Rosiglitazone Inhibits Proliferation, Motility, and Matrix Metalloproteinase Production in Keratinocytes

Narasimharao Bhagavathula,* Kamalakar C. Nerusu,* Ashish Lal,* Charles N. Ellis,† Amar Chittiboyina,‡ Mitchell A. Avery,‡ Christopher I. Ho,§ Stephen C. Benson,§ Harrihar A. Pershadsingh,§§¶ Theodore W. Kurtz,# and James Varani*

*Department of Pathology and †Department of Dermatology, University of Michigan Medical School, Ann Arbor, Michigan; ‡Department of Medicinal Chemistry, University of Mississippi, University, Mississippi; §Department Biological Sciences, California State University, Hayward, California; §§Departments of Family Medicine, Kern Medical Center, and University of California, Irvine, California; ¶Bethesda Pharmaceuticals, Inc., Bakersfield, California; and #Department of Laboratory Medicine, University of California, San Francisco, California, USA

This study was undertaken to evaluate the effects of thiazolidinediones (TZD) on keratinocyte proliferation, motility, and matrix metalloproteinase (MMP) production. Rosiglitazone (a potent TZD) inhibited both proliferation and motility as well as elaboration of MMP-1 and MMP-9. Inhibition was obtained with keratinocytes in monolayer culture and human skin in organ culture. There were significant concentration–response differences in sensitivity of the three keratinocyte responses to treatment with rosiglitazone. In contrast to keratinocytes, dermal fibroblasts were resistant to the effects of rosiglitazone. Treatment of keratinocytes with rosiglitazone did not suppress epidermal growth factor receptor autophosphorylation, but inhibited signaling through the extracellular regulated kinase mitogen-activated protein kinase pathway without a concomitant effect on pathways that lead to c-*jun* activation. Pioglitazone, another TZD, also suppressed keratinocyte proliferation, although it was less effective than rosiglitazone. An experimental TZD (BP-1107) inhibited keratinocyte proliferation at a much lower concentration than either rosiglitazone or pioglitazone. Because enhanced keratinocyte motility and increased MMP production as well as increased keratinocyte proliferation are thought to contribute to the phenotype of psoriatic lesional skin, we propose that interference with these keratinocyte responses contributes to the previously reported antipsoriatic activity of TZD.

Key words: hyperplasia/psoriasis/peroxisome proliferator-activated receptor-gamma (PPAR-gamma)/epidermal growth factor/thiazolinedione.
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The peroxisome proliferator-activated receptor-γ (PPAR-γ) and the two other PPAR isoforms (PPAR-α and PPAR-β) are known to be necessary for physiological epidermopoiesis (Rivier *et al*, 1998; Komuves *et al*, 2000; Rivier *et al*, 2000). Endogenous PPAR-γ ligands identified to date have low affinity and are fatty acid-like compounds such as prostanoid derivatives of archadonic acid (15-deoxy-$\Delta^{12,14}$PGJ2) and polyunsaturated fatty acids such as linoleic acid (Forman *et al*, 1995; Kliewer *et al*, 1995; Nagy *et al*, 1998). Synthetic agents known as thiazolidinediones (TZD) have been discovered to be high-affinity ligands for PPAR-γ, and two of these agents (rosiglitazone and pioglitazone) are currently approved for the treatment of type 2 diabetes (Henry, 1997; Olefsky, 2000). TZD have proven to be effective inhibitors of proliferation and promoters of differentiation in a number of malignant tumors (Kubota *et al*, 1998; Mueller *et al*, 1998; Sarraf *et al*, 1998), and recent studies indicate that psoriasis, a benign epidermal hyperplasia, is also amenable to treatment with TZD (Ellis *et al*, 2000; Malhotra *et al*, 2002).

How TZD function to ameliorate the psoriatic phenotype is not known. Given the ability of these agents to inhibit proliferation of malignant epithelial cells (Kubota *et al*, 1998; Mueller *et al*, 1998; Sarraf *et al*, 1998), we assessed the effects of these agents on keratinocyte proliferation as well as other keratinocyte functions that are abnormally expressed in psoriasis and thought to contribute to the pathophysiology of the disease (Sawa *et al*, 2002).

Abbreviations: EGF, epidermal growth factor; KBM, keratinocyte basal medium; KGM, keratinocyte growth medium; IL-1β, interleukin-1β; MAP, mitogen-activated protein; MMP, matrix metalloproteinase; PPAR, peroxisome proliferator-activated receptor; TZD, thiazolidinedione.

Results

Effects of rosiglitazone on proliferation of human epidermal keratinocytes in monolayer culture In the first series of experiments, rosiglitazone was examined for effects on keratinocyte proliferation in the presence or absence of exogenous EGF. Keratinocyte growth was observed under both conditions, but (as expected) the increase was less in the absence of exogenous EGF than when EGF was present (6.6-fold increase in KBM over a 4-d

period vs. 8.9-fold increase in EGF-supplemented KBM). In spite of this difference, rosiglitazone suppressed keratinocyte proliferation in basal medium as effectively as in the presence of exogenous EGF. Under both conditions, inhibition was time-dependent. Minimal (not statistically significant) inhibition of growth was observed 1 d after treatment. Statistically significant differences between control and rosiglitazone-treated cells were seen on days 2, 3, and 4 (Fig 1*A,B*). Inhibition of keratinocyte proliferation was concentration-responsive between 4 and 40 μM. At 20 μM, 52% inhibition was observed in the absence of EGF and 56% inhibition was observed in the presence of EGF. At 40 μM, approximately 70% inhibition was achieved under both conditions (Fig 1*C*). Although 5×10^4 cells per well were normally utilized in proliferation assays, experiments were conducted in which cells were plated at 1×10^4, 2×10^4, 3×10^4, and 4×10^4 cells per well and examined for sensitivity to rosiglitazone. Comparable inhibition was seen at all starting cell concentrations (data not shown). Additional studies showed that inhibition of keratinocyte proliferation by rosiglitazone was reversible. When keratinocytes were treated for 2 d with 20 μM rosiglitazone and then washed thoroughly to remove the agent, proliferation resumed. The growth response between days 2 and 4 in cells that had been treated with rosiglitazone was not significantly different from that of control cells (Fig 1*D*). Similar results to those shown here were observed with adult skin keratinocytes and with the HaCaT line of immortalized keratinocytes (data not shown).

Keratinocyte growth inhibition with rosiglitazone was not the result of toxicity. The lack of cytotoxicity was demonstrated using a ^{51}Cr-release assay in conjunction with a viable cell assay. In control cells, 28.4% of the incorporated ^{51}Cr was released whereas in cells treated with 20 μM rosiglitazone, the released ^{51}Cr was 30.1% of the total. In the viable cell assay based on replating efficiency, 78% of the control cells and 77% of the rosiglitazone-treated cells were viable. Differences between control and treated cells were not statistically different in either assay.

In contrast to its effects on keratinocyte proliferation, rosiglitazone concentrations as high as 40 μM did not significantly affect human dermal fibroblast growth (Fig 2).

Effects of rosiglitazone on epidermal thickness of human skin in organ culture Previous studies showed that human skin could be maintained in organ culture for several days. Under serum-free, growth factor-free conditions, normal histologic structure and biochemical function were preserved (Varani *et al*, 1993; Varani *et al*, 1994). Nevertheless, in the presence of EGF (10 ng/mL), epidermal keratinocytes underwent a proliferative response (Fligiel and Varani, 1993). This was accompanied by erosion of the basement membrane and down-growth of cells into the dermal space. Abnormal epidermal differentiation, with hyperkeratosis and acantholysis, was also prevalent in growth factor-treated skin. Histologic features of growth factor-treated normal skin were very similar to the histologic features of psoriatic skin maintained in the absence of exogenous growth factors (Varani *et al*, 1998). Figure 3 demonstrates the effects of rosiglitazone (20 μM) on epidermal structure of EGF-treated skin. In the presence of EGF (but without rosiglitazone), epidermal thickness was increased by 23% (from approximately 30 ± 12 μm to 37 ± 18 μm; $n = 9$). In the presence of EGF and rosiglitazone, there was a 50% reduction in epidermal thickness (from 32 ± 15 μm to 16 ± 5 μm; $n = 8$). These differences are consistent with what we have reported previously for induction of epidermal hyperplasia with all-*trans*-retinoic acid and inhibition with an EGF receptor antagonist (Varani *et al*, 2001). The reduction in epidermal thickness was accompanied by a corresponding (partial) reduction in the abnormal features of differentiation (acanthosis and hyperkeratosis). Concentrations of rosiglitazone that reduced epidermal thickness in organ culture (10–40 μM) were

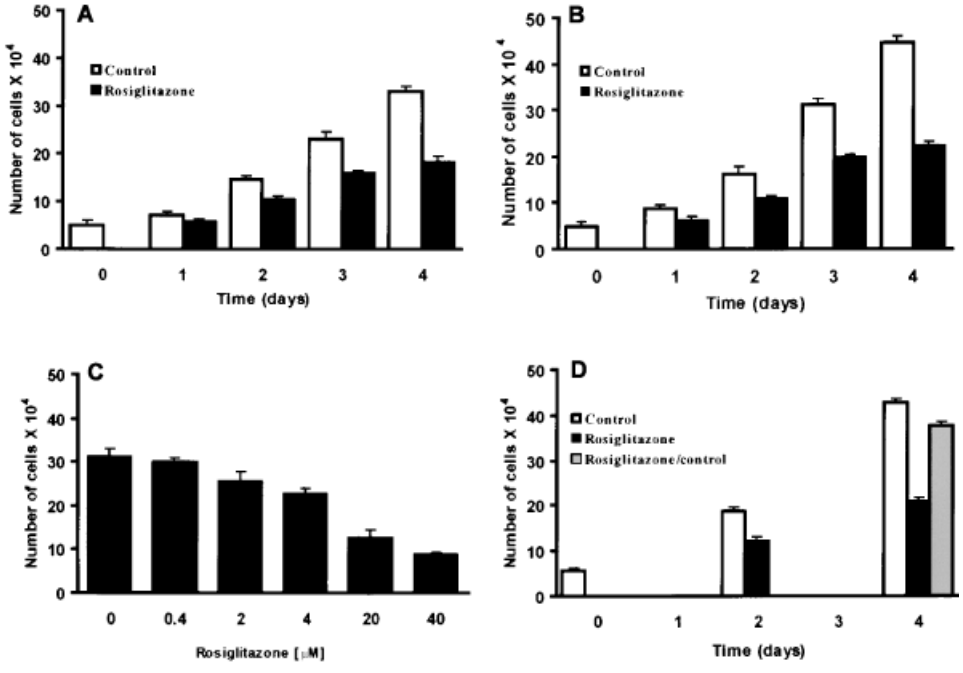

Figure 1
Effects of rosiglitazone on keratinocyte proliferation in monolayer culture. (*A,B*) Time-dependent inhibition of keratinocyte proliferation. Keratinocytes were incubated in KBM or EGF-supplemented KBM and left as control or treated with 20 μM rosiglitazone on day 0. Cell counts were taken at the indicated time points. (*C*) Concentration-dependent inhibition of keratinocyte proliferation. Keratinocytes were incubated with different concentrations of rosiglitazone and cell proliferation was assessed on day 4. (*D*) Reversal of growth inhibition. Cells were left as control or treated with 20 μM rosiglitazone for 2 d. At the end of day 2, rosiglitazone-treated cells were washed thoroughly, and fresh medium with vehicle was added. Cell counts were taken on day 4. Bars and error bars represent means and SEM of six independent experiments performed in quadruplicate. In *A* and *B*, rosiglitazone-treated cells were statistically different from control cells on days 3 and 4. In *C*, rosiglitazone-treated cells were statistically different from the control cells at 20 and 40 μM. In *D*, cells treated with rosiglitazone for 2 d and then washed were statistically different from cells treated for 4 d but were not different from controls.

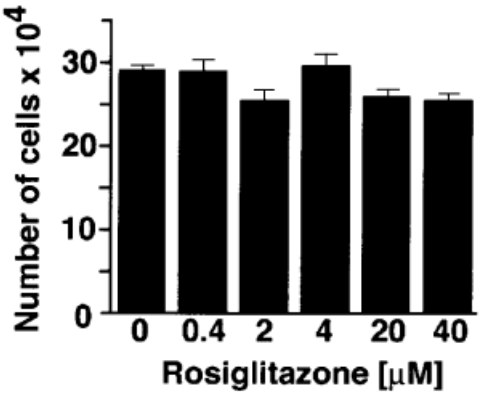

Figure 2
Effects of rosiglitazone on fibroblast proliferation in monolayer culture. Primary human dermal fibroblasts were incubated with different concentrations of rosiglitazone and cell proliferation was assessed on day 4. Bars and error bars represent means ± SEM based on three independent experiments.

similar to the concentrations that inhibited keratinocyte proliferation in monolayer culture. Rosiglitazone treatment had no measurable effect of histologic features of control skin maintained in the absence of exogenous growth factor (Fig 3).

Effect of rosiglitazone on keratinocyte motility EGF-induced epithelial cell proliferation is associated with an induction of motility (Barrandon and Green, 1987). It was of interest therefore to determine if rosiglitazone inhibition of keratinocyte proliferation would be accompanied by inhibition of motility. As seen in Fig 4, keratinocyte migration in response to EGF was inhibited by rosiglitazone in a concentration-dependent manner. Approximately 85% inhibition was observed at a 40 μM concentration. Even at a concentration as low as 0.1 μM, 25% inhibition was still achieved. Thus, keratinocyte motility appears to be even more sensitive to inhibition by rosiglitazone than is proliferation. In the absence of exogenous growth factors (i.e., in serum-free, growth factor-free KBM), there was essentially no keratinocyte motility (<10 μm on day 2). The presence or absence of rosiglitazone had no effect on this (data not shown).

Inhibition of MMP-1 and MMP-9 production by rosiglitazone Previous studies have demonstrated that the same concentrations of EGF that induce epidermal thickening in organ culture and epidermal keratinocyte proliferation/motility in monolayer culture also stimulates MMP production (Zeigler *et al*, 1996a, 1996b). Because rosiglitazone inhibited both proliferation and migration of keratinocytes, it was of interest to determine whether MMP elaboration would also be inhibited. Human skin organ cultures and keratinocyte monolayer cultures were treated for 3 d with EGF and different concentrations of rosiglitazone. At the end of the incubation period, culture fluids were assessed for MMP-1 by western blotting and MMP-9 by gelatin zymography. As seen in Figs 5 and 6 and in Table I, rosiglitazone inhibited production of both MMP-1 and MMP-9. Inhibition was observed at 40 and 20 μM concentrations, but not at lower doses. The degree of MMP inhibition was much greater in organ culture than in keratinocyte monolayer culture. With MMP-9, there was essentially no inhibition in cell culture. Of interest in regard to MMP suppression, both the active and the latent forms of the enzymes were reduced in the presence of rosiglitazone. This strongly suggests that enzyme production rather than activation was inhibited.

Effects of rosiglitazone on intracellular signaling events that underlie proliferation, motility, and MMP production in keratinocytes Previous studies have demonstrated the importance of mitogen-activated protein (MAP) kinase signaling and, in particular, signaling through the ERK pathway, to EGF-induced biologic events in keratinocytes (Zeigler *et al*, 1999). To determine whether the inhibitory effects of rosiglitazone on keratinocyte function could be

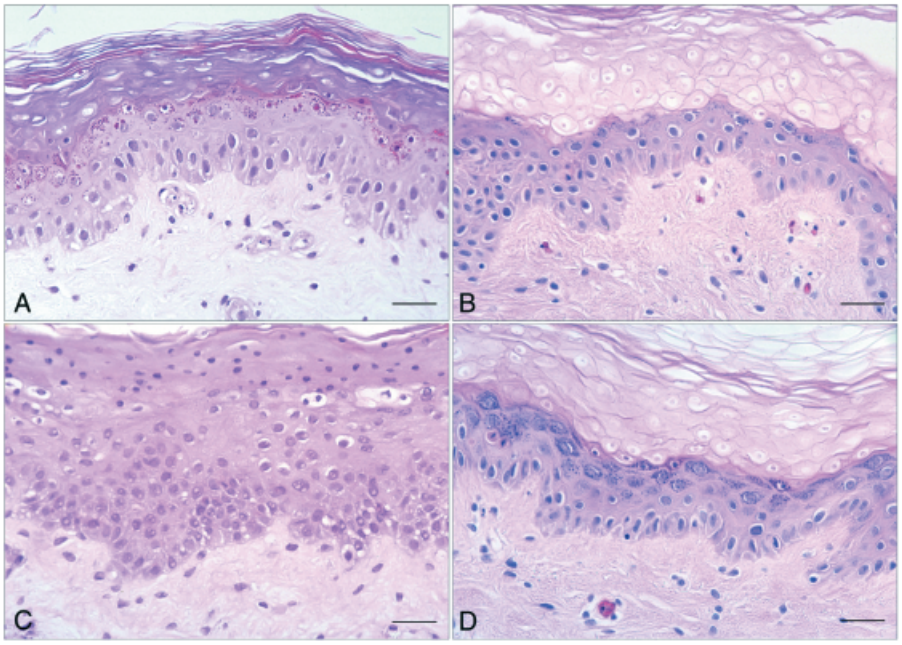

Figure 3
Effects of rosiglitazone on histologic structure of human skin in organ culture. Histologic appearance of normal skin after 8 d in organ culture. (*A*) KBM + Ca^{2+}; (*B*) KBM + Ca^{2+} and 20 μM rosiglitazone; (*C*) 10 ng per mL EGF; (*D*) 10 ng/mL EGF and 20 μM rosiglitazone. The appearance of the skin sections shown here was representative of results obtained with tissue from seven different donors (*bar*, 8.75 μm).

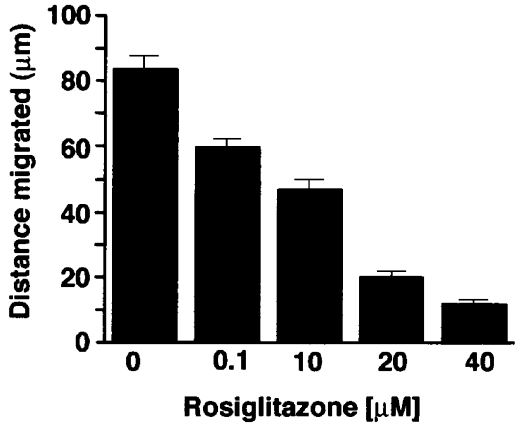

Figure 4
Effects of rosiglitazone on keratinocyte motility. Keratinocytes were treated with vehicle or different concentrations of rosiglitazone, and motility was assessed after 48 h. Data are expressed as distance migrated and represent means ± SEM from four independent experiments. Rosiglitazone-treated cells were statistically different from the controls at 0.1 to 40 μM.

related to interference with MAP kinase signaling, we assessed the effects of rosiglitazone on ERK phosphorylation, as well as on upstream and downstream events (i.e., EGF receptor phosphorylation and cyclin D protein levels, respectively) in EGF-stimulated keratinocytes. Figure 7*A* shows the effects of rosiglitazone (20 μM) on autophosphorylation of the EGF receptor at two time points after EGF stimulation. There was no measurable effect. Note that in addition to assessing EGF receptor phosphorylation, we also used antibodies to EGF receptor protein to demonstrate equivalent amounts of total EGF receptor in the blots (Fig 7*A*).

The effects of rosiglitazone on ERK phosphorylation in EGF-treated keratinocytes are demonstrated in Fig 7*B*. In contrast to the complete lack of effect on EGF receptor phosphorylation, ERK phosphorylation was inhibited in the presence of rosiglitazone. Inhibition was seen as early as 15 min after EGF stimulation and extended through the 4-d incubation period. The effects of U0126, a potent inhibitor of ERK phosphorylation, is shown for comparison in the same cells. U0126 treatment resulted in a complete lack of ERK phosphorylation whereas inhibition in the presence of rosiglitazone was partial.

We next examined the effects of rosiglitazone on cyclin D1 expression. At early time points (i.e., up to 6 h) there was no measurable effect. By 1 d, however, a detectable decrease in cyclin D1 protein level occurred, and by 2 d, the decrease was substantial (Fig 7*C*). The effects of U0126 on cyclin D1 expression were also assessed. As expected, cyclin D1 was completely inhibited in the presence of U0126 (not shown).

In a final set of experiments, we examined the ability of rosiglitazone to reduce c-*jun* phosphorylation. In contrast to what was observed in regard to ERK, treatment with rosiglitazone produced essentially no change in the amount of phosphorylated c-*jun* seen in keratinocytes over a 6-h

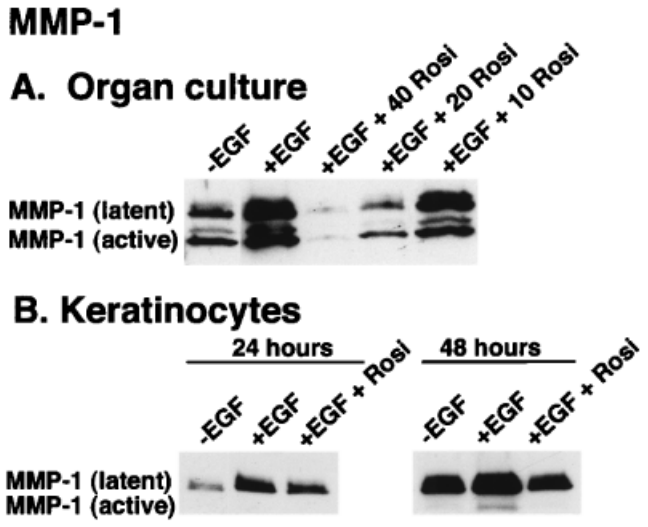

Figure 5
Effects of rosiglitazone on MMP-1 elaboration in organ cultured skin and keratinocytes. (*A*) Western blot immunoreactivity of organ culture fluid with antibody to MMP-1. Adult human skin was maintained under control conditions or treated with 10 ng per mL EGF and different concentrations of rosiglitazone (*Rosi*). Organ culture fluid was collected on day 3 and assayed. (*B*) Western blot immunoreactivity of conditioned medium from neonatal keratinocytes. Human neonatal keratinocytes were maintained under control conditions or treated with 10 ng per mL EGF with or without 20 μM rosiglitazone. Conditioned medium was collected at 24 and 48 h and assayed. The blots presented in the figure are representative of results obtained with tissue from seven different donors and results obtained in three replicate cell culture experiments.

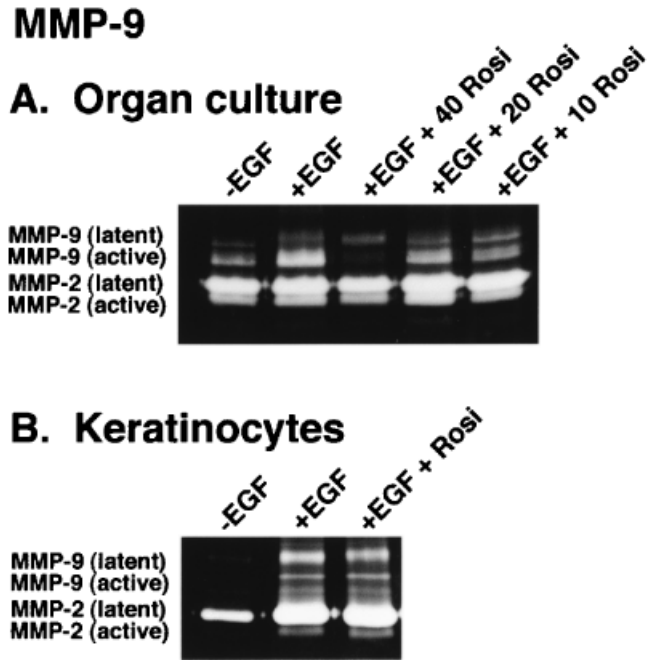

Figure 6
Effects of rosiglitazone on MMP-9 elaboration in organ-cultured skin and keratinocytes. (*A*) Assessment of MMP-9 in of organ culture fluid by gelatin zymography. Adult human skin was maintained under control conditions or treated with 10 ng per mL EGF and different concentrations of rosiglitazone (*Rosi*). Organ culture fluid was collected on day 3 and assayed. (*B*) Assessment of MMP-9 in conditioned medium from keratinocytes by gelatin zymography. Human neonatal keratinocytes were maintained under control conditions or treated with 10 ng per mL EGF with or without 20 μM rosiglitazone. Conditioned medium was collected at 48 h and assayed. The blots presented in the figure are representative of results obtained with tissue from seven different donors and results obtained in three replicate cell culture experiments.

Table I. Suppression of MMP production by rosiglitazone[a]

		Percentage of inhibition		
Group	**Rosiglitazone concentration (μM)**	**MMP-1**	**MMP-2**	**MMP-9**
Organ culture	40	92 ± 5	13 ± 10	45 ± 19
	20	72 ± 6	<5	40 ± 15
	10	30 ± 8	<5	43 ± 8
Cell culture	20	49 ± 6	<5	<5

[a]Zymograms were scanned. Negative images of the zones of hydrolysis were digitized, and percentage of inhibition was determined using the formula %Inhibition = $1 - EGF^{(+)}$ and $Rosi - EGF^{(-)}/EGF^{(+)} - EGF^{(-)} \times 100$. Values shown are means ± SEM based on triplicate experiments.

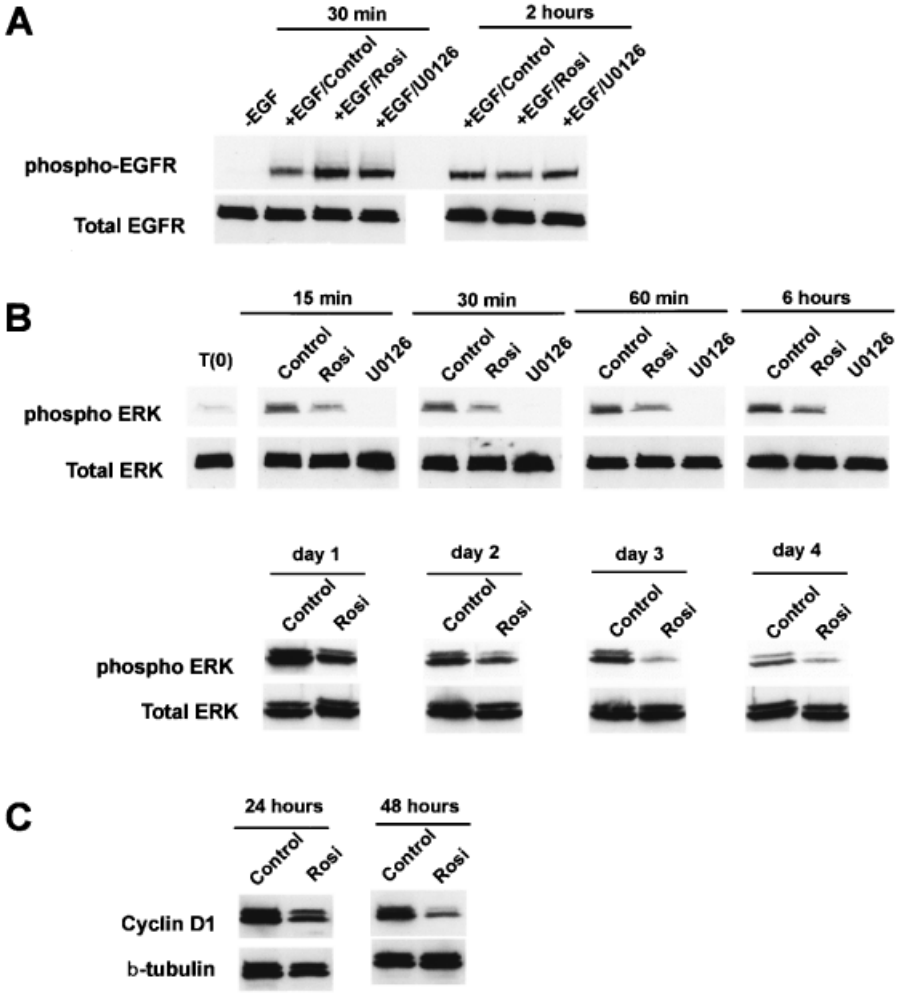

Figure 7
Effects of rosiglitazone on ERK pathway signaling. (*A*) Phosphorylation of EGF receptor. Keratinocytes were left as control or stimulated with 10 ng per mL EGF. EGF-treated cells were incubated alone or treated with 20 μM rosiglitazone (*Rosi*) or 10 μM U0126. Cell lysates were prepared at the indicated time points. Forty micrograms of lysate protein was resolved on 10% SDS-PAGE and probed for the phosphorylated form of EGF receptor with a phospho-specific anti-EGF receptor antibody. The results are representative of three independent experiments. When the same lanes were probed with an antibody to the EGF receptor protein, there were no differences. (*B*) Phosphorylation of ERK. Keratinocytes were stimulated with EGF (10 ng/mL) and incubated alone or treated with 20 μM rosiglitazone or 10 μM U0126. Cell lysates were prepared at the indicated time points. Forty micrograms of lysate protein was resolved on 10% SDS-PAGE and probed for phosphorylated ERK with a phospho-specific ERK antibody. The bottom panel shows the same blot stripped and reprobed with antibody recognizing total ERK protein. The results are representative of three independent experiments. (*C*) Cyclin D. Keratinocytes were stimulated with EGF (10 ng/mL) and incubated alone or treated with 20 μM rosiglitazone. Cell lysates were prepared at the indicated time points. Forty micrograms of lysate protein was resolved on 10% SDS-PAGE and probed for cyclin D1 protein. The results represent three independent experiments. When the same lanes were probed with an antibody to β-tubulin, there were no differences.

time period following EGF stimulation. Results from the 60-min time period are shown in Fig 8. Consistent with past reports (Zeigler *et al*, 1999), EGF itself did not induce a major change in c-*jun* phosphorylation in keratinocytes. Nevertheless, even when the potent inducer of c-*jun* activation, IL-1β, was used to stimulate c-*jun*, rosiglitazone had no effect (Fig 8). As part of the same experiments, we demonstrated equivalent amounts of total protein (western blotting for β-tubulin; not shown) and total c-*jun* (western blotting for c-*jun* protein; Fig 8) in the blots. Finally, it was demonstrated that rosiglitazone had no significant inhibitory effect on P38 MAP kinase (either phosphorylated P38 or total P38) when examined over a 6-h time-period following stimulation with EGF or IL-1β (data not shown).

Effects of two other TZD on proliferation of human epidermal keratinocytes and dermal fibroblasts in monolayer culture Two additional TZD (pioglitazone and BP-1107) were used in a final series of experiments. BP-1107 is an adamantyl TZD derivative that, based on results in a reporter-gene assay with CV-1 cells, is a potent PPAR-γ agonist (the EC_{50} of BP-1107 for PPAR-γ activation is 26 pM, approximately three orders of magnitude less than the EC_{50} of rosiglitazone (64 nM), which is the most potent TZD in clinical use). The EC_{50} value of pioglitazone for PPAR-γ activation has previously been reported to be in the range of 550 nM (Willson *et al*, 2000). The effects of these TZD on keratinocyte proliferation are shown in Fig 9. All three TZD inhibited keratinocyte proliferation in a concentration-

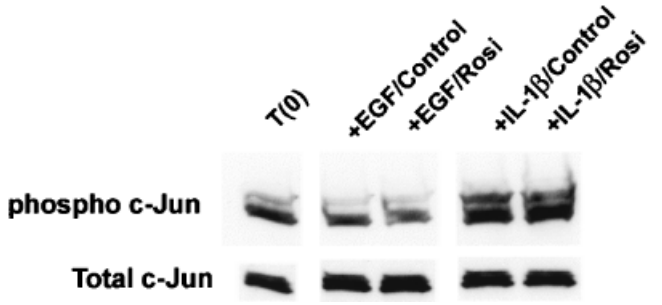

Figure 8
Effects of rosiglitazone on c-Jun. Keratinocytes were stimulated with either 10 ng per mL EGF or 1 ng per mL IL-1β and treated with 20 μM rosiglitazone (*Rosi*) for 60 min. Cell lysates were prepared, and 40 μg of lysate protein was probed for phosphorylated form of c-Jun. The results are representative of findings in three independent experiments. When the same lanes were probed with an antibody to total c-*jun* protein, there were no differences among the lanes.

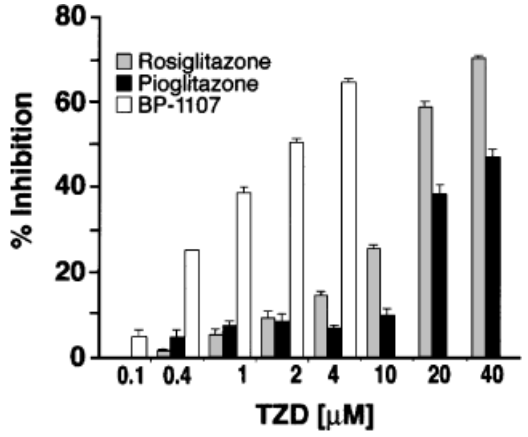

Figure 9
Comparison of pioglitazone and BP-1107 with rosiglitazone for effects on keratinocyte proliferation. Primary human keratinocytes were incubated with different concentrations of each reagent and cell proliferation assessed on day 4. Bars and error bars represent means ± SEM of three independent experiments performed in quadruplicate. Percentage of inhibition values were calculated using the formula $\%\text{Inhibition} = 1 - \text{Exp}_{t48} - \text{Ctrl}_{t0} / \text{Ctrl}_{t48} - \text{Ctrl}_{t0} \times 100$. Rosiglitazone-treated cells were statistically different from the controls at 10, 20, and 40 μM. Pioglitazone-treated cells were statistically different from the controls at 20 and 40 μM, and BP-1107-treated cells were statistically different from the controls at 0.4 μM and above. At 20 and 40 μM, BP-1107 was cytotoxic to keratinocytes.

dependent fashion. The rank order for the inhibitory effects of these compounds on keratinocyte proliferation was BP-1107 > rosiglitazone > pioglitazone. At the highest concentrations of BP-1107 (20 and 40 nM), keratinocyte cytotoxicity was observed. When fibroblast growth was examined, pioglitazone, like rosiglitazone, did not suppress growth at concentrations as high as 40 μM (the highest concentration examined). With BP-1107, fibroblast growth inhibition was seen at 20 and 40 μM (not shown).

Discussion

This study was undertaken to evaluate the effects of TZD on human epidermal keratinocyte functions (proliferation, motility, and MMP elaboration) in monolayer culture and in whole skin organ culture. All three functions were inhibited. Although there was no direct evidence from our studies that these PPAR-γ ligands suppressed keratinocyte functions via PPAR-γ activation, the presumption is that they did since (in the proliferation assay, at least) there was a direct relationship between inhibition of growth and rank order of potency for PPAR-γ activation. Although all three keratinocyte functions were inhibited, differences among the three responses were seen. Specifically, keratinocyte proliferation and MMP elaboration both occurred in the absence of exogenous growth factor stimulation and both responses were further stimulated when exogenous EGF (10 ng/mL) was added. In contrast, keratinocyte motility was only observed when exogenous EGF was included in the culture medium. Although proliferation and MMP production occurred in the absence of exogenous growth factors, we suggest that a sufficient amount of endogenously produced EGF receptor ligands were available to "drive" the responses. Keratinocytes are a source of several ligands for the EGF receptor, including transforming growth factor-α, amphiregulin, and heparin-binding EGF (Piepkorn *et al*, 2003), and past studies have shown that interference with EGF receptor function suppresses keratinocyte proliferation in basal medium (Pittelkow *et al*, 1993). Of interest, the virtually complete lack of a motility response in the absence of exogenous stimulation (i.e., under conditions that support proliferation and MMP production) suggest that motility is under more exquisite control than are the other two responses. The fact that rosiglitazone suppressed motility at concentrations too low to have an effect on proliferation or MMP production is consistent with this.

In keratinocytes, proliferation, motility, and MMP production can all be stimulated by EGF (as well as by other growth factors) (Zeigler *et al*, 1996a, 1996b) and follow from intracellular signaling through MAP kinase pathways (Zeigler *et al*, 1999). The disparity among the three responses in regard to concentrations of rosiglitazone needed for inhibition provides possible insight into mechanisms of TZD inhibition. Past studies have shown that motility (like proliferation) is stimulated in keratinocytes primarily through EGF receptor activation (Barrandon and Green, 1987) with signaling through the ERK MAP kinase pathway (Zeigler *et al*, 1999). The mitogenic and motogenic signals are believed to be on a common pathway leading to MAP kinase activation. The pathways may diverge, however, based on the ability of ERK to translocate to the nucleus (Gonzalez *et al*, 1993; Chen *et al*, 1994) and/or localize in the cytoplasm with cytoskeletal elements (Gonzalez *et al*, 1993; Reszka *et al*, 1995). Thus, growth factors may lead to direct activation of the intracellular motility machinery independent of (or in addition to) *de novo* gene transcription (Klemke *et al*, 1997). Mitogenic responses, on the other hand, depend on translocation to the nucleus and new gene transcription (Khokhlatchev *et al*, 1998; Brunet *et al*, 1999). Suppression of cytoplasmic ERK function by rosiglitazone may be more readily achieved.

In contrast to both proliferation and motility (which can be mediated through ERK activation, independent of signaling through other MAP kinase pathways (Zeigler *et al*, 1999; Moon *et al*, 2002)), transcription of MMP genes appears to require c-*jun* activation as well (Lim *et al*, 1998;

Reunanen *et al*, 1998; Westermarck *et al*, 1998; Zeigler *et al*, 1999; Brauchle *et al*, 2000; Moon *et al*, 2002). Formation of the activation protein-1 complex, which serves as a transcription factor for a number of MMP genes (including MMP-1 and MMP-9), consists of c-*jun* homodimers or heterodimers of c-*fos*, c-*jun*, and ATF2 (Karin *et al*, 1997; Whisler *et al*, 1997). Ligands that act through a number of different receptors (e.g., tumor necrosis factor-α and IL-1β) as well as certain non-receptor-mediated stimuli (ultraviolet irradiation, oxidant stress) are capable of activating the JNK or P38 signaling pathways, leading to c-*jun* activation (Fisher *et al*, 1998; Chung *et al*, 2000; Moon *et al*, 2002). In ultraviolet-induced MMP production, activation of c-*jun* appears to be rate-limiting (Fisher *et al*, 1998). Our finding that rosiglitazone was less effective in inhibiting MMP elaboration than in inhibiting proliferation or motility is consistent with the finding that TZD treatment did not appear to have a significant impact on c-*jun* phosphorylation. Taken together, these findings are consistent with the suggestion that TZD might function most effectively as cytoplasmic inhibitors of ERK activity.

The findings presented here may help understand how TZD such as troglitazone (Ellis *et al*, 2000), pioglitazone (Malhotra *et al*, 2002), and rosiglitazone (H.A. Pershadsingh, unpublished observation) work in psoriasis. The pathophysiology of psoriasis is complex and multifaceted. T cells are involved in the initiation of psoriatic lesions, but abnormalities in keratinocyte function also appear to be important to the overall pathophysiology of the disease. Keratinocytes from psoriatic lesional skin have been shown to be less responsive than keratinocytes from normal skin to the growth-inhibitory effects of interferon-γ (Nickoloff *et al*, 1989), and differences in cytokine generation between normal and psoriatic keratinocytes have also been documented (Gottlieb *et al*, 1988; Nickoloff *et al*, 1994). In particular, ligands for the EGF receptor, including transforming growth factor-α, amphiregulin, and heparin-binding EGF, are elevated in psoriatic lesional skin and/or in psoriatic keratinocytes relative to control skin/cells (Gottlieb *et al*, 1988; Elder *et al*, 1989; Cook *et al*, 1992; Piepkorn *et al*, 1998; Piepkorn *et al*, 2003). These EGF receptor ligands are epidermal-derived. In addition, transgenic mice overexpressing amphiregulin develop a psoriasiform phenotype (Cook *et al*, 1997). A direct role for EGF receptor function in psoriatic keratinocyte proliferation is suggested by our own previous study (Varani *et al*, 1998), which demonstrated that treatment of psoriatic skin in organ culture with a function-blocking anti-EGF receptor antibody normalized histologic features. Most importantly, it was demonstrated in the same study that when skin from control subjects or nonlesional skin from individuals with psoriasis was exposed to EGF in organ culture, the histologic features mimicked those of psoriatic lesional skin. Based on this information, it can be hypothesized that although the keratinocyte responses that underlie expression of the psoriatic phenotype (Krueger *et al*, 1984; Fry, 1988; Varani *et al*, 1998) are triggered by multiple growth factors, in psoriasis, ligands acting through the EGF receptor probably play a predominant role. Although TZD do not appear to interfere with the initial ligand receptor-mediated events, by interfering with downstream signaling events, TZD prevent the cellular responses that constitute the abnormal phenotype. Of course, this does not rule out the possibility that TZD could independently affect T cell function. Determining the effects of these agents on immune function is beyond the scope of this work.

In summary, the findings presented here demonstrate that TZD inhibit a number of keratinocyte responses. They do so without demonstrating toxicity and without a significant effect on dermal fibroblast function. These findings provide insight into how this class of synthetic agents may suppress abnormal proliferative responses in the epidermis without the toxicity associated with many other agents that are used to control excess epidermal growth. Recently, a role for EGF receptor activation was demonstrated to play a role in retinoid-induced hyperplasia (Varani *et al*, 2001). Perhaps, TZD may have use in abnormal proliferative responses other than psoriasis.

Materials and Methods

Reagents Rosiglitazone was obtained from GlaxoSmithKline (Research Triangle Park, NC). Pioglitazone was obtained from Lilly (Indianapolis, IN) and BP-1107, a novel, high-affinity PPAR-γ ligand, was provided by Bethesda Pharmaceuticals, Inc. (Bakersfield, CA). Reagents used in intracellular signaling studies included: antibodies to phospho-extracellular regulated kinase (ERK), total-ERK, phospho-c-Jun, total-c-Jun, phospho-p38, total-p38, cyclin D1, phospho-epidermal growth factor (EGF) receptor, and total-EGF receptor (obtained from Cell Signaling Technologies, Inc., Beverly, MA); c-*fos* antibody (obtained from Upstate Biologicals Inc., Waltham, MA); and antibody to β-tubulin (obtained from Santa Cruz Biotechnology, Santa Cruz, CA). Antibody to matrix metalloproteinase-1 (MMP-1) was obtained from Chemicon International, Inc. (Temecula, CA). EGF and interleukin-1β (IL-1β) were from R & D Systems (Minneapolis, MN). U0126 was obtained from Calbiochem (San Diego, CA).

Human skin organ cultures Replicate 2-mm full-thickness punch biopsies of sun-protected hip skin were obtained from young adult volunteers. Immediately upon biopsy, the tissue was immersed in culture medium consisting of keratinocyte basal medium (KBM) (Cambrex Bioscience., Walkersville, MD). KBM is a low-Ca^{2+}, serum-free modification of MCDB-153 medium optimized for high-density keratinocyte growth. It was supplemented with $CaCl_2$ to bring the final Ca^{2+} concentration to 1.4 mM. After transport to the laboratory on ice, the biopsies were incubated in a 24-well dish containing 250 μL of Ca^{2+}-supplemented KBM with or without additional treatments. Cultures were incubated at 37°C in an atmosphere of 95% air and 5% CO_2. Other than to maintain the tissue in a minimal volume of medium, nothing further was done to maintain a strict air–liquid interface. Incubation was for 8 d, with change of medium containing the various treatments every second or third day. At the end of the incubation period, tissue was fixed in 10% buffered formalin and examined histologically after staining with hematoxylin and eosin. Epidermal thickness measurements were made at several sites in each tissue section and averaged. Overall average thickness values for the control and rosiglitazone-treated groups were then determined. Supernatant fluids were collected for MMP measurements. The participation of human subjects in this project was approved by the University of Michigan Institutional Review Board, and all subjects provided written informed consent before their inclusion in the study. Foreskin tissue obtained from neonatal circumcisions was also used. Organ culture preparations from neonatal foreskin tissue were processed in a similar way to that of adult biopsy tissue. The use of foreskin tissue in this project was approved by the University of Michigan Institutional Review Board.

Human epidermal keratinocytes and dermal fibroblasts in monolayer culture Normal human epidermal keratinocytes were isolated from either neonatal foreskin or adult skin as described previously (Varani *et al*, 1994). Primary and early passage cells were maintained in keratinocyte growth medium (KGM) (Cambrex Bioscience). KGM contains the same basal medium as KBM but is further supplemented with a mixture of growth factors including 0.1 ng per mL EGF, 0.5 μg per mL insulin, and 0.4% bovine pituitary extract. In some experiments, the HaCaT line of immortalized human epidermal keratinocytes (Boukamp *et al*, 1988) was used in place of normal keratinocytes. HaCaT cells were maintained in exactly the same manner as keratinocytes and used interchangeably with keratinocytes. Fibroblasts obtained from neonatal foreskin were grown in monolayer culture using Dulbecco's modified minimal essential medium supplemented with nonessential amino acids and 10% fetal bovine serum. Both keratinocytes and fibroblasts were maintained at 37°C in an atmosphere of 95% air and 5% CO_2. Cells were subcultured by exposure to trypsin/ethylenediaminetetraacetic acid and used at passages 2 to 3.

Proliferation assays For concentration–response studies, keratinocytes were seeded at 5×10^4 cells per well in a 24-well plate using KGM as culture medium. The cells were allowed to attach overnight. The next day, they were washed and then incubated in KBM with or without EGF (10 ng/mL) and different concentrations of the agents under study as indicated in the figure legends. Proliferation was measured on day 4 by releasing the cells with trypsin/ethylenediaminetetraacetic acid and enumerating them using a particle counter (Coulter Electronics, Hialeah, FL). For the time-course studies, 5×10^4 cells were seeded in a 24-well plate in KGM and allowed to attach overnight. The next day, cells were washed two times and incubated in KBM with or without 10 ng per mL EGF and treated with 20 μM rosiglitazone. Cell counts were made on days 1 to 4. Fibroblast proliferation studies were conducted in the same manner except KBM supplemented with 1.4 mM Ca^{2+} was used as culture medium. Our previous studies have shown that Ca^{2+}-supplemented KBM is optimal for maintenance of human skin in organ culture (Varani *et al*, 1993) and for assessing fibroblast proliferation (Varani *et al*, 1994).

Cytotoxicity assays Keratinocytes were plated at 5×10^4 cells per well and incubated for 1 d with 1 μCi of $^{51}CrO_4$. At the end of the incubation period, the cells were washed two times to remove unincorporated radioactivity. The cells were then incubated for 2 d in KBM- or EGF-supplemented KBM with or without 20 μM rosiglitazone. At the end of the incubation period, the percentage of incorporated ^{51}Cr that was released into the culture medium was assessed. Cells exposed to 1% Triton X-100 detergents were used to determine a "100% release value." In parallel, cells were incubated under the same conditions, but without ^{51}Cr. After exposure to control conditions or rosiglitazone, the cells were harvested, counted, and replated in growth medium. Four hours later, the percentage of cells that had reattached and spread was determined. The ability of cells to reattach and spread after treatment was used as a measure of viable cells. The use of the two assays in conjunction to assess epithelial and endothelial cell injury has been described in our past reports (Varani *et al*, 1985, 1988, 1996).

Motility assay For motility assays, 4×10^6 to 5×10^6 keratinocytes were centrifuged into a pellet and suspended in KBM supplemented with 2% Seaplaque agarose (FMC Bio Products; Portland, ME). Small drops (approximately 2 μL) of cell suspension were plated in wells of 96 well tissue culture plates and allowed to solidify at 4°C. After solidifying (approximately 15 min), agarose drops were overlayed with KBM with or without 10 ng per mL EGF and different concentrations of rosiglitazone. The cells were then incubated at 37°C in an atmosphere of 95% air and 5% CO_2. Cells in the dense agarose suspension migrated out from the pellet across the surface of the cell culture well. After 2 d, the distance between the edge of the agarose drop and the leading edge of migrating cells was measured using a microscope with a calibrated grid in the eyepiece. This assay has been described in the past (Nickoloff *et al*, 1988).

MMP assays Conditioned medium collected from organ cultures on day 2 or monolayer cultures on day 3 was assayed for MMP activity. Organ culture medium from different groups was compared on a "per-volume" basis, but cell culture medium from different groups was normalized to cell number at the end of the experiment. SDS-PAGE substrate embedded enzymography (zymography) was used to identify enzymes with gelatinase activity. Assays were carried out as described in a previous report (Gibbs *et al*, 1999). Briefly, denatured but nonreduced culture fluid samples were resolved in 10% SDS-PAGE gels prepared with incorporation of gelatin (1 mg per mL) before casting. After electrophoresis, gels were washed twice for 15 min in 50 mM Tris buffer containing 1 mM Ca^{2+}, 0.5 mM Zn^{2+}, and 2.5% Triton X-100. The gels were then incubated overnight in Tris buffer with 1% Triton X-100 and stained the following day with brilliant blue R-250. Following destaining, zones of enzyme activity were detected as regions of negative staining against the dark background. Gelatin zymography is used for detection of MMP-2 (72-kDa gelatinase A) and MMP-9 (92-kDa gelatinase B).

The same conditioned medium was assayed for MMP-1 (interstitial collagenase) by Western blotting. Briefly, samples were separated in 10% SDS-PAGE under denaturing and reducing conditions and transferred to nitrocellulose membranes. After blocking with a 5% nonfat milk solution in Tris-buffered saline with 0.1% Tween at 4°C overnight, membranes were incubated for 1 h at room temperature with a rabbit polyclonal antihuman MMP-1 antibody, diluted 1:6000 in 0.5% nonfat milk/0.1% Tween-TBS. Thereafter the membranes were washed with TTBS and bound antibody detected using the Phototope-HRP western blot detection kit (Cell Signaling Technologies, Inc., Beverly, MA). The western blotting procedure has been described previously (Varani *et al*, 2000).

For zymography, negative images were scanned and digitized. For western blots, the positive images were scanned and digitized. The digitized images were used to provide a relative quantitative assessment of changes in enzyme levels.

Preparation of cell lysates and immunoblot analysis of signaling intermediates Keratinocytes were plated at 3×10^5 cells per well in wells of a six-well dish using KGM as culture medium. The cells were allowed to attach overnight. The next day, they were washed and then incubated in KBM with or without EGF (10 ng/mL) and different concentrations of the agents under study as indicated in the figure legends. After incubation for the desired amount of time, cells were lyzed in 1× cell lysis buffer consisting of 20 mM Tris-HCl (pH 7.4); 2 mM sodium vanadate; 1.0 mM sodium fluoride: 100 mM NaCl; 1% Nonidet P-40; 0.5% sodium deoxycholate; 25 μg per mL each aprotinin, leupeptin, and pepstatin; and 2 mM ethylenediaminetetraacetic acid and ethylene glycol bis tetraacetic acid. Lysis was performed at 4°C by scraping the cells into lysis buffer and sonicating the samples. Cell lysates were incubated on ice for 30 min and then cleared by microcentrifugation at $16{,}000 \times g$ for 15 min. The supernatant fluids were collected and protein concentration was estimated using the Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA).

Cell extracts containing equivalent amounts of protein (40 μg of total protein per lane) were electrophoresed in 10% SDS-polyacrylamide gels. Western blotting for signaling intermediates was carried out as described above for MMP-1.

PPAR-γ transactivation assay PPAR-γ activity was determined using a cell-based transactivation assay. CV-1 cells were maintained in Dulbecco's modified minimal essential medium supplemented with nonessential amino acids and 10% fetal bovine serum. The cells were plated 2×10^5 cells per well in 24-well dishes. Twenty-four hours after plating, the medium was replaced

with Dulbecco's modified minimal essential medium containing 0.5% (w/v) charcoal-dextran treated FBS (Hyclone). Forty-eight hours after plating, the cells were transfected with 200 ng PPAR-γ receptor expression plasmid pGAL4-mPPAR-γ LBD and 1 μg luciferase reported plasmid pUAS-tk-luc along with 400 ng pCMVSport β-gal plasmid (Gibco, Grand Island, NY) as an internal control. Transfections were performed using the GenePorter reagent (Gene Therapy Systems, San Diego, CA) according to the manufacturer's instructions. Twenty-four hours after transfection, cells were treated with varying concentrations of the ligands (BP-1107 or rosiglitazone) and incubated for an additional 24 h. Cell extracts were prepared and assayed for luciferase and β-galactosidase activity using the Promega (Madison, WI) luciferase and β-galactosidase assay systems according to the manufacturer's instruction. All treatments were performed in triplicate and normalized for α-galactosidase activity. The agonist concentrations yielding half maximal activation (EC_{50} values) were calculated using GraphPad Prism version 3.03 (GraphPad Software, Inc., San Diego, CA).

Statistical analysis Data from proliferation and motility assays are expressed as means $\pm$ SEM. Statistical analyzes were carried out by ANOVA followed by paired group comparisons; $p < 0.05$ was considered statistically significant.

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Address correspondence to: James Varani, PhD, Department of Pathology, The University of Michigan, 1301 Catherine Road/Box 0602, Ann Arbor, MI 48109. Email: varani@umich.edu

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