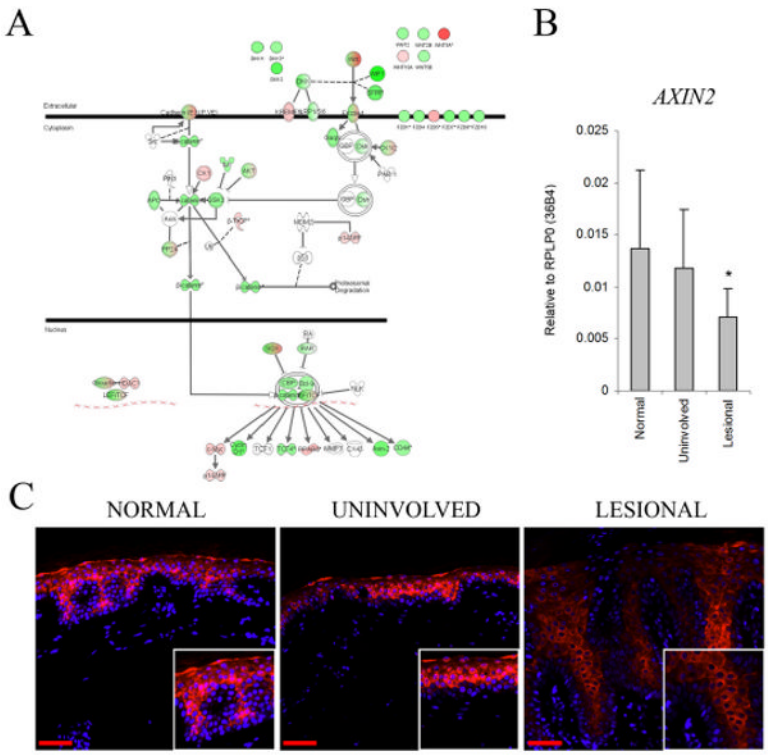

Figure 3. Pathway analysis reveals global down-regulation of nearly all members of the canonical Wnt signaling pathway in psoriasis

Global gene expression differences between normal and lesional psoriatic skin were overlaid onto a global molecular and pathway network within the Ingenuity Pathway Knowledge Base (A). This revealed near global down-regulation (green) of nearly all members of the canonical signaling pathway. However, several genes, associated with the non-canonical Wnt pathway, were upregulated (pink) including; *WNT5A* and *FZD5*. Decreased activity of the canonical Wnt-pathway was confirmed by QRT-PCR for *AXIN2*, a marker of canonical Wnt signaling (B). Bars indicate mean ± S.D, * $p < 0.05$. Immunofluorescent microscopy of normal, uninvolved and psoriatic skin (C) (20X, n=3).

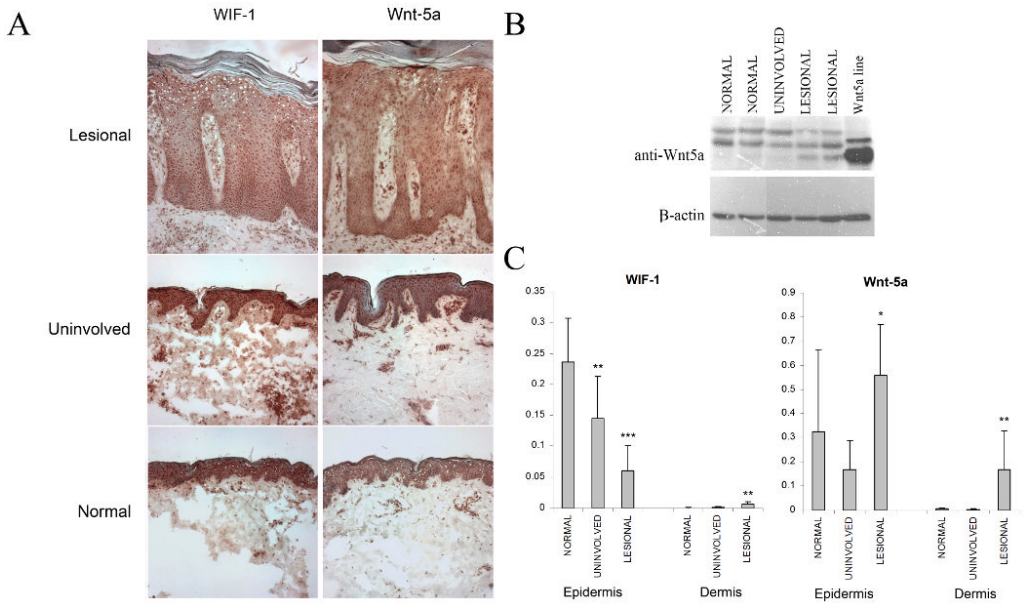

Figure 4. Immunohistochemistry of normal, uninvolved and lesional psoriasis skin revealed increased Wnt-5a and decreased WIF-1 tissue expression

Immunohistochemical staining was performed on fresh frozen sections of skin for Wnt-5a (n=5) and WIF-1 (n=5) (A). Western-blot of normal, uninvolved and lesional psoriasis n=3). Lysate from a Wnt-5a transgenic cell line was used as a positive control (B). Differences in the expression of these proteins were confirmed with computer-assisted image quantification (C). Bars indicate mean ± S.D. Magnifications 100X. Isotype-control antibodies were used and did not show any staining (not shown).

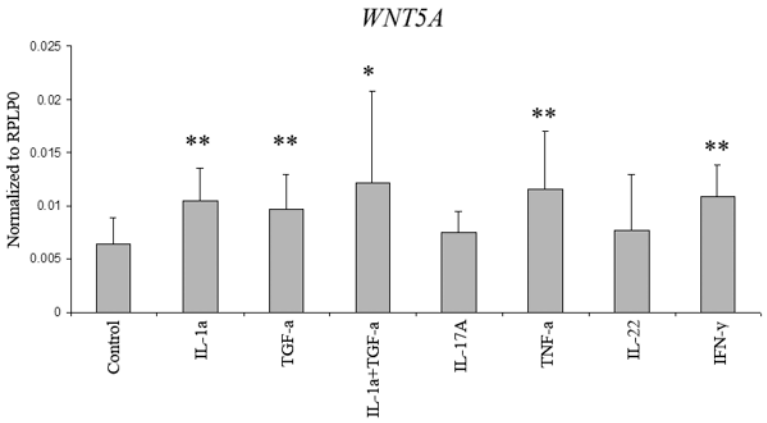

Figure 5. Expression of *WNT5A* by normal human keratinocytes can be induced by pro-inflammatory cytokines

WNT5A expression could be induced by 24hours' treatment with TNF-α (10ng/ml), IFN-γ (20ng/ml), IL-1α (10ng/ml) or TGF-α (24ng/ml) after 24 hours. Expression could be further induced by the additive response to IL-1α and TGF-α. Neither IL-17A (20ng/ml) nor IL-22 (20ng/ml) had any effect on WNT5A expression. Data are expressed as fold-change relative to unstimulated NHK. Bars indicate mean ± S.D (n= 3 in duplicate wells). * $p<0.05$, ** $p<0.01$.

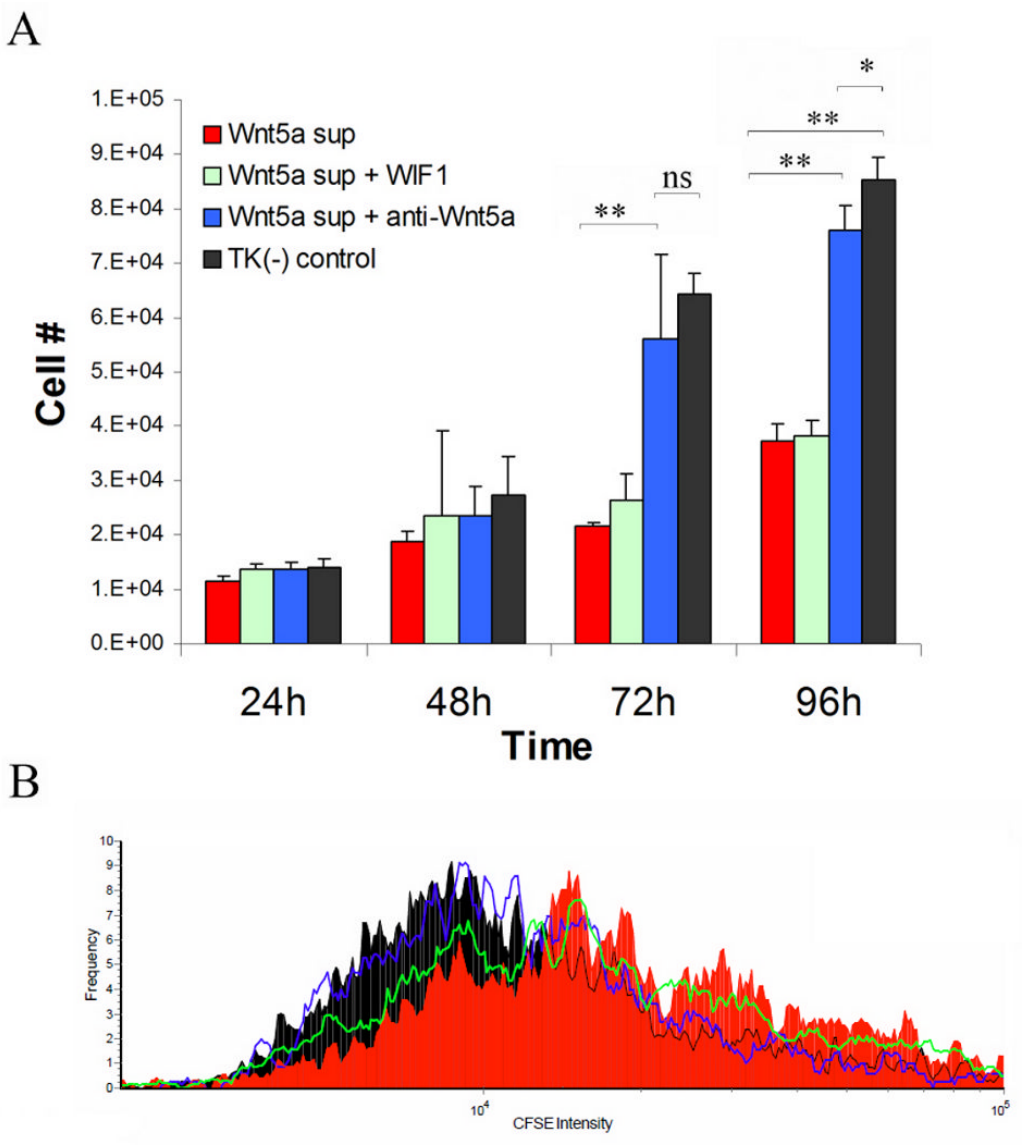

Figure 6. Biological effects of Wnt-5a and WIF-1 on normal human keratinocytes

Conditioned medium containing Wnt-5a was obtained from a transfected cell line and diluted 1:4 in fresh M154CF culture medium. Control medium was obtained from the untransfected parental cell line. Wnt5a had growth suppressive effect on NHK that could be neutralized with anti-Wnt-5a antibodies but no effect was seen with exogenous WIF-1. Bars indicate mean ± S.D (n=3 in duplicate wells). * $p<0.05$, ** $p<0.01$ (A). CFSE staining by flow cytometry correlated with cell counts (B).

TABLE 1

Fold changes between lesional psoriatic (PP) and control (NN) skin for Wnt family members and Fz receptors present on the HU133 PLUS 2.0 microarray. P-value is corrected for multiple testing (FDR; false discovery rate).

Gene	Fold change PP vs. NN	P-value (FDR)	Probe id
WNT1	1.03	ns	208570_at
WNT2	0.74	2.54E-23	205648_at
WNT2B	0.77	2.86E-20	206458_s_at
WNT3	1.08	ns	221455_s_at
WNT4	1.01	ns	208606_s_at
WNT5A	5.01	3.19E-176	205990_s_at
WNT5B	0.82	7.75E-08	221029_s_at
WNT6	1.05	ns	221609_s_at
WNT7A	1.11	9.94E-05	210248_at
WNT7B	0.774	2.80E-15	217681_at
WNT8A	1.00	ns	224259_at
WNT8B	1.04	ns	207612_at
WNT9A	0.94	ns	230643_at
WNT9B	1.03	ns	1552973_at
WNT10A	1.31	4.69E-17	223709_s_at
WNT11	0.92	ns	206737_at
WNT16	0.96	ns	221113_s_at
FZD1	0.74	1.50E-14	204451_at
FZD2	1.16	4.41E-06	210220_at
FZD3	0.95	ns	219683_at
FZD4	0.66	1.66E-19	218665_at
FZD5	1.27	4.13E-14	206136_at
FZD6	1.04	ns	203987_at
FZD7	0.58	5.19E-26	203705_s_at
FZD8	0.66	3.86E-29	227405_s_at
FZD9	1.07	ns	207639_at
FZD10	0.68	9.17E-17	219764_at