A Characteristic Subset of Psoriasis-Associated Genes Is Induced by Oncostatin-M in Reconstituted Epidermis

Alix Gazel[1,2], Martin Rosdy[2], Béatrice Bertino[2], Carine Tornier[2], Florent Sahuc[2] and Miroslav Blumenberg[1,3,4]

The pathological manifestations of psoriasis are orchestrated by many secreted proteins, but only a handful, tumor necrosis factor-alpha, IFN-γ and IL-1, have been studied in great detail. Oncostatin-M (OsM) has also been found in psoriatic skin and we hypothesized that it makes a unique and characteristic contribution to the psoriatic processes. To define in-depth the molecular effects of OsM in epidermis, we used high-density DNA microarrays for transcriptional profiling of OsM-treated human skin equivalents. We identified 374 unambiguously OsM-regulated genes, out of 22,000 probed. OsM suppressed the expression of the "classical" epidermal differentiation markers, but strongly and specifically induced the S100A proteins. Cytoskeletal and complement proteins, proteases, and their inhibitors were also induced by OsM. Interestingly, a large set of genes was induced by OsM at early time points but suppressed later; these genes are known regulatory targets of IFN and thus provide a nexus between the OsM and IFN pathways. OsM induces IL-4 and suppresses the T-helper 1-type and IL-1-responsive signals, potentially attenuating the psoriatic pathology. The data suggest that OsM plays a unique role in psoriasis, different from all other, more thoroughly studied cytokines.

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INTRODUCTION

Psoriasis is a paradigm of inflammatory diseases of the skin, which are mediated by white blood cells through an orchestrated interplay of secreted cytokines and chemokines that act on epidermal keratinocytes disrupting their differentiation and causing hyperplasia (Nickoloff and Nestle, 2004; Bowcock and Krueger, 2005). The cytokines most commonly studied in the context of psoriasis are tumor necrosis factor-alpha (TNF-α), IFN-γ, and IL-1 (Nickoloff, 1984; Groves *et al.*, 1994); indeed, TNF-α-targeted therapies show great promise for treatment of patients with psoriasis (Gottlieb *et al.*, 2005; Strober, 2005). Additionally, growth-related cytokine alpha, IL-6, IL-8 macrophage inflammatory protein 3-alpha, transforming growth factor-alpha, and its analogs have been assigned prominent roles in the various aspects of the pathology of psoriasis (Schon and Boehncke, 2005). However, Oncostatin-M (OsM) has received much less attention, although it has been detected in the psoriatic skin (Bonifati *et al.*, 1998) and it may hamper the treatment of the disease by inducing resistance to drugs (Dreuw *et al.*, 2005). OsM also plays a role in the initial phases of wound healing (Goren *et al.*, 2006). Subcutaneous injection of OSM in mice causes an acute inflammatory reaction (Modur *et al.*, 1997). Keratoacanthomas and squamous cell carcinomas express OsM and OsM is the major autocrine growth factor for Kaposi sarcomas (Nair *et al.*, 1992).

OsM was initially identified for its ability to inhibit proliferation of tumor cell lines, but not healthy cells (Zarling *et al.*, 1986; Kligman and Thorne, 1990). It is produced by activated T-lymphocytes, monocytes, and macrophages, and perhaps hepatocytes and endothelial cells (Grant and Begley, 1999). OsM belongs to the IL-6/leukemia-inhibiting factor cytokine family and mediates its activity through their common gp130 receptor (Naar *et al.*, 1991; Auguste *et al.*, 1997; Grant and Begley, 1999). The binding of OsM activates JAKs, receptor-associated kinases that phosphorylate signal transducers and activators of transcription (STATs), which, when phosphorylated, translocate to the nucleus and regulate transcription (Darnell *et al.*, 1994; Narazaki *et al.*, 1994). Activation of JAK1 can activate the Raf-1/mitogen-activated protein kinase pathway as well (Stancato *et al.*, 1997; Ihn and Tamaki, 2000).

Because of the specific signaling pathways and molecular responses to OsM in keratinocytes, we hypothesized that among the panoply of symptoms and processes seen in

[1]*Department of Dermatology, New York University School of Medicine, New York, New York, USA;* [2]*SkinEthic Laboratories, Nice, France;* [3]*Department of Biochemistry, New York University School of Medicine, New York, New York, USA and* [4]*NYU Cancer Institute, New York University School of Medicine, New York, New York, USA*

Correspondence: Dr Miroslav Blumenberg, Department of Dermatology, New York University School of Medicine, 550 First Avenue, New York, New York 10016, USA. E-mail: blumem01@med.nyu.edu

Abbreviations: OsM, Oncostatin-M; STAT, signal transducers and activators of transcription; Th, T-helper; TNF-α, tumor necrosis factor-alpha

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psoriasis, OsM is responsible for a unique and characteristic subset. Previously, we used high-density DNA microarrays for transcriptional profiling in OsM-treated monolayer cultures of human epidermal keratinocytes (Finelt *et al.*, 2005). We found that OsM strongly and specifically affects the expression of many genes, in particular those involved with innate immunity, angiogenesis, adhesion, motility, tissue remodeling, cell cycle, and transcription. Secreted cytokines and growth factors and their receptors, as well as nuclear transcription factors, are primary targets of OsM regulation, and these, in turn, effect the secondary changes.

The above studies, however, could not fully represent the epidermal responses to OsM because keratinocyte monolayers do not fully recapitulate the multilayered differentiation of the epidermis. Therefore, in this study we examined the transcriptional effects of OsM in human skin equivalents consisting of epidermal cells grown on air–liquid interface, that is, stratified structures that completely recreate a differentiating epidermis, including a fully formed stratum corneum (Rosdy and Clauss, 1990; Gazel *et al.*, 2003). Our aim is to use skin equivalents made with healthy human cells to create a reproducible model of psoriasis *in vitro*. Similar skin reconstruction models with cells from psoriatic patients have been used to replicate aspects of psoriatic phenotype (Barker *et al.*, 2004). We found that in the stratified skin equivalents OsM, in addition to the already identified genes, specifically suppresses ''classical'' differentiation markers, involucrin, loricrin, and filaggrin, while strongly inducing the S100A family of calcium-binding proteins.

RESULTS

At the beginning of the treatment, after 12 days of growth at the air–liquid interface, the cultures are fully differentiated, with a six to eight-cell-thick living layer, including a basal, spinous, granular, and even a fully formed cornified layer (Figure 1a). Between days 12 and 19, the extent of differentiation in these cultures does not appreciably change. These cultures do not senesce for at least another several weeks. Therefore, during our experimental period, we see no pronounced changes in the organotypic cultures. The cultures are treated with OsM at three different times, on day 12 for 7 days, on day 17 for 48 hours, and on day 18 for 24 hours. They are all harvested at the same time and processed together to avoid technical variability.

Phenotypically, the OsM-treated and the -untreated skin equivalents appear similar, although after 7 days of treatment with OsM, the living layers are more preserved and there is attendant reduction in cornified cells (Figure 1b). The granular layer also appears different in OsM-treated cultures, both morphologically and biochemically, with significantly reduced expression of filaggrin (see Figure 4). Under appropriate conditions (M Rosdy, in preparation), even parakeratosis can be observed (Figure 1c).

To determine which genes are regulated by OsM in differentiating keratinocytes, we performed DNA microarray analysis on skin equivalent samples that were treated with OsM for 1 hour, 4, 24 and 48 hours, and 7 days, and on

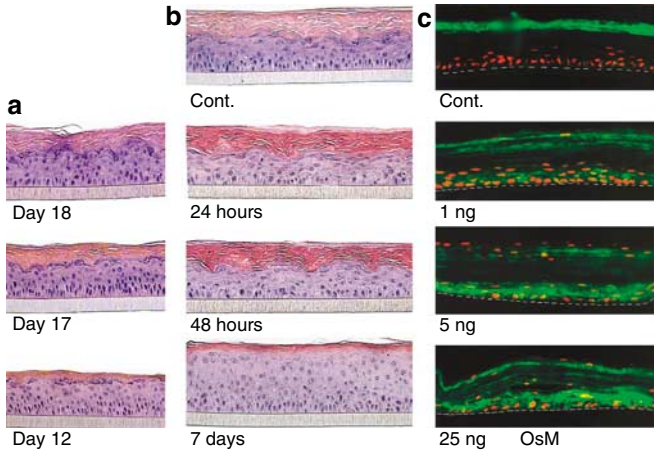


Figure 1. Morphological differences and parakeratosis in skin equivalents after the treatment with OsM. (**a**) At the beginning of the treatment, days 12, 17, and 18, that is, 7, 2 days, and 1 day before harvesting, the organotypic cultures are fully differentiated and have a functional stratum corneum. (**b**) The samples were harvested simultaneously, after 19 days of growth at air–liquid interface, which makes them identical in all aspects of growth, except for the presence of OsM in the medium. (**c**) In certain treated cultures, parakeratosis can be seen, with nuclei in the stratum corneum staining with propidium iodide and keratin 17 induction detected using green immunofluorescence. The dashed line represents the top of the artificial substrate on which the basal cells are attached (original magnification × 200).

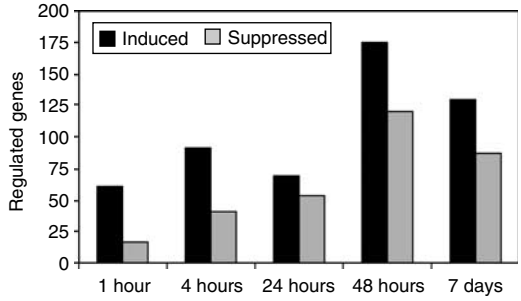


Figure 2. The time course of gene regulation by OsM. The number of induced and suppressed genes is plotted at each time point. The number of suppressed genes at 1 hour time point may be underestimated because the long half-life RNAs may not decay appreciably in the short time.

their corresponding controls. We selected the differentially expressed genes that were induced or suppressed at least 2-fold at two or more consecutive time points. We excluded genes of unknown function and eliminated duplicates. Using these stringent criteria, we found that of the approximately 12,000 genes present on the microarray chips, approximately 374 are regulated by OsM (Figure 2). Of these, 187 were induced, 140 suppressed by OsM, while 47 genes were both suppressed and induced, although at different time points. The greatest number of genes, 265, was regulated at the 48 hours time point.

The genes regulated by OsM in skin equivalents are listed in Figure 3. A more detailed presentation of the regulated genes is given in the Figure S1. We grouped the regulated genes according to a hierarchical list of cellular functions for

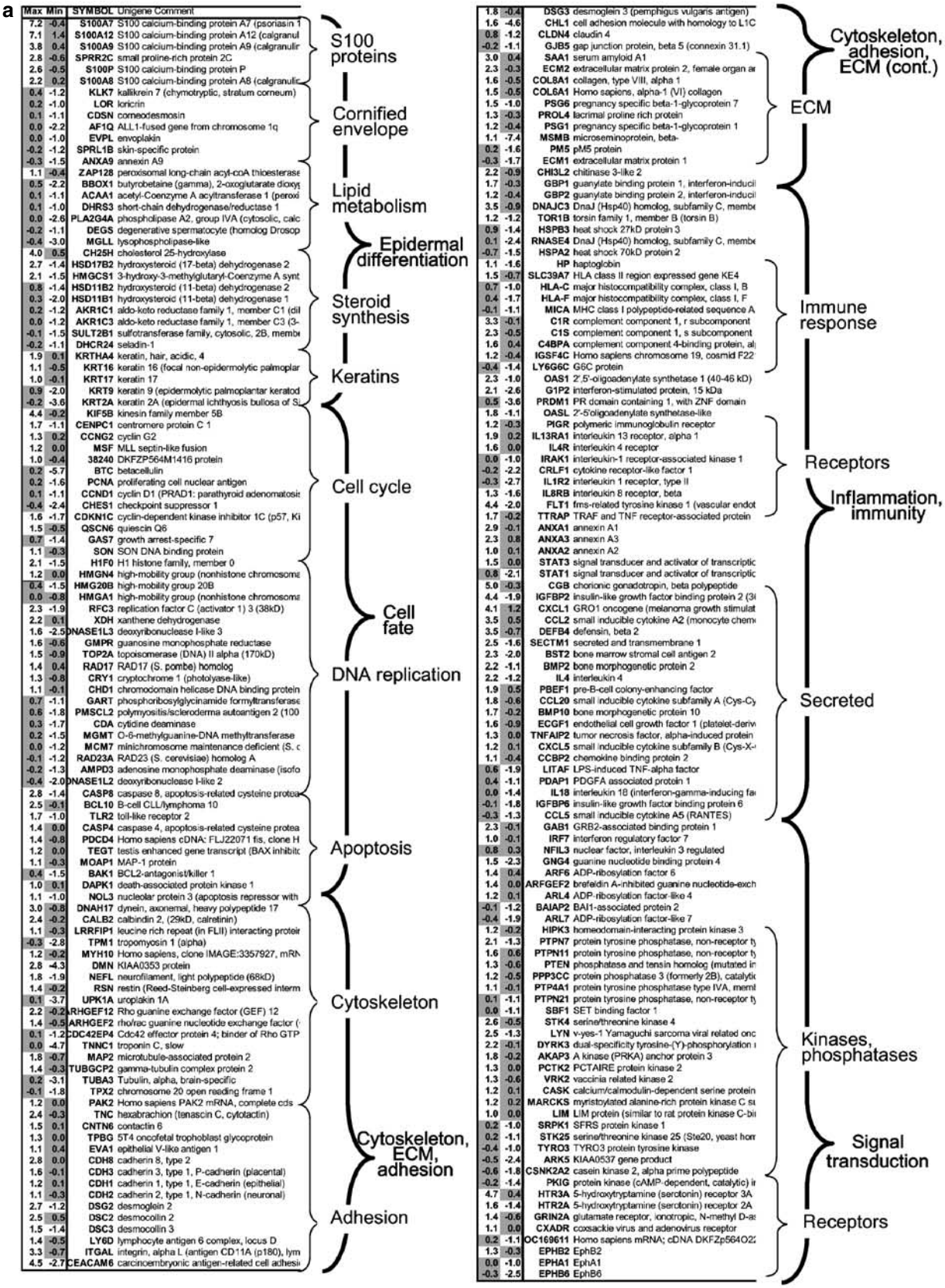


Figure 3. OsM-regulated genes in skin equivalents. The columns on the left show the maximum and minimum fold regulation at any time point, on a log 2 scale. Gene symbols and Unigene comments are given next. The genes are grouped according to their functional categories marked with brackets. Shaded values do not reach the cutoff of 2-fold regulation.

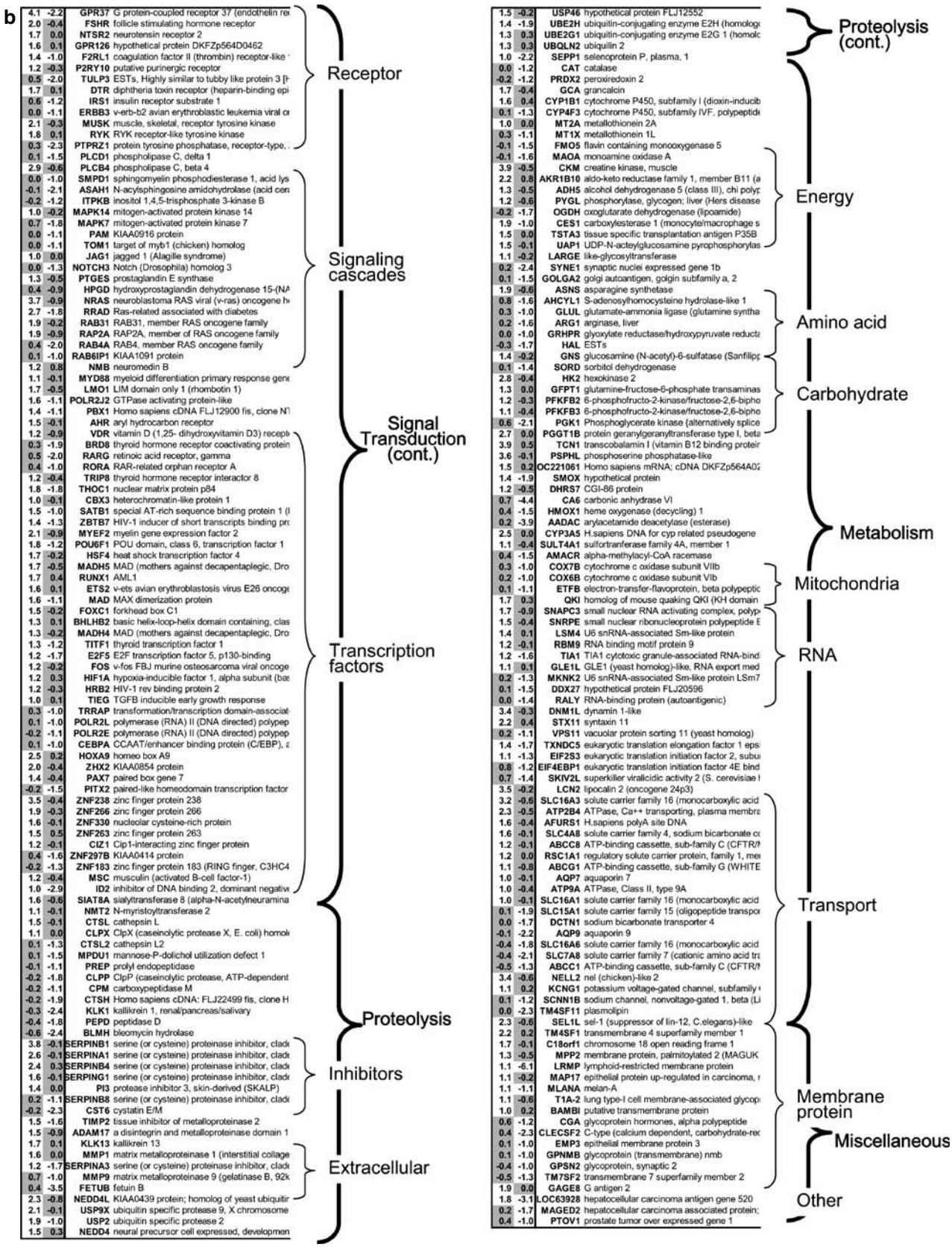


Figure 3. (continued)

easy overview of the biological processes affected by OsM. The higher-order groups include: (1) epidermal differentiation, (2) cell fate, (3) structural proteins, (4) inflammation and immunity, (5) signal transduction, (6) proteolysis, and (7) metabolism. Within each, there are several more specific categories. Although we attempted to categorize the regulated genes with their most salient characteristics, we recognize that the designations are somewhat arbitrary because some genes belong to multiple categories (e.g. STAT1 and STAT3 are transcription factors, but here are grouped with inflammation/immunity category).

We were particularly interested in the specific effects of OsMs on differentiating keratinocytes, and therefore we compared the results in Figure 3 with the published list of OsM-regulated genes in keratinocyte monolayer cultures (Figure S2; Finelt *et al.*, 2005). We find that, in general, OsM regulates similar sets of genes in monolayers and in stratified cultures, provided the genes of interest are expressed in the monolayers. For example, the membrane proteins group contains roughly equal numbers of induced and suppressed genes and most of them are expressed both in monolayer cultures and in stratified skin equivalents. Within this group, the induced genes tend to be induced both in stratified skin equivalents and in the monolayer cultures, and the parallel holds for the suppressed genes as well (Figure S2).

In contrast, the epidermal differentiation markers are generally not expressed in monolayer cultures (Figure S3). In this functional category, we find a complex and intriguing result. Half of the epidermal differentiation marker genes are induced, whereas half are suppressed by OsM (Figure S3). Upon closer look, the markers divide into two clearly distinct categories: the S100A proteins are induced, whereas the ''classical'' markers, loricrin, filaggrin, involucrin, and late envelope proteins xp32 and xp33, are suppressed. The suppression of these markers may correlate with the inhibition of cornification by OsM, as shown in Figure 1. Many of the regulated genes are located in the epidermal differentiation complex on chromosome 1q21 (Zhao and Elder, 1997). Similarly suppressed are corneodesmosin, envoplakin, and many enzymes of lipid and steroid metabolism, genes that are specifically induced during epidermal differentiation, but are not encoded in the epidermal differentiation complex (Elias, 2005). The overall conclusion from these results is that OsM suppresses the expression of epidermal differentiation markers, except for the S100A class.

Cell proliferation is among the important processes regulated by extracellular influences and therefore we specifically examined the associated functional categories, including cell cycle and DNA replication. These functional categories are usually co-regulated and strong proliferative and antiproliferative signals can synchronously induce or suppress a large set of these genes (Banno *et al.*, 2003). We compared the OsM-regulated genes to those affected by IFN-γ, a strong suppressor of proliferation (Banno *et al.*, 2003), or TNF-α, a mild enhancer (Banno *et al.*, 2004). No clear trends were seen; OsM does not regulate many genes in these categories and the numbers of induced and suppressed genes is balanced. The data suggest that OsM does not affect keratinocyte proliferation. Similarly, only a few apoptosis-associated genes are regulated by OsM and, while more genes are induced than suppressed, apoptosis seems not to be clearly affected by OsM. We note, however, that the CFLAR, an essential antiapoptotic gene, was induced by OsM, which may inhibit apoptosis in keratinocytes. We conclude that OsM is not a direct regulator of keratinocyte cell fate in the epidermis.

Several structural proteins, both extracellular and cytoplasmic, are regulated by OsM. Although not many cytoskeletal proteins are affected, OsM strongly induced adhesion proteins. In particular, cadherins and basal cell-specific desmosomal proteins desmogleins 2, desmocolin 2, and desmocolin 3 are induced (Yin *et al.*, 2004). Furthermore, several extracellular matrix proteins are induced. Conversely, very few of the genes in these functional categories are suppressed by OsM. Apparently, OsM boosts the cellular adhesion of the epidermal basal layer by inducing the production of the components of the desmosomal and adherens junctions, and their extracellular counterparts.

Importantly, a large proportion of genes associated with inflammation and immunity are regulated by OsM (Figure 3). Three complement proteins are induced, providing a hitherto unrecognized link between the complement and OsM. Components of the IL-1 pathway are suppressed by OsM while annexins are induced. The T-helper (Th)2-associated cytokine IL-4 and its receptor IL-4R are induced by OsM, whereas IL-18, an inducer of INF-γ, is suppressed. STAT3, OsM-specific responder transcription factor, is induced, whereas STAT1, the primary IFN-γ responder, is suppressed. The overall effect of OsM may be to quiet down the inflammatory and immune responses in psoriatic epidermis.

Among the signal-transduction proteins affected by OsM, we found several protein kinases and phosphatases, G-protein-coupled receptors and G-regulated proteins, as well as transcription factors. Although more genes are induced than suppressed, the regulated proteins do not point to a specific pathway or process. For example, mitogen-activated protein kinase and dual-specificity phosphatase family kinases and phosphatases are not regulated by OsM and many of the well-characterized regulated transcription factors, for example, the NF-κB, activator protein 1 and the nuclear receptor family, are scarcely represented among the regulated genes. These results are in stark contrast with those we saw in TNF-α- or IFN-γ- treated keratinocytes, where the signal-transduction proteins are among the most conspicuous regulated functional categories (Banno *et al.*, 2003, 2004).

Prominent among the OsM-regulated genes are those encoding proteases and their inhibitors. Proteases and peptidases are, in general, suppressed, whereas proteolysis inhibitors of the SERPIN family are induced. Matrix metalloproteinase 1, interstitial collagenase, is induced, whereas matrix metalloproteinase 9, 92 kD gelatinase, is suppressed. Several ubiquitin pathway proteins are induced, with none suppressed, suggesting that certain intracellular substrates are specifically targeted for degradation through the ubiquitin

pathway by OsM. Overall, proteolysis is one of the processes significantly altered by OsM in skin equivalents.

Metabolic pathways affected by OsM include energy-producing enzymes. The carbohydrate metabolism enzymes are induced, whereas the amino-acid metabolism ones are suppressed. Mitochondrial and detoxifying proteins are suppressed. Many transporter proteins are regulated by OsM; approximately similar numbers are induced and suppressed.

We noticed that a relatively large group of genes was found to be induced by OsM at the early time points, 1 hour and 4 hours post-treatment, and then suppressed at the late time points, 2 and 7 days post-treatment (Figure S4). To investigate this phenomenon more closely, we identified the 34 genes with a similar expression pattern (Figure S4). Most of these show suppression at the 24 hour time point already. For 8/34, the function is not known and their only ontological category is that they are regulated by IFN-γ. For the remainder, careful individual analysis showed that many are known to be regulated by IFN (MX1, MAD2L1, IRS1, IGFBP2, ANGPT1, IFI27, STAT1, and TRIM22), whereas others (e.g., C1D, POLA, PLOD2, LRMP, GPR19, LSM6, BST2, SEMA3C, LAMP3, POLR3F, SLC26A1, and SLC16A5) were not known to be IFN-regulated; we predict that the regulation by IFN-γ will be established for these genes as well. These genes identify a parallel between the OsM and IFN signaling. Curiously, two proteins associated with melanocytes are in this category, TYRP1 and SILV (Figure S4). The significance of this phenomenon is at present unknown, but it may reflect the presence of a small number of melanocytes in our cultures. In contrast, the few genes suppressed early and induced late by OsM are not known to be regulated by IFN. The only exception is heat-shock protein A1B, which was shown to be suppressed by IFN-γ (Saile *et al.*, 2004). The reciprocal regulation of this gene reinforces the connection between the OsM and IFN signaling pathways.

To cross-reference the OsM-regulated genes with genes regulated under other circumstances, we used the L2L set of comparison programs. The lists of OsM-regulated genes were compared with the gene ontology categories of Biological Process, Molecular function, and Cellular Component, as well as with a set of lists of regulated genes in the L2L database (Figure S5). These analyses can lead to understanding of the biological processes and functions affected by OsM.

The biological processes induced by OsM at the 1 hour time point include genes responsible for the immune, inflammatory, and defensive responses to pathogens and stress (Figure S5). Similar biological processes are induced by OsM when all time points are analyzed in aggregate, with the addition of the genes associated with the responses to wounding and with chemotaxis. Interestingly, OsM suppressed the genes that belong to the NF-κB signal transduction at the 1 hour time point. At later time points, the biological processes concerning steroid and lipid metabolism are suppressed, these are differentiation-associated processes.

The genes induced by OsM at early, and suppressed at late time points also identify the associated biological processes with very high statistical probability, and these processes include JAK-STAT signaling cascade. These results further corroborate the nexus between the IFN signaling and OsM signaling.

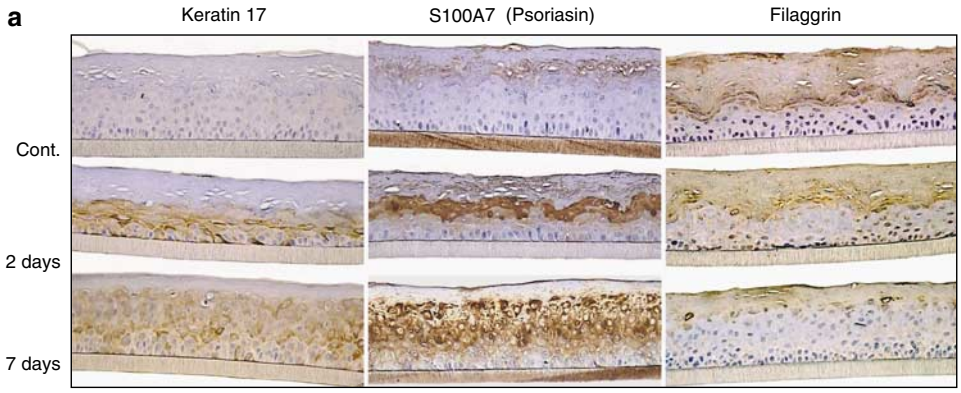
The molecular functions associated with the OsM-induced genes concern transcription and cytoskeletal proteins, while protein degradation seems suppressed especially at the early time points (Figure S5). Antiviral responses comprise the most certain molecular function of the induced/suppressed genes, paralleling the IFN-responsive molecular functions.

The cellular components induced by OsM include extracellular and intermediate filament-associated proteins, while the suppressed ones include junctional proteins, perhaps in conjunction with the increased taxis and motility of the OsM-treated cells (Figure S5).

We compared the lists of OsM-regulated genes with a large collection of lists of genes regulated by other agents (Newman and Weiner, 2005). Although the induced and suppressed genes did not reach high probability of relatedness to any gene list tested, the genes described in Figure S4, that is, those induced early and suppressed late, did achieve very high *P*-values (Figure S5). Specifically, the highest probability of relatedness, $P = 4 \times 10^{-22}$, was found between these genes and the genes induced by the ectopic expression of IRF3 (a.k.a. NF90) (Krasnoselskaya-Riz *et al.*, 2002). In addition, the IFN-induced and transforming growth factor-beta- and TNF-α-induced genes are significantly related to the up-then-down OsM-regulated genes (Figure S5).

To determine whether the OsM-regulated genes have an overabundance of a particular transcription factor binding site in the DNAs of their promoters, we used the oPOSSUM set of programs (Ho Sui *et al.*, 2005). We did not find a highly probable set of binding sites, and SPI-B and cEBP sites barely reached statistical significance for the set of all OsM-regulated genes, while Irf-1 barely reached statistical significance for the genes induced at 1 hour and 4 hours post-OsM treatment (Figure S6). We note that the most current version of oPOSSUM does not list STAT binding sites.

To verify the effects of OsM in skin equivalents, we focused on the induction of the S100 proteins by OsM because of their association with psoriasis. The quantitative real-time reverse transcription (RT)-PCR of S100A7 (psoriasin 1) and S100A12 (calgranulin C) mRNA shows time-dependent increase for both genes, confirming the microarray results described above (Figure 4). The increase of the psoriasin 1 mRNA during the OSM treatment kinetics was steady and smooth, whereas the calgranulin C mRNA was increased to extraordinarily high levels after 7 days of the OSM treatment. In both cases, the 7 days OSM treatment led to the strongest mRNA induction: 40.6 times for psoriasin 1 and 46.8 times for calgranulin C. The induction was confirmed at the protein level, using Western blotting (Figure 4). Whereas phenotypically, the treated and the untreated samples appeared very similar, OsM strongly induced the expression of S100A7 in the treated samples (Figure 4). Importantly, while S100A7 was induced to a high level in the granular layer, the induction of keratin 17 begins, and seems the strongest in the spinous layer. These data not only confirm



b

Fold induction	S100A7-JID	S100A12
Control	1	1
4 hours	1.2	1.3
24 hours	7.5	2.2
48 hours	14.1	4.3
7 days	40.6	46.8

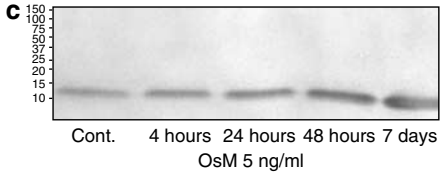


Figure 4. S100A7 is induced by OsM in skin equivalents. (**a**) Immunohistological detection of S100A7 (psoriasin) and keratin 17 in skin equivalents after the treatment with OsM. The expression of filaggrin is reduced (original magnification × 200). (**b**) RT-PCR showing S100A7 and S100A12 mRNAs are induced by OsM in skin equivalents. The numbers show fold increase in the OsM-treated tissue, over the untreated controls. (**c**) Western blots confirm the induction of S100A7 by OsM at the protein level.

our findings in microarray and RT-PCR analysis at the protein level but also demonstrate that the induction of the epidermal genes in skin equivalents occurs in the appropriate layer of the epidermis.

DISCUSSION

The results presented here demonstrate that, indeed, in the complex orchestration of the psoriasiform phenotype, the OsM strikes a specialized, important, and unique note. Specifically, OsM inhibits the expression of ''classical'' epidermal differentiation markers, while simultaneously inducing the expression of psoriasin and other S100A calcium-binding signaling proteins. In keratinocytes, OsM primarily affect proteolysis, the differentiation process, the cytoskeleton, and the adhesion structures. The effects of OsM are thus different from the effects of IFN-γ, which primarily stimulates an antiviral state, induces production of cytokines and chemokines and inhibits keratinocyte proliferation. They are also different from the effects of TNF-α, which also induces the expression of secreted signaling proteins, induces apoptotic proteins, and cytoskeletal components associated with increased motility. Furthermore, we show that the skin equivalents, with their three-dimensional architecture and faithful reproduction of epidermal differentiation process, represent an excellent model to study the psoriasiform changes caused by OsM and, presumably, other growth factors and cytokines.

OsM seems to have both proinflammatory and anti-inflammatory properties in skin equivalents. The proinflammatory effects of OsM are stimulated in part through the induction of the S100A proteins, which can act as secreted signaling messengers in inflammation, in tumors and other pathological conditions (Roth *et al.*, 2003; Cross *et al.*, 2005; Hermani *et al.*, 2006). We were particularly impressed with the extremely high induction of the S100A proteins by OsM; the over 100-fold induction of any gene by any extracellular agent in keratinocytes is, to our knowledge, unprecedented. We note that the values in Figure 3 are on log2 scale; the actual maximum fold induction for S100A7 and S100A12 are 144- and 137-fold, respectively. Because the induction of S100A8 and S100A9 pair was found to be among the earliest molecular changes in an animal model resembling psoriatic eruptions (Zenz *et al.*, 2005), and because OsM causes such drastic induction of the S100A proteins, it is possible that OsM plays a much more important role in the induction of S100A proteins in the psoriatic phenotype than IL-1 and TNF-α.

The S100A proteins activate the NF-κB and mitogen-activated protein kinase pathways; they are chemotactic and proinflammatory, and play a role in angiogenesis, and squamous and basal cell carcinomas (Roth *et al.*, 2003; Semov *et al.*, 2005; Hermani *et al.*, 2006). The S100A proteins are encoded at the epidermal differentiation complex, on chromosome 1q21; they have been found incorporated into cornified envelopes and associated with epidermal differentiation (Robinson *et al.*, 1997). The expression of S100A genes is associated with other processes besides epidermal differentiation; they serve different functions and have a different regulatory set of influences (Broome *et al.*, 2003; Eckert *et al.*, 2004; Cross *et al.*, 2005; Jackson *et al.*, 2005). Thus, the borders of the epidermal differentiation locus proper may not include the S100A genes. However, the epidermal differentiation-specific regulatory circuits of epidermal differentiation complex may influence the neighboring genes (Elder and Zhao, 2002). The S100A genes may

use such circuits in the epidermis to limit their expression to the differentiating layers.

The anti-inflammatory effects of OsM include the suppression of Th1-type signaling molecules and induction of IL-4, a Th2-type cytokine. Psoriasis is a distinctly Th1-associated disease (Lew *et al.*, 2004) and by inducing a Th2-type cytokine OsM may be acting to inhibit the Th1 psoriatic processes. Furthermore, OsM suppresses the expression of STAT1, transcription factor specifically responsive to IFN-γ, a Th1-type cytokine, while inducing the expression of STAT3, the OsM-responsive one. In addition, by suppressing IL-1 signals, OsM may be suppressing the proinflammatory psoriatic processes. The combined effects of OsM may reduce the severity of psoriasis, an interesting hypothesis that deserves further exploration.

Particularly intriguing is the discovery of the large set of genes that are induced at the early but suppressed at the later time points after the OsM treatment. Although the differences between the early and late effects may be due to the changes inherent in organotypic culture, it is more likely that these represent a nexus between the IFN-γ and the OsM signaling pathways. The nexus may be owing to a partial overlap of the STAT1- and STAT3-targeted genes. However, the genes with this particular regulatory pattern also overlap a set of genes induced by NF90, a double-stranded RNA-binding protein that mediates a subset of IFN-γ responses (Krasnoselskaya-Riz *et al.*, 2002). Therefore, OsM may have an intriguing and so far unexplored role in viral infections; this contention is strengthened by the known association of OsM with Kaposi's sarcomas (Nair *et al.*, 1992).

Importantly, while the analysis of the keratinocytes cultured in monolayers yielded a trove of information regarding the molecular and biological effects of OsMs, it is clear that certain important aspects of OsM biology in the epidermis could not be seen in the undifferentiating cultures. Therefore, skin equivalents, such as the ones used here, represent a major improvement in our ability to reproduce *in vitro* a fuller spectrum of *in vivo* epidermal responses to external influences.

MATERIALS AND METHODS

Production and maintenance of skin equivalents

The reconstituted human epidermis (SkinEthic Laboratories, Nice, France) consists of a fully differentiated three-dimensional multilayered keratinocyte structure grown on the air–liquid interface at 37°C, 5% CO_2, and saturated humidity for 19 days on modified chemically defined medium (MCDB) 153 supplemented with 1 ng/ml EGF, 0.4 μg/ml hydrocortisone, and 5 μg/ml insulin (growth medium) (Rosdy and Clauss, 1990; Rosdy *et al.*, 1997; Bernard *et al.*, 2000). Samples for RT-PCR, Western blots, and histology sections and staining originated from several different skin donors. All cells were expanded and treated according to the SkinEthic quality-controlled production procedures.

OsM tissue treatment

Growth medium was supplemented with 5 ng/ml OsM (R&D Systems, Abingdon, UK) for 1 hour, 4, 24, and 48 hours, and 7 days before harvesting. Note that the 24, 48, and 72 hours samples were treated on days 18, 17, and 12 of culture, respectively. At day 19, all tissues were harvested for microarray analysis, immunohistochemical studies, Western blotting, and quantitative real-time RT-PCR analysis. We chose this treatment because it allowed us to harvest all samples on the same day, that is, at the exactly the same stage of growth, differentiation, and cornification. The untreated tissues were used as negative controls.

Preparation and hybridization of probes

The inserts were placed on 4 ml dispase II (Roche, Meylan, France) in a 100 mm Petri dish at room temperature. After 3 minutes, the epidermal tissues were removed from the polycarbonate filter inserts with fine RNase-free forceps. RNA was isolated using the Qiagen kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. Approximately 8 μg of total RNA was reverse-transcribed, amplified, and labeled as described (Li *et al.*, 2001;Banno *et al.*, 2004). The labeled cRNA was hybridized to HGU95Av2 arrays (Affymetrix, Santa Clara, CA) with the capacity to display transcript levels of approximately 12,000 human genes. Arrays were washed, stained with anti-biotin streptavidin–phycoerythrin-labeled antibody, and scanned using the Gene Array Scanner system (Hewlett-Packard, Palo Alto, CA), as described by Affymetrix.

Gene array data analysis

Intensity values were obtained using GCOS Microarray Suite version 5.0. (Affymetrix), and normalized by setting the overall signal for each array to 500 arbitrary units. To eliminate genes not expressed in keratinocytes, or genes expressed at such low levels that their measurements were unreliable, we first determined the signal intensity value that is 1 SD above average for all genes scored as ''absent'' in the samples, specifically 150 U. We removed from analysis all genes with expression levels below 150 in every sample and genes not scored as ''present'' in at least one of the 10 samples. Affymetrix' GCOS programs was used to derive scatter-plots, perform the *t*-test and the Mann–Whitney test, and calculate fold change in preliminary data analyses.

Genes were considered regulated if their expression levels differed 2-fold or more between control and treated samples, or if they were deemed increased or decreased according to the GCOS criteria. To reduce the number of false positives, we used very strict selection criteria and only retained for analysis the genes that are regulated in the same direction in at least two consecutive time points. As a result, the consecutive time points serve as pseudo-duplicates. The regulated genes were functionally classified according to an extensive gene annotation table describing the molecular function and biological category of the genes present on the chip. The table is based on the Gene Ontology Consortium http://cgap.nci.nih.gov/Genes/GOBrowser.

The list of Ch1q21 genes in the epidermal differentiation locus was obtained from National Center for Biological Information Map Viewer http://www.ncbi.nlm.nih.gov/mapview. To compare the lists of OsM-regulated genes with lists of gene ontology annotated genes, we used the L2L microarray analysis tool, http://depts.washington.edu/l2l (Newman and Weiner, 2005). We used the 2005-06-15 version. The transcription factor binding sites were searched using oPOSSUM tool http://www.cisreg.ca/cgi-bin/oPOSSUM/opossum (Ho Sui *et al.*, 2005).

The data are deposited with the GEO, http://www.ncbi.nlm.nih.gov/geo/, under the access number GSE2822.

Quantitative real-time RT-PCR analysis

After dispase treatment (see above), total RNAs were extracted from three epidermal tissues using Trizol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, and then treated with Turbo Deoxyribonuclease I (Turbo DNA-free, Ambion, Austin, TX). Purified RNAs (1.5 μg) were incubated in a solution containing 50 ng of random hexanucleotides, 500 ng Oligo$(dT)_{12-18}$ and 200 U of SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) in a final volume of 20 μl at 42°C, for 2 hours to synthesize the cDNAs. Two units of *E. coli* RNAse H (Invitrogen, Carlsbad, CA) were added, and samples were incubated 20 minutes at 37°C.

S100A7 and S100A12 expression analysis was performed by quantitative real-time PCR procedure using an ABY Prism 7700 real-time PCR machine (Perkin-Elmer, Boston, MA). The primer sequences were as follows: S100A7-JID, GenBank NM002963, FP: 5′-CCCAACTTCCTTAGTGCCTGTG, RP: 5′-AAGACGTCGGCGAG GTAATTT (Ebihara *et al.*, 2005); S100A7-EJP, FP: 5′-CTTCCTTAGTG CCTGTGACAAAAA; RP: 5′-AAGGACAGAAACTCAGAAAAATCAA TCT (Franssen *et al.*, 2005); S100A12, GenBank NM005621, FP: 5′-CACTGCTGGCTTTTTGCTGTAG, RP: 5′-TTAACCCCTCAATGCAC AGGA (Ebihara *et al.*, 2005); and TBP, FP: 5′-ACCCTTCACCAATGA CTCCTATG, RP: 5′-ATGATGACTGCAGCAAATCGC (Bezy *et al.*, 2005). The optimal primer concentration (300, 600, 900 nM) for A7 and A12 genes was chosen to achieve maximum amplification and minimum nonspecific amplification. All primers were obtained from Eurogentec SA (Seraing, Belgium). The real-time PCR assays were performed using 1.33 μl of first-strand cDNA as template and 300 nM of paired primers. Reactions were performed according to the manufacturer's instructions by using SYBR green PCR Master Mix (Perkin-Elmer, Boston, MA). The expression of S100 calcium-binding protein A7 and A12 was normalized to the expression to TBP.

Immunohistology

Epidermal tissues were fixed in 10% formalin neutral buffered solution and embedded in paraffin. Four micrometer vertical sections were stained with hematoxylin/eosin for standard histology. For immunohistology, antigen retrieval was performed on de-paraffinized sections by cooking in citrate buffer (pH 6.0). Then, sections were incubated with primary monoclonal antibodies for psoriasin (psoriasin/HID-5 clone 47C1068, Imgenex, San Diego, CA) or keratin 17 (Keratin 17 clone E3), filaggrin (clone FLG01, Neomarkers, Fremont, CA), for 1 hour at room temperature. Subsequently, the sections were stained with horseradish peroxidase-conjugated secondary antibody for psoriasin, whereas for keratin 17 the sections were stained with Ultravision LP System (HRP polymer, Neomarkers, Freemont, CA), visualized with 3′-diaminobenzodine tetrahydrochloride chromogen, and counterstained with hematoxylin.

For immunofluorescence, epidermal cultures were frozen directly at −80°C. Before being cut, they were embedded in octamer motif-binding compound (Miles, Elkhart, IN). Vertical sections were air-dried, rinsed in phosphate-buffered saline and incubated with a primary monoclonal antibody for keratin 17 clone E3, (Dako, Denmark), then with a fluorescent secondary antibody (Dako, Denmark) and counterstained with a propidium iodide solution (50 μg/ml) (Sigma, St Louis, MO).

Western blotting

After dispase treatment (as above), three epidermal tissues of 0.5 $cm^{2.}$ for each time point or control were transferred to a 1.5 ml microtube containing 1 ml lysis buffer (20 mM Tris (pH 7.4), 150 mM NaCl, 1 ml EDTA 1% NP-40, and 1% protease inhibitor cocktail). After 30 minutes of incubation on ice, the samples were sonicated and centrifuged at 10.000 × g for 10 minutes. Protein concentration was determined using Bradford Bio-Rad protein assay (Bio-Rad, Hercules, CA). Five micrograms of proteins were electrophoresed in 15% SDS-PAGE, and then transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA).

The membrane was blocked for 30 minutes with phosphate-buffered saline (pH 7.2) solution, containing 0.05% Tween 20 and 5% low-fat milk, and then incubated overnight at 4°C with primary antibody (mAb to psoriasin, HID-5 clone 47C1068, Imgenex, San Diego, CA or polyclonal antibody to calgranulin C, C20, Santa Cruz Biotechnology, Santa Cruz, CA). After phosphate-buffered saline washing, the membrane was incubated at room temperature for 1 hour with horseradish peroxidase-conjugated secondary antibody for psoriasin (Sigma, St Louis, MO). Visualization was performed using 3′-diaminobenzodine tetrahydrochloride as the chromogen.

CONFLICT OF INTEREST

Drs Rosdy, Bertino, Tornier, Mas, and Sahuc are employed by Skinethic; the skin equivalents used in this study were produced by Skinethic and were provided grants for experimentation.

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SUPPLEMENTARY MATERIAL

Figure S1. OsM-regulated genes in skin equivalents.

Figure S2. Comparison of the regulated genes by OsM in skin equivalents and in monolayer cultures.

Figure S3. The epidermal differentiation complex on chromosome 1.

Figure S4. OsM-regulated genes induced at the early, and suppressed at the late time points.

Figure S5. Comparison of the lists of OsM-regulated genes with Gene Ontology categories and specific lists of genes identified in other systems.

Figure S6. Transcription factor binding sites in the promoters of the OsM-regulated genes.

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