

Assessment of the Psoriatic Transcriptome in a Large Sample: Additional Regulated Genes and Comparisons with *In Vitro* Models

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To further elucidate molecular alterations in psoriasis, we performed a gene expression study of 58 paired lesional and uninvolved psoriatic and 64 control skin samples. Comparison of involved psoriatic (PP) and normal (NN) skin identified 1,326 differentially regulated transcripts encoding 918 unique genes (549 up- and 369 downregulated), of which over 600 are to our knowledge previously unreported, including S100A7A, *THRSP*, and *ELOVL3*. Strongly upregulated genes included *SERPINB4*, *PI3*, *DEFB4*, and several S100-family members. Strongly downregulated genes included Wnt-inhibitory factor-1 (*WIF1*), β -cellulin (*BTC*), and *CCL27*. Enriched gene ontology categories included immune response, defense response, and keratinocyte differentiation. Biological processes regulating fatty acid and lipid metabolism were enriched in the down-regulated gene set. Comparison of the psoriatic transcriptome to the transcriptomes of cytokine-stimulated cultured keratinocytes (IL-17, IL-22, IL-1 α , IFN- γ , TNF- α , and OSM) showed surprisingly little overlap, with the cytokine-stimulated keratinocyte expression representing only 2.5, 0.7, 1.5, 5.6, 5.0, and 1.9% of the lesional psoriatic dysregulated transcriptome, respectively. This comprehensive analysis of differentially regulated transcripts in psoriasis provides additional insight into the pathogenic mechanisms involved and emphasizes the need for more complex yet tractable experimental models of psoriasis.

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INTRODUCTION

Psoriasis is a genetically determined chronic inflammatory disease of the skin characterized by sharply demarcated scaly red plaques commonly located on the extensor surfaces of the skin. The most characteristic feature of psoriasis is marked hyperproliferation and altered differentiation of the epidermis. Additionally, psoriasis has complex immunological, biochemical, vascular, and neurological alterations. Psoriasis

has been shown to be immune-mediated as targeted treatments against T-cells (Ellis *et al.*, 1986) or principal inflammatory mediators such as tumor necrosis factor- α (TNF- α) (Gottlieb *et al.*, 2003; Leonardi *et al.*, 2003) lead to near-complete remission of the disease. Recent genetic association studies of psoriasis support this concept, but suggest that the story is more complex, with variants in genetic loci regulating barrier function (Zhang *et al.*, 2009; de Cid *et al.*, 2009) and antimicrobial defenses (Hollox *et al.*, 2008) in addition to antigen presentation (Nair *et al.*, 2006), NF- κ B signaling (Nair *et al.*, 2009), and T-cell polarization (Chang *et al.*, 2008; Nair *et al.*, 2009) all having a role in its pathogenesis.

Earlier studies of the functional pathways involved in the pathogenesis of psoriasis were limited to one or a few genes or proteins at a time. Examples include transforming growth factor- α (Elder *et al.*, 1989), S100A7 (Madsen *et al.*, 1991), S100A8 and S100A9 (Kelly *et al.*, 1989), protease inhibitor-3 (PI3/elafin/skin-derived anti-leukoproteinase (SKALP)) (Schalkwijk *et al.*, 1990), IFN- γ (Bjerke *et al.*, 1983), and IL-8 (CXCL8) (Christophers *et al.*, 1989). Recently, array-based techniques that profile multiple genes at the same time have become important methods to characterize molecular alterations. The first array-based gene expression study of psoriasis identified 159 genes in eight psoriasis patients that were differentially regulated between uninvolved and

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Abbreviations: *BTC*, β -Cellulin; *CCL*, chemokine (C-C motif) ligand; *DAVID*, Database for Annotation, Visualization and Integrated Discovery; *DEFB*, human β -defensin; *EGF*, epidermal growth factor; *IL1F*, IL-1-family member; *NN*, non-psoriatic normal; *OSM*, oncostatin-M; *PN*, psoriatic normal; *PI*, protease inhibitor; *PP*, psoriatic plaque; *QTR-PCR*, quantitative reverse transcription-PCR; *SERPINB*, serine protease inhibitor, clade-B; *SPRR(P)*, small proline-rich (proteins); *THRSP*, thyroid hormone-responsive spot; *TNF*, tumor necrosis factor; *WIF1*, Wnt-inhibitory factor-1

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lesional skin (Oestreicher *et al.*, 2001). Likewise, a similar study of 15 psoriasis patients identified a total of 177 genes that differed in expression in lesional versus normal skin (Bowcock *et al.*, 2001). Another study of 15 atopic dermatitis patients and 14 psoriasis patients identified 62 genes upregulated in psoriatic as compared with that in atopic skin (Nomura *et al.*, 2003). More recent array-based studies have indicated that over 1,000 genes may be differentially regulated in psoriatic lesions as compared with that in normal skin (Zhou *et al.*, 2003; Haider *et al.*, 2006). Interestingly, a number of these genes are differentially expressed in the lesional skin of psoriasis relative to that in chronic atopic dermatitis, despite comparable epidermal hyperplasia in these two settings (Nomura *et al.*, 2003; de Jongh *et al.*, 2005).

These studies provide a detailed but complex picture of the gene expression events in lesional psoriatic skin. To aid in the interpretation of such data, gene expression maps based on cytokine stimulation of keratinocytes have recently been published (Banno *et al.*, 2004; Finelt *et al.*, 2005; Gazel *et al.*, 2006; Bando *et al.*, 2007; Mee *et al.*, 2007; Haider *et al.*, 2008b; Nograles *et al.*, 2008). These genomic maps provide insights into the cellular sources of the gene-expression signature and the complex positive and negative feedback signaling pathways occurring between different cell types. However, these model systems use monolayer keratinocyte cultures and therefore may not fully recapitulate the differentiated and multicellular environment present in normal epidermis, uninvolved psoriatic skin, and psoriatic plaques (PPs).

We have previously used the data set presented here to explore the roles of the sonic hedgehog (Gudjonsson *et al.*, 2009a) and Wnt5a pathways in psoriasis (Gudjonsson *et al.*, submitted). We have also studied the expression of genes mapping to confirmed susceptibility loci in psoriasis (Nair *et al.*, 2009), as well as the profile of differential gene expression comparing uninvolved psoriatic (PN) and normal (NN) skin (Gudjonsson *et al.*, 2009b). The aims of this study were (i) to further delineate the gene expression profile of lesional psoriatic (PP) relative to PN and NN skin in a large data set; (ii) to compare our results with those reported in previously published microarray studies of psoriasis; and (iii) to explore how well the published transcript maps derived from cytokine-stimulated cultured keratinocytes represent the alterations observed in lesional psoriatic skin.

RESULTS

Alterations in gene expression in lesional psoriatic skin

Unsupervised principal-component analysis (Figure 1) of the 58 paired PP and PN and 64 NN samples showed near-complete separation of the PN and NN samples from the PP samples (Gudjonsson *et al.*, 2009b). As described previously (Zhou *et al.*, 2003; Gudjonsson *et al.*, 2009b), there was substantial overlap between the PN and NN samples. These results show a distinct gene expression profile of PP skin that is markedly different from those of PN and NN skin. On the basis of our criteria for differentially regulated transcripts (see Materials and Methods), we identified 1,326 probes, encoding 918 unique genes, that differed in expression between the

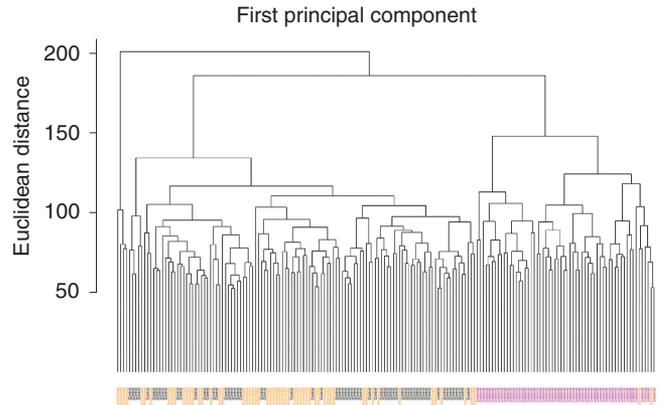


Figure 1. Hierarchical clustering of the entire data sample. This figure has been presented previously (Gudjonsson *et al.*, 2009b) and is presented here for comparison. Unsupervised hierarchical clustering showed near-complete separation of the PP ($n=58$) samples from the PN and NN samples, whereas there was some overlap between the PN ($n=58$) and NN ($n=64$) samples (Gudjonsson *et al.*, 2009b).

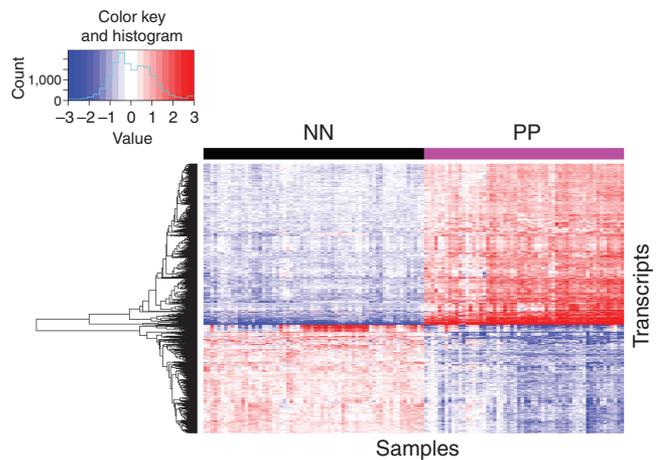


Figure 2. Heatmap of differentially regulated transcripts between PP and NN skin. Differentially regulated transcripts between PP and NN skin ($n=1,326$) are shown in a heatmap image. The blue color indicates low expression levels whereas red indicates high expression levels. The black bar above the heatmap indicates NN skin and the purple bar above the heatmap indicates PP skin. Note that the number and intensity of upregulated transcripts exceeds the number and intensity of downregulated transcripts. Clustering was performed only on rows and based on row means, for column samples were grouped into NN or PP groups without any clustering.

PP and NN samples (549 genes upregulated and 369 genes downregulated), and 1,085 probes, detecting 758 unique genes, between the PP and PN samples (Figure 2). There was considerable overlap between the different groups of genes (Figure 3). More genes were differentially expressed in the PP versus NN skin than in the PP versus PN skin, suggesting that the PN skin has subtle changes in the gene expression pattern that make it more similar to PP skin than NN skin is (see Supplementary File 1 online). The raw microarray data of this study have been deposited in the NCBI Gene

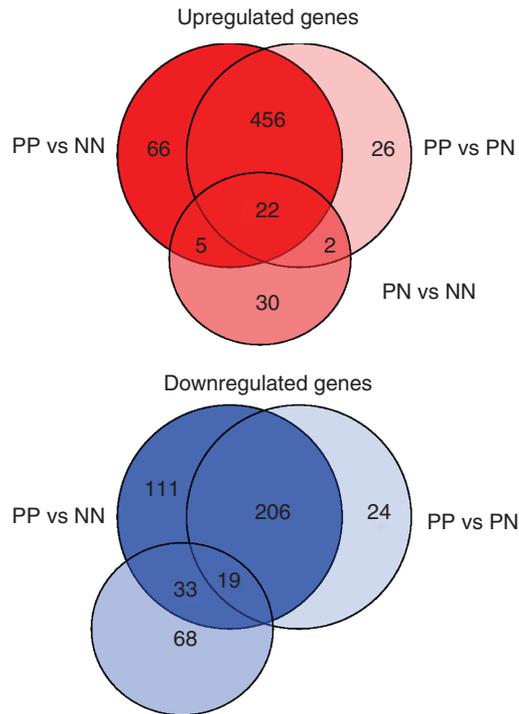


Figure 3. A Venn diagram of up- and downregulated genes in psoriasis skin. The diagram shows a comparison of the overlap between PP versus NN, PP versus PN, and PN versus NN genes for all up- and downregulated genes. Not unexpectedly, there is a large overlap between the PP versus NN and PP versus PN data sets. There was a larger overlap of PN versus NN with PP versus NN (27 upregulated genes and 52 downregulated genes) as compared with the PP versus PN (24 upregulated genes and 19 downregulated genes) data set, suggesting that PN skin is more similar to PP skin.

Expression Omnibus (GEO; <http://www.ncbi.nlm.nih.gov/geo>) and is accessible through GEO Series accession number GSE13355.

The most highly up- and downregulated genes

Several genes were increased more than 50-fold in PP as compared with that in NN skin (Table 1A). These included the peptidase inhibitors serpin peptidase inhibitor, clade-B, member-4 (*SERPINB4*) and (*SERPINB3*), *PI3*, and the antimicrobial peptide human β -defensin-2 (*DEFB4*). Other upregulated genes included members of the S100 family of proteins such as *S100A7L1*, *S100A12*, *S100A9*, and *S100A7*; keratin-16; and *IL-8*. Only two genes showed more than 10-fold downregulation in PP skin: Wnt-inhibitory factor-1 (*WIF1*) and β -cellulin (*BTC*). Other notable downregulated genes included thyroid hormone-responsive spot-14 (*THRSP*), IL-1-family member-7 (*IL1F7*), and chemokine (C-C motif) ligand-27 (*CCL27*) (Table 1B).

QRT-PCR confirmation of differentially regulated genes

We confirmed the up- and downregulation of several genes in PP versus PN and NN skin by quantitative reverse transcription-PCR (QRT-PCR). On average, *DEFB4* and *S100A7L1* (*S100A7A*) were upregulated more than 2,000- and 4,000-fold in PP versus NN skin, respectively, whereas *PI3* was upregulated more than 1,300-fold, *S100A12* about 63-fold,

small proline-rich-2C (*SPRR2C*) about 15-fold, and *IL-8* about 150-fold (Figure 4). Likewise, we found that genes *ELOVL3* and *BTC* were downregulated by 30-fold in PP versus NN skin, whereas *THRSP* was downregulated by \sim 8-fold (Figure 4).

Comparison to previously published microarray studies

We compared our list of unique up- and downregulated genes to those of Zhou *et al.* (2003), Oestreicher *et al.* (2001), Nomura *et al.* (2003), and Haider *et al.* (2006) using the same criteria of significance (>2.0 -fold change, $P < 0.05$) but removing duplicate genes, hypothetical transcripts, and unnamed expressed sequence tags. Of the 549 unique upregulated genes in our data sample, only 136 were present in the data set of Zhou *et al.* (2003) (Supplementary Figure S1). Likewise, of the 369 downregulated transcripts in our data set, only 57 genes were found to be present in both data sets (Supplementary File 2). Notable among the replicated upregulated genes were *RNASE7* (upregulated 3.1-fold, $P = 5.6E-24$) and *KLK8* (upregulated 2.5-fold, $P = 7.8E-75$). Reproducibly downregulated genes included *IL17D* (downregulated 2.0-fold, $P = 7.1E-23$) and *CD207* (encoding langerin, downregulated 2.9-fold, $P = 1.2E-29$). Of the 159 genes that Oestreicher *et al.* (2001) identified in PP skin, 153 encoded unique genes. Of those, we could confirm the expression of 49 in our data set (32%) (Supplementary Figure S1). We also compared our data set to that of Nomura *et al.* (2003). This study was limited to genes that were differentially expressed between atopic dermatitis skin and psoriatic skin. Of the 59 unique genes described we found 36 (61%) to be present in our upregulated gene list (data not shown). Comparison to the results of Haider *et al.* (2006) showed even greater overlap. This analysis was limited to genes that were upregulated in both squamous cell carcinoma and psoriasis and were more than >2 -fold upregulated in PP versus NN skin. Of the 102 unique genes that fulfilled these criteria, 84 (82%) were present in our upregulated PP versus PN and NN gene list (Supplementary File 2). Finally, we compared our differentially expressed gene lists with those of Yao *et al.* (2008). We found that among 721 upregulated probesets (PP vs PN) in our list, 666 (92.4%) were present in that of Yao *et al.*, and among 364 downregulated transcripts (PP vs PN), 323 (88.7%) were also present in that of Yao *et al.*

Gene ontology and pathway analyses

Gene ontology analysis was focused on three major categories, biological processes, cellular compartments, and molecular function. As the gene expression profiles of PN and NN skin are very similar (Gudjonsson *et al.*, 2009b), we compared the gene-expression signature of PP skin to that of NN and PN skin combined to increase the statistical power. For the upregulated genes the processes most significantly enriched for upregulated transcripts in the combined comparisons of PP versus NN and PP versus PN skin were "immune response", "defense response", and "response to wounding" (Figure 5). Our analysis identified 90 upregulated genes that were annotated with immune response (Figure 5),

Table 1. The top 15 up- and downregulated genes in PP versus NN skin

Gene symbol	Gene title	Fold change	FDR P-value	Mean in control (log2)	Mean in uninvolved (log2)	Mean in lesional (log2)
<i>(A) Upregulated PP versus NN</i>						
<i>SERPINB4</i>	Serpin peptidase inhibitor, clade-B, member-4	377	0	4.9	5.5	13.5
<i>DEFB4</i>	Defensin, β 4	197	0	6.2	6.8	13.9
<i>S100A7L1</i>	S100 calcium-binding protein-A7-like-1	150	0	5.0	5.2	12.2
<i>PI3</i>	Peptidase inhibitor-3, skin-derived (SKALP)	131	0	6.5	7	13.5
<i>SERPINB3</i>	Serpin peptidase inhibitor, clade-B, member-3	64	0	7.0	7.4	13.0
<i>SPRR2C</i>	Small proline-rich protein-2C	58	2.2E-264	4.4	4.6	10.3
<i>AKR1B10</i>	Aldo-keto reductase family-1, member-B10	58	0	5.9	6.4	11.8
<i>S100A12</i>	S100 calcium-binding protein-A12 (calgranulin-C)	57	0	4.7	4.9	10.6
<i>S100A9</i>	S100 calcium-binding protein-A9 (calgranulin-B)	57	0	8.7	9.3	14.5
<i>C10orf99</i>	Chromosome-10 open reading frame-99	32	0	6.0	6.9	11.4
<i>KYNU</i>	Kynureninase (L-kynurenine hydrolase)	25	0	6.1	6.1	10.8
<i>LCE3D</i>	Cornified envelope-3D	24	0	8.9	9.7	13.5
<i>S100A7</i>	S100 calcium-binding protein-A7 (psoriasin-1)	18	3.3E-114	9.9	10.7	14.1
<i>IL-8</i>	Interleukin-8	17	1.3E-53	4.3	4.4	8.4
<i>KRT16</i>	Keratin-16	17	5.7E-272	9.4	9.4	13.5
<i>(B) Downregulated PP versus NN</i>						
<i>WIF1</i>	Wnt-inhibitory factor-1	-14.0	4.5E-91	9.6	9.4	5.8
<i>BTC</i>	β -Cellulin	-13.9	5.6E-167	8.4	8.6	4.6
<i>THRSP</i>	Thyroid hormone responsive spot-14	-9.4	1.6E-24	11.1	10.5	7.9
<i>IL1F7</i>	Interleukin-1 family, member-7 (zeta)	-8.4	3.5E-79	9.4	9.7	6.3
<i>CCL27</i>	Chemokine (C-C motif) ligand-27	-7.7	7.5E-76	9.9	10.1	7.0
<i>KRT1B</i>	Keratin-1B	-7.6	4.9E-112	11.6	11.7	8.7
<i>MSMB</i>	Microseminoprotein, β	-6.5	2.4E-55	7.3	7.1	4.6
<i>ELOVL3</i>	Elongation of very long chain fatty acids-like-3	-6.5	1.4E-18	8.9	8.0	6.2
<i>GAL</i>	Galanin	-6.4	1.1E-16	9.4	8.5	6.7
<i>FABP7</i>	Fatty acid binding protein 7, brain	-6.0	1.7E-19	9.5	9.1	6.9
<i>ACSBG1</i>	Acyl-CoA synthetase bubblegum family member-1	-5.7	3.8E-19	9.2	8.5	6.6
<i>MLSTD1</i>	Male sterility domain containing-1	-5.2	9.8E-20	8.8	7.8	6.3
<i>HS3ST6</i>	Heparan sulfate (glucosamine) 3-O-sulfotransferase-6	-4.2	2.9E-79	8.6	8.6	6.5
<i>WDR72</i>	WD repeat domain-72	-4.2	5.3E-46	8.3	8.1	6.2
<i>SERPINA12</i>	Serpin peptidase inhibitor, member-12	-4.1	1.5E-38	11.2	11.5	9.2

Abbreviations: FDR, false discovery rate; NN, non-psoriatic normal; PP, psoriatic plaque.

as compared with 25 genes in the largest previous analysis of PP versus NN skin (Zhou *et al.*, 2003). Despite the fact that epidermal hyperproliferation and altered differentiation are the most obvious histological features of psoriasis, the *P*-value obtained for “immune response” was 6 orders of magnitude more significant than that obtained for “mitotic cell cycle”; 9 orders of magnitude more significant than that obtained for “ectodermal development”; and 14 orders of magnitude more significant than that obtained for “epidermal cell differentiation”. The main cellular compartments

involved included the extracellular space and the cornified envelope. Chemokine and cytokine activity predominated in the molecular function of the upregulated transcripts. The downregulated categories involved biological processes regulating fatty acid and lipid metabolism, organ development, and blood circulation. Moreover, Ingenuity Pathway Analysis identified “dendritic cell maturation” as the canonical pathway most significantly and markedly enriched within the differentially regulated transcripts, with 25 of 165 pathway components being differentially regulated

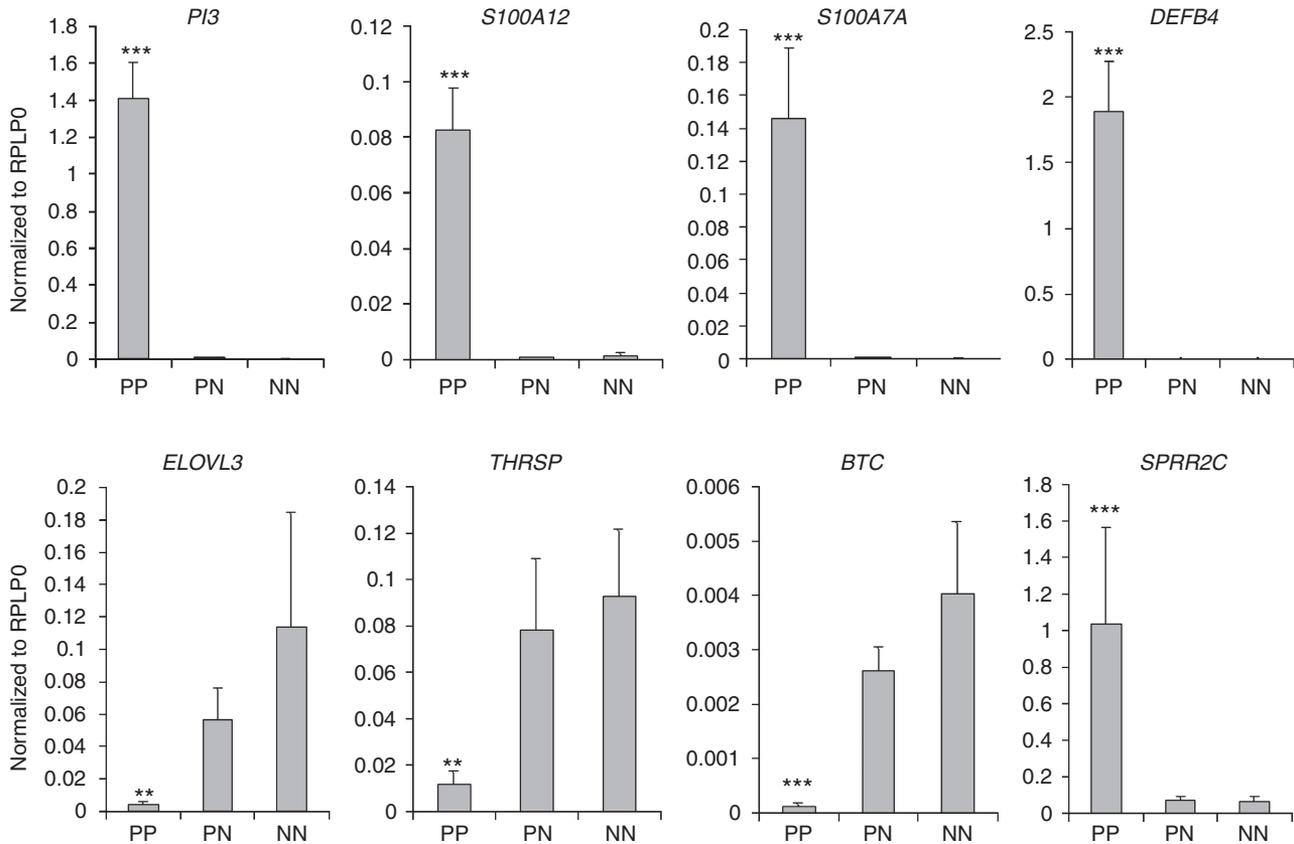


Figure 4. QRT-PCR confirmation of several of the differentially expressed genes. *PI3* (*SKALP*), *DEFB4*, *S100A12*, *SPRR2C*, and *S100A7L1* (*S100A7A*) were upregulated 1,300; 2,200; 63; 15; and 4,500-fold, respectively, in PP versus NN skin, whereas *ELOVL3*, *THRSP*, and *BTC* were downregulated 30, 8, and 36-fold, respectively, in PP skin. The results are shown relative to the expression of the housekeeping gene *RPLP0* (*36B4*) and indicate the mean + SEM; $n=30$. Statistical significance was tested using Student's two-tailed *t*-test assuming equal variances and are indicated as ** $P<0.01$ and *** $P<0.001$, for PP versus PN skin.

($P=1.3 \times 10^{-8}$). Likewise, canonical pathways involving LXR/RXR activation ($P<1.0 \times 10^{-6}$), IL-10 signaling ($P<1.0 \times 10^{-5}$), pattern-recognition receptors ($P<1.0 \times 10^{-4}$), and IFN signaling ($P<1.0 \times 10^{-4}$) were highly enriched (Supplementary File 3). These results are in excellent agreement with those reported in a previous gene ontology analysis of the psoriatic transcriptome (Zhou *et al.*, 2003).

Comparison to published cytokine-stimulated keratinocyte transcriptomes

We compared the differentially regulated genes that we found in PP skin versus PN and NN skin to previously published data sets describing cytokine-induced gene expression changes in cultured keratinocytes (Banno *et al.*, 2004; Finelt *et al.*, 2005; Bando *et al.*, 2007; Mee *et al.*, 2007; Nograles *et al.*, 2008). These published analyses were focused on cytokines previously implicated in the pathogenesis of psoriasis, including IL-1 α (Mee *et al.*, 2007), oncostatin-M (OSM) (Finelt *et al.*, 2005), TNF- α (Banno *et al.*, 2004), IFN- γ (Mee *et al.*, 2007), and the Th17 cytokines IL-22 and IL-17 (Nograles *et al.*, 2008). For this analysis, we used the same criteria for change that we used for the comparison of PP skin versus PN and NN skin (>2 -fold change, $P<0.05$).

Several of the cytokine-induced transcripts observed in these studies showed considerable overlap with that of the PP transcriptome. Thus, IL-22 treatment caused upregulation of four genes (*S100A7*, *SERPINB4*, *S100P*, *SERPINB1*) all of which were also upregulated in the PP transcriptome (Figure 6). Likewise there was substantial overlap between IL-17-induced genes (Nograles *et al.*, 2008) and lesional gene expression, with 69% of IL-17-induced genes also being significantly upregulated in the PP transcriptome. The upregulated genes included *DEFB4*, the S100-family members *S100A7* and *S100A12*, and the chemokines *CCL20*, *CXCL1*, *CXCL3*, and *IL-8*. The fold induction of these genes in keratinocytes was commonly comparable to that observed in PP skin. Thus, IL-17 treatment of keratinocytes induced the expression of *DEFB4* (238-fold), *CXCL8* (14-fold), and *CCL20* (28-fold), compared with 197, 17, and 7.8-fold, respectively, in PP skin (Table 1). Interestingly, some of the genes were induced more highly in keratinocyte cultures as compared with that in PP skin. Thus, IL-22 and IL-17 stimulated *S100A7* expression by a factor of 458 and 189, respectively (Nograles *et al.*, 2008), compared with 18-fold induction in PP skin, possibly because basal levels of *S100A7* are very low in keratinocytes (Elder and Zhao, 2002) and the heterogenous

GO categories for upregulated genes

Biological process			
Term	Count	%	P-value
Immune response	90	13.91%	4.57E-20
Defense response	68	10.51%	1.00E-19
Response to wounding	57	8.81%	7.56E-19
Mitotic cell cycle	42	6.49%	7.08E-14
Ectoderm development	27	4.17%	1.40E-11
Keratinocyte differentiation	13	2.01%	6.28E-10

Cellular compartment			
Term	Count	%	P-value
Extracellular space	53	8.19%	4.95E-13
Spindle	17	2.63%	3.26E-09
Vesicular fraction	24	3.71%	2.07E-08
Microsome	23	3.55%	5.67E-08
Cornified envelope	9	1.39%	1.05E-07

Molecular function			
Term	Count	%	P-value
Chemokine activity	13	2.01%	4.42E-08
Chemokine receptor binding	13	2.01%	5.58E-08
Catalytic activity	246	38.02%	1.97E-07
Cytokine activity	26	4.02%	7.36E-07

GO categories for downregulated genes

Biological process			
Term	Count	%	P-value
Multicellular organismal development	99	21.02%	4.94E-13
System development	78	16.56%	4.06E-11
Fatty acid metabolic process	21	4.46%	4.66E-10
Lipid metabolic process	42	8.92%	1.33E-08
Organ development	55	11.68%	2.16E-07
Blood circulation	16	3.40%	1.68E-06

Cellular compartment			
Term	Count	%	P-value
Extracellular region part	46	9.77%	1.14E-08
Proteinaceous extracellular matrix	24	5.10%	2.18E-06
Cytoskeleton	45	9.55%	1.70E-04

Molecular function			
Term	Count	%	P-value
Cytoskeletal protein binding	30	6.37%	1.88E-08
Protein binding	190	40.34%	1.85E-06
Structural molecule activity	39	8.28%	1.35E-05

Figure 5. The enriched gene ontology categories for up- and downregulated transcripts.

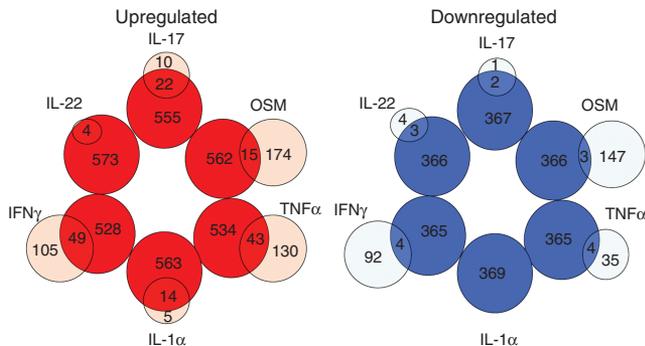


Figure 6. Comparison of the psoriatic transcriptome to cytokine-stimulated keratinocytes. We compared the PP versus combined PN and NN transcriptome to published genomic maps obtained from cytokine-stimulated keratinocytes using the same criteria that was used for the PP transcriptome (≥ 2 -fold change). This analysis was focused on cytokines previously implicated in the pathogenesis of psoriasis, such as IL-1 α (Mee *et al.*, 2007), OSM (Finelt *et al.*, 2005), TNF- α (Banno *et al.*, 2004), IFN- γ (Mee *et al.*, 2007), IL-22, and IL-17 (Nogralles *et al.*, 2008). Note that there is no overlap between the downregulated genes in PP versus NN/PN and IL-1 α downregulated genes. Several of the cytokines showed moderate overlap with the psoriatic transcriptome, although this never represented more than a very small fraction of the entire PP transcriptome (maximum 53 of 649 = 5.6% for IFN- γ).

source of RNA in psoriatic lesional skin as compared with these homogenous keratinocyte cultures could dilute the keratinocyte-derived RNA fraction.

There was also considerable overlap between the expression of IFN- γ -induced genes (Mee *et al.*, 2007) and PP gene

expression. IFN- γ induced the expression of several chemokines such as CXCL9 and CXCL10 that are found to be increased in PP skin, but suppressed several genes involved in cell proliferation that were upregulated in PP skin, including the cyclin-A2, B1, and B2 (*CCNA2*, *CCNB1*, *CCNB2*, respectively) and cell-cycle genes *CDC2* and *CDC20* (Mee *et al.*, 2007). These data are consistent with the observation that IFN- γ is one of the principal cytokines mediating the growth-promoting effect of psoriatic T-cells (Bata-Csorgo *et al.*, 1995), even though it has a growth-suppressive effect on cultured keratinocytes in monolayer cultures (Nickoloff *et al.*, 1984). Not surprisingly, there was also some overlap between the expression of TNF- α -induced genes and PP gene expression (Banno *et al.*, 2004). TNF- α treatment of cultured keratinocytes induced the expression of a number of chemokines and cytokine receptors that are found to be upregulated in PP skin, including IL-8 (*CXCL8*), *CXCL1*, *CXCL2*, *CCL20*, *IL1B*, *IL7R*, and *IL4R*.

Although we found *OSM* mRNA to be elevated in PP skin (1.3-fold, $P < 0.0001$), there was minimal overlap between genes upregulated in PP skin and genes induced by *OSM* in keratinocyte monolayers (Finelt *et al.*, 2005; Figure 6). Interestingly, however, when epidermal constructs were used instead of keratinocyte monolayer cultures, the number of overlapping genes increased from 15 of 189 (7.9%) (Finelt *et al.*, 2005) to 50 of 279 (17.9%) (Gazel *et al.*, 2006).

There was a much smaller overlap between downregulated genes in cytokine-stimulated keratinocytes and that of the PP downregulated transcriptome. Thus, only four of the transcripts downregulated by IFN- γ were also downregulated

in PP skin, along with only four of the TNF- α -downregulated, three of the IL-22-downregulated, three of the OSM-downregulated, and only two of the IL-17-downregulated genes (Figure 6). As was observed for the corresponding upregulated genes, the number of overlapping downregulated genes in the OSM-stimulated skin equivalent model increased from 2% (3 out of 150) to 6.5% (14 out of 216) (Gazel *et al.*, 2006) (Supplementary File 4).

For several of the cytokines, there was an overlap between the downregulated genes in treated keratinocytes and upregulated genes in PP skin. Thus, for IFN- γ , out of 96 downregulated genes, 23 were present on the PP upregulated gene list. Similarly, for OSM-treated keratinocyte monolayers (Gazel *et al.*, 2006), out of 150 downregulated genes, 19 were present on the PP upregulated gene list. Reminiscent of the effects of IFN- γ on cultured keratinocytes (Nickoloff *et al.*, 1984), many of these genes encoded cyclins (*CCNA2*, *CCNB1*) and other components involved in cell-cycle regulation such as *CDC2*, *CDC20*, and *CDKN3*. Whereas there are no reports of the effects of OSM on the proliferation of monolayer keratinocytes, it has been shown to induce migration of monolayer keratinocytes and induce hyperplasia of keratinocytes in reconstituted human epidermis (Boniface *et al.*, 2007).

DISCUSSION

This is the largest study of global gene expression in psoriasis to date and provides a global view of the biological changes that occur in lesional skin. Many of the changes that we observed are confirmatory of previous observations (Oestreicher *et al.*, 2001; Zhou *et al.*, 2003; Yao *et al.*, 2008), but the data presented here expand these in scope and detail and provide a greater in-depth insight into the biological changes that occur in psoriatic lesions.

Several factors could be responsible for the somewhat limited overlap between our study and these other studies. The first of these is study design. In the work of Nomura *et al.* (2003) the aim was to compare the gene expression of psoriasis with that of atopic dermatitis, whereas in that of Haider *et al.* (2006), the PP transcriptome was compared to that of squamous cell carcinoma. Second, the larger sample size of this study provides increased power to identify differentially regulated genes. Third, these studies were performed using different microarray platforms, representing overlapping but non-identical visualizations of the human transcriptome, although there is greater overlap with the more recent arrays possibly reflecting a larger number of probesets that these assays have in common. Thus, several of the genes reported by Zhou *et al.* represented expressed sequence tags and hypothetical genes that are no longer annotated as genes on the microarray that we used. Moreover, several of the genes reported by Zhou *et al.* later were shown to be different probes targeting the same genes, leading to overestimation of the number of genes involved. One other cause of discrepancy between the data sets is batch effect (Akey *et al.*, 2007). In comparing our data set to that of Yao *et al.* (2008) we noted that pairs of non-lesional/lesional skin samples were not always processed together at

the same time, therefore creating a batch effect inflating the number of differentially expressed genes. We avoided this confounding effect by running the paired uninvolved and lesional samples at the same time. Overall, most of our differentially expressed transcripts were present in the data set of Yao *et al.* indicating that the data of Yao *et al.* included truly differentially expressed genes along with those “noisy” genes that were introduced due to the batch effect in the data.

One of the canonical pathways that was upregulated in our data set involved members of the IFN-signaling pathway, with 8 of 29 pathway components upregulated. The IFNs consist of two classes: type-I, which includes IFN- α and IFN- β , which have an important role in antiviral defenses and are almost exclusively derived from plasmacytoid dendritic cells, and type-II, which includes IFN- γ , which is the prototypical Th1 cytokine. Subcutaneous injection of IFN- γ has been shown to induce localized psoriasis in the uninvolved skin of psoriatic patients (Fierlbeck *et al.*, 1990). More recently, plasmacytoid-derived type-I IFNs were shown to have a central role in the onset of psoriatic lesions in a spontaneous xenograft model of psoriasis (Nestle *et al.*, 2005). Consistent with previously published studies (Nestle *et al.*, 2005) we could not detect upregulation of type-I IFNs in established chronic plaque lesions. However, the type-II IFN, IFN- γ , was upregulated 1.4-fold. The most strongly upregulated genes belonging to the IFN-signaling pathway were *STAT1* (2.8-fold), *MX1* (4.5-fold), *IRF1* (2.0-fold), *IRF9* (2.1-fold), and *OAS1* (4.4-fold). Moreover, at least 40 other IFN-inducible transcripts were upregulated at least two-fold in PP versus NN skin, including *OASL* (9.9-fold), *OAS2* (9.0-fold), *RGS1* (7.0-fold), *IFI27* (8.0-fold), *CXCL10* (7.1-fold), *PBEF1* (5.3-fold), *IFI6* (5.0-fold), and *CXCL1* (5.8-fold). These results confirm and extend the results of earlier studies indicative of a strong IFN-response signature in psoriasis (Zhou *et al.*, 2003; Lew *et al.*, 2004; Yao *et al.*, 2008). Besides activating IFN signature genes, IFN- γ can influence other cytokine pathways. We have recently shown that IFN- γ produced by T-cells is a major stimulus for the production of IL-23 by macrophages or dendritic cells (Kryczek *et al.*, 2008), which in turn stimulates the development of Th17 cells (Harrington *et al.*, 2005; Kryczek *et al.*, 2008).

Several lines of evidence indicate that TNF- α also has a central role in the pathogenesis of psoriasis. Treatments that block the action of TNF- α have profound therapeutic effects on psoriasis (Lowes *et al.*, 2007), and polymorphisms of two variants that regulate TNF- α signaling were recently identified in a genome-wide association study of subjects with psoriasis and point to a possible altered NF- κ B-signaling pathway in psoriasis (Nair *et al.*, 2009). The predominant source of TNF- α in psoriatic skin is activated dendritic cells (Boyman *et al.*, 2004), and whereas TNF- α mRNA was only increased by 1.3-fold in PP versus NN skin, several genes known to be upregulated by TNF- α treatment were markedly induced, including *AKR1B10* (aldose reductase, 57.6-fold; Iwata *et al.*, 1999), *IL1F9* (IL-1 ϵ , 36.3-fold; Debets *et al.*, 2001), *MMP12* (macrophage elastase, 9.1-fold; Feinberg *et al.*, 2000), *CCL20* (MIP-3 α , 7.8-fold), *IL1F5* (6.6-fold; Debets *et al.*, 2001), and *CXCL9* (MIG, 6.1-fold; Rottman *et al.*, 2001). TNF- α has

recently been shown to have a role in maintaining the Th17 subset of T-cells in psoriatic skin (Zaba *et al.*, 2007) and may synergize with IFN- γ in the induction of proinflammatory mediators from mononuclear leukocytes (Haider *et al.*, 2008a). Thus, there is a strong synergy between TNF- α and IFN- γ signaling in the course of T-cell activation by dendritic cells, as has been found for a variety of cell types (Boehm *et al.*, 1997).

As mentioned earlier, the newly identified T-cell subset Th17 has recently been implicated in the pathogenesis of psoriasis and other disease involving epithelial barriers (Bettelli *et al.*, 2006; Zaba *et al.*, 2007). Although the transcripts directing the synthesis of IL-12 and IL-23 did not reach the threshold of twofold change, this likely being due to the low sensitivity of microarrays for less abundantly expressed transcripts, we found highly significant changes ($P < 10^{-10}$) in *IL12B* (p40), being upregulated 1.2-fold, and *IL23A* (p19), upregulated 1.2-fold, but no change in expression for *IL12A* (p35). IL-17 strongly induces the expression of several chemokines by keratinocytes, including *CXCL1*, *CXCL3*, *CXCL5*, *CXCL6*, *CXCL8* (IL8), and *CCL20* (Nogralles *et al.*, 2008), and also induces the expression of antimicrobial peptides such as *DEFB4* (Wilson *et al.*, 2007). Of the 47 genes that were found to be upregulated in keratinocytes by IL-17 (Nogralles *et al.*, 2008), we could confirm 36 to be upregulated in lesional skin, although several of these had a less than twofold change as seen by microarray analysis. Moreover, IL-17 suppresses TNF- α -induced *CCL27* production by keratinocytes (Kanda *et al.*, 2005); in agreement with previously published data (Nomura *et al.*, 2003) *CCL27* is among the most markedly downregulated transcripts in PP skin (Table 1). In contrast to IL-17, IL-22 is a relatively weak inducer of chemokine expression in keratinocytes, but has more marked effect on the differentiation and proliferation of stratified epithelium and the expression of antimicrobial proteins such as the S100 family of proteins (Wolk *et al.*, 2006; Sa *et al.*, 2007). Of the 21 unique genes induced or suppressed in keratinocytes by IL-22 (Nogralles *et al.*, 2008), 17 were found to be similarly changed in PP skin. The high expression of antimicrobial peptides is characteristic for psoriasis; this is in contrast to atopic dermatitis, which despite comparable epidermal proliferation, is not characterized by a similar overexpression of these genes (de Jongh *et al.*, 2005; Buchau and Gallo, 2007). Many of the most highly upregulated transcripts we have identified in psoriasis encode antimicrobial peptides, including *DEFB4* (197-fold), *S100A7* (18-fold), *S100A8* (12-fold), *S100A9* (57-fold), *S100A12* (57-fold), *S100A7L1* (also known as *S100A15* and is now officially designated *S100A7A*) (150-fold), and *LCN2* (17-fold) (Table 1).

Despite being limited by the lack of many of the cellular players in psoriasis, including inflammatory cells, fibroblasts, and endothelial cells, keratinocyte cultures have been used as a models to gain insights into cytokine-driven gene expression changes in psoriatic skin either using keratinocytes in monolayer or as epidermal constructs. These cytokines include IL-1 (Mee *et al.*, 2006; Yano *et al.*, 2008), OSM (Finelt *et al.*, 2005; Boniface *et al.*, 2007), TNF- α

(Banno *et al.*, 2004, 2005), and IFN- γ (Banno *et al.*, 2003; Mee *et al.*, 2006, 2007). Whereas treatment with either IL-1 or OSM upregulated several of the most strongly upregulated transcripts observed in PP skin, including *SERPINB4*, *DEFB4*, *IL1F9*, *PI3*, *S100A7*, *S100A8*, *S100A9*, and *S100A12*, other strongly upregulated transcripts, such as *CXCL9* and *CXCL10* were not upregulated; rather, these transcripts were strongly induced by IFN- γ . Conversely, several transcripts that were strongly induced in culture were not strongly upregulated in psoriasis. Although some of this discrepancy is influenced by the heterogenous mixture of cells involved in lesional psoriatic skin as compared with the homogenous keratinocyte cultures, it is likely that the psoriatic transcriptome reflects exposure of the epidermis to a medley of multiple cytokines, producing a response that can be partially but not completely mimicked by any single cytokine alone as was recently shown in a novel skin-equivalent system (Tjabringa *et al.*, 2008). Likewise, it appears that the model system used has important implications for the gene expression pattern observed. This is clearly shown by the OSM studies (Finelt *et al.*, 2005; Gazel *et al.*, 2006) where the overlap between gene-expression signature and PP skin increased from 7.9 to 17.9% by using reconstituted epidermal constructs instead of semi-confluent keratinocyte cultures. Finally, one study examined a panel of 33 cytokines singly and in various combinations, and found that IL-1 α and IL-1 β , IL-6, IL-17, IL-20, IL-22, IL-24, and TNF- α could provoke *S100A7* and *DEFB4* responses similar to that elicited by OSM. They also observed marked synergy between IL-17, TNF- α , and OSM as the major inducers of *S100A7* and *DEFB4* (Boniface *et al.*, 2007).

Many of the most highly upregulated genes that we encountered reside in the epidermal differentiation complex located on chromosomal band 1q21. In addition to the S100 genes involved in the innate immune response, these include the *SPRR* and the late cornified envelope gene families whose members have important roles keratinocyte terminal differentiation (Gibbs *et al.*, 1993; Eckert *et al.*, 2004; Jackson *et al.*, 2005). These gene families are structurally distinct and rapidly evolving (Ravasi *et al.*, 2004; Jackson *et al.*, 2005), suggesting that this distinctive chromosomal region is under some form of long-range epigenetic regulation (Segre, 2006). Other clustered and highly upregulated genes encode the serine peptidase inhibitors *SERPINB3* (upregulated 64-fold), *SERPINB4* (upregulated 377-fold), and *SERPINB13* (upregulated 7.5-fold) on chromosome 18q21.3, and the kallikrein serine proteases *KLK6* (upregulated 9.2-fold) and *KLK13* (upregulated 9.8-fold) on chromosome 19q13. The mechanism(s) through which these clustered genes are coordinately upregulated in psoriasis remain to be determined.

Psoriasis is characterized by a markedly altered balance of proteolytic and antiproteolytic activities involved in keratinocyte turnover (Magert *et al.*, 2005), consistent with the observation that catalytic activity was one of the most highly upregulated molecular functions upregulated in PP skin (Figure 5). In addition to the clustered proteases and inhibitors described above, the *PI3* gene (upregulated 131-fold) encoding protease inhibitor-3 (SKALP/elafin) is

markedly overexpressed in psoriasis (Alkemade *et al.*, 1994). Several of these proteases were stimulated by cytokines in keratinocyte cultures, particularly PI3 and members of SERPINB. Thus, IL-1 α , IL-17, and IL-22 induced the expression of *SERPINB4* (Bando *et al.*, 2007; Nograles *et al.*, 2008) in keratinocytes. OSM, IFN- γ , and TNF- α increased the expression of *SERPINB1* (Banno *et al.*, 2004; Finelt *et al.*, 2005; Mee *et al.*, 2007), and IL-1 α -induced the expression of *SERPINB3* and *PI3* (Bando *et al.*, 2007), whereas TNF- α induced *SERPINB8* expression (Banno *et al.*, 2004). Interestingly, OSM's stimulation of reconstituted epidermal constructs greatly increased the number of proteases and protease inhibitors overlapping with that of PP skin (*KLK13*, *PI3*, *SERPINB1*, *SERPINB4*, *SERPINA1*, and *SERPINA3*) (Gazel *et al.*, 2006). In all instances the expression observed in keratinocytes or reconstituted epidermis was lower than that observed in PP skin. This indicates that expression of proteases and protease inhibitors is rather a function of the stratified epithelia in contrast to keratinocyte monolayer cultures. Furthermore, the dramatic overexpression of these proteases and antiproteases shows that control of the proteolytic environment is a crucial element of epidermal homeostasis and barrier function (Meyer-Hoffert, 2009).

Interestingly we observed large alterations in genes involved in metabolism, particularly lipid and fatty acid metabolism (Figure 5), and amino-acid metabolism (Supplementary File 3). Kynureninase (*KYNU*) is one of the genes that was among the most highly upregulated genes in psoriatic skin in our data set (Table 1). Upregulation of *KYNU* has been reported previously in psoriasis (Nomura *et al.*, 2003; Ito *et al.*, 2004) as well as atopic dermatitis (Ito *et al.*, 2004). The product of this gene is involved in tryptophan metabolism and 11 other genes involved in the tryptophan pathway were found to be differentially regulated in psoriatic skin (Supplementary File 3). Importantly, activity of this pathway is induced by IFN- γ (Taylor and Feng, 1991; Ito *et al.*, 2004). The exact role of this pathway in psoriasis is unknown, but upregulation of tryptophan catabolism has been shown to confer antibacterial effector functions on multiple cell types, including epithelial cells (Daubener and MacKenzie, 1999). The changes we observed for genes involved in lipid and fatty acid metabolism are similar to changes that we have previously reported in uninvolved and lesional psoriatic skin, and suggests a defect involving the lipid barrier of the epidermis of psoriatic skin (Gudjonsson *et al.*, 2009b).

Taken together, these results are broadly consistent with the model for development and maintenance of psoriatic lesions that has emerged in recent years. Thus complex two-way interactions between activated dendritic cells, T-cells, and keratinocytes, are largely mediated by cytokines and chemokines, and punctuated by periodic bursts of neutrophilic infiltration of the epidermis (Lowe *et al.*, 2007). Interestingly, several of the most highly upregulated genes in PP skin drive neutrophil infiltrations, including *IL-8* (*CXCL8*) (upregulated 17 fold vs NN) and *CXCL1* (upregulated 5.8-fold vs NN). However, neutrophils appear to be dispensable for the development of psoriasis in a transgenic mouse

model (Stratis *et al.*, 2006), focusing attention on mononuclear cells.

We observed fewer downregulated than upregulated transcripts in PP skin, compared with that in either PN or NN skin. Of the downregulated transcripts observed in PP skin (Table 1) *WIF1*, encoding Wnt-inhibitory factor-1, and *BTC*, encoding *BTC*, were the most prominent. WIF-1 is a secreted protein that binds to Wnt proteins and inhibits their activities (Hsieh *et al.*, 1999). Taken together with increased expression of the non-canonical Wnt member, *WNT5A*, that we and others (Zhou *et al.*, 2003; Reischl *et al.*, 2007) have observed, we have proposed that the non-canonical Wnt pathway may be activated in psoriasis (Gudjonsson *et al.*, submitted). *BTC* is one of several epidermal growth factor (EGF)-receptor ligands expressed in the skin, along with amphiregulin, heparin-binding EGF, transforming growth factor- α , and epiregulin. In contrast to the other EGF-family ligands, many of which have been reported to be upregulated (Elder *et al.*, 1989; Shirakata *et al.*, 2007), *BTC* is downregulated in psoriasis, as shown by immunohistochemistry (Piepkorn, 1996; Piepkorn *et al.*, 2003) and by a previous microarray analysis (Zhou *et al.*, 2003). These results suggest that *BTC* may have a role in the maintenance of the differentiated phenotype of the epidermis (Piepkorn *et al.*, 2003). In addition to *CCL27* discussed earlier, interesting transcripts that were markedly downregulated in PP versus NN skin included *THRSP*, encoding a nuclear protein involved in fatty acid synthesis (Cunningham *et al.*, 1998), *GAL*, encoding galanin, a vasoactive peptide that mediates vasoconstriction and inhibition of blood flow (Schmidhuber *et al.*, 2007), *IL1F7*, which has been shown to be expressed in fully differentiated keratinocytes in the stratum granulosum of the epidermis (Busfield *et al.*, 2000), and *MSH5*, thought to participate in mitotic DNA repair (Her *et al.*, 2007). There was very small overlap between genes downregulated by individual cytokines and those downregulated in PP skin. Interestingly, this overlap was larger for the only study of cytokine-stimulated reconstituted epidermis (Gazel *et al.*, 2006) published so far. In that study one of the transcripts that was downregulated, encoded for *BTC*, which is a member of the EGF ligand family and one of the most strongly downregulated transcripts in PP skin, but its expression is undetectable in keratinocyte monolayers (Johnston, unpublished observation). Thus, apart from the more complex situation in PP skin where various combinations and two-way interactions of cytokines and activated immune cells may affect gene expression, this indicates that lack of stratification may be one of the reasons for the small overlap observed between downregulated genes in PP skin and that of cytokine-stimulated keratinocyte monolayers. Finally, another explanation for this discrepancy may relate to differences in epidermal and keratinocyte responses between psoriasis and healthy controls that have been proposed to be genetically determined (Zeeuwen *et al.*, 2008).

In conclusion, this is the most comprehensive analysis of differentially regulated transcripts in psoriasis and provides a global view of the psoriatic transcriptome. This study identified a group of genes that are dysregulated in lesional

psoriatic skin, large proportion of which have not been described before. Our comparison of the PP transcriptional genome and the limited overlap with genomic maps obtained from previously published cytokine-stimulated keratinocytes using monolayer systems indicates the limitations of using this approach and suggests that greater insights into the pathogenesis of psoriasis and the effect of individual key cytokines might be obtained by using reconstituted epidermal constructs or other more elaborate tissue models. Overall, these data provide novel insights into the pathogenic mechanisms involved in psoriasis and more accurately define the biochemical changes and pathways involved.

MATERIALS AND METHODS

Subjects

The criterion for entry of a case was the presence of at least one well demarcated, erythematous, scaly PP that was not limited to the scalp. In those instances where there was only a single PP, the case was only considered if the plaque occupied more than 1% of the total body surface area. We enrolled 58 patients with psoriasis and 64 normal healthy controls to the study. This study was approved by the Institutional Review Board of the University of Michigan Medical School and was conducted according to the Declaration of Helsinki Principles. Informed written consent was obtained from all study subjects. Study subjects did not use any systemic anti-psoriatic treatments for 2 weeks before topical anti-psoriatic treatments for 1 week before biopsy. Two biopsies were taken under local anesthesia from each psoriatic subject; one 6-mm punch biopsy was obtained from PP skin and the other from PN skin sampled at least 10 cm away from any active plaque. One or two biopsies were obtained from the normal skin of healthy controls (NN skin). Gender was balanced in both case and control cohorts. The mean age of the controls was 41.1 years (range 18–75), whereas the mean age of the cases was 48.5 years (range 21–69). Patients' demographic characteristics are shown in Supplementary File 5.

RNA processing and microarray hybridization

After removal from the skin, biopsies were snap-frozen in liquid nitrogen and stored at -80°C until use. Biopsies were pulverized with a hammer while still frozen and total RNA isolation was performed using a commercial kit (RNeasy, Qiagen, Chatsworth, CA), using glass beads (Biospec, Bartlesville, OK) for homogenization. RNA quantity and quality were measured using an Agilent 2,100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Only samples yielding intact 18S and 28S ribosomal RNA profiles were used. cDNA and *in vitro* transcription for probe biotinylation were performed using 5 μg of total RNA according to the manufacturer's protocols (Affymetrix, Foster City, CA). Samples were run on HU133 Plus 2.0 arrays to query the expression of > 54,000 probes.

Quantitative reverse transcription-PCR

Reverse transcription reaction was performed using 0.15 μg of RNA template and cDNA was synthesized using a High Capacity cDNA Reverse Transcription kit as per manufacturer's protocols (Applied Biosystems, Foster City, CA). The primers for genes *IL-8*, *DEFB4*, *S100A7A*, *S100A12*, *PI3*, *THRSF*, *ELOVL3*, and *BTC* were obtained from Applied Biosystems. Results were normalized to the expression of the housekeeping gene *RPLP0/36B4*, encoding ribosomal protein, large,

P0 (Minner and Poumay, 2009). QRT-PCR was performed using an Applied Biosystems 7900HT Fast Real Time PCR System. Fold changes were calculated comparing normal versus lesional psoriatic skin.

Microarray data analysis and statistics

The Robust Multichip Average (RMA) method (Irizarry *et al.*, 2003) was used to process the raw data from 180 microarrays. The data were adjusted to account for gender and batch effects. Specifically, we used a linear regression model to estimate the gender and batch effects, and then subtracted them from the RMA expression values to obtain the adjusted data. Hierarchical clustering (using a "complete" agglomeration method) and principal-component analysis were performed using the adjusted expression data using the publicly available software R (www.r-project.org). Gene expression was contrasted between PP versus PN (by using one-sample *t*-tests on the difference between paired PP, PN samples) or PN versus NN (by using two-sample *t*-tests) on the basis of the following criteria: ≥ 2.0 -fold change in the means of expression in the two groups and a false discovery rate *P*-value ≤ 0.05 . Transcripts with small variation across samples were not filtered out. But when the estimated variance for a gene in the *t*-test was less than the median variance for all genes, we used the median variance in the *t*-test. In doing so, we prevented genes from being designated differentially expressed if they had very small variation. Gene ontology category enrichment analysis was performed using DAVID (Database for Annotation, Visualization and Integrated Discovery; <http://david.abcc.ncifcrf.gov/>; Bethesda, MD). For this exploratory analysis, *P*=0.001 was chosen as a stringent significance criterion and *P*=0.05 as a relaxed significance criterion. QRT-PCR data were tested for significance using Student's two-tailed *t*-test assuming equal variances and *P*-values ≤ 0.05 were considered to be significant.

Ingenuity pathway analysis

The Ingenuity Pathway Analysis software package (Ingenuity Systems, Redwood City, CA) was used to analyze the differentially regulated transcripts. For network and pathway generation, a data set containing gene identifiers and the corresponding expression values was uploaded into the application. Each gene identifier was mapped to its corresponding gene object in the Ingenuity pathways knowledge base.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/jid>

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