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Ciglitazone negatively regulates CXCL1 signaling through MITF to suppress melanoma growth

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We have previously demonstrated that the thiazolidinedione ciglitazone inhibited, independently of PPARγ activation, melanoma cell growth. Further investigations now show that ciglitazone effects are mediated through the regulation of secreted factors. Q-PCR screening of several genes involved in melanoma biology reveals that ciglitazone inhibits expression of the *CXCL1* chemokine gene. CXCL1 is overexpressed in melanoma and contributes to tumorigenicity. We show that ciglitazone induces a diminution of CXCL1 level in different human melanoma cell lines. This effect is mediated by the downregulation of microphthalmia-associated transcription factor, MITF, the master gene in melanocyte differentiation and involved in melanoma development. Further, recombinant CXCL1 protein is sufficient to abrogate thiazolidinedione effects such as apoptosis induction, whereas extinction of the CXCL1 pathway mimics phenotypic changes observed in response to ciglitazone. Finally, inhibition of human melanoma tumor development in nude mice treated with ciglitazone is associated with a strong decrease in MITF and CXCL1 levels. Our results show that anti-melanoma effects of thiazolidinediones involve an inhibition of the MITF/CXCL1 axis and highlight the key role of this specific pathway in melanoma malignancy.
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Cutaneous melanoma is an aggressive skin cancer that originates from epidermal melanocytes. Melanoma development is accomplished through the accumulation of genetic alterations in growth control pathways including oncogenic mutations or gene amplification. For example, constitutive activation of the Ras/MAPkinase-signaling pathway is frequently observed in melanomas, as a consequence of activating mutations of the B-Raf and N-Ras genes.[1–3] Melanoma progression is also accompanied by generation of autocrine and paracrine loops associated with the aberrant production and secretion of growth factors and chemokines that sustain growth, survival and invasion.[4]

In humans, melanoma is one of the most lethal cancers among young adults. Melanoma has a high capability of invasion and rapid metastasis to other organs. The prognosis of metastatic melanoma is extremely dismal, as the various treatments have not shown survival benefit.[5] It appears thus necessary to develop approaches enabling the discovery of new molecular targets, candidates for specific biotherapy treatment of this disease.

Thiazolidinediones (TZDs) regulate transcriptional activity of the nuclear receptor peroxysome proliferator activated receptor γ (PPARγ) and are currently used in type II diabetes treatment. More recently, TZD have been reported to inhibit proliferation and survival of numerous cancer cells.[6] Furthermore, we have previously demonstrated that ciglitazone, which belongs to TZD family, inhibits growth and viability of melanoma cells without affecting normal melanocyte growth.[7]

To further investigate the molecular events elicited by ciglitazone and to better understand the implication of TZD signaling in melanoma biology, we examined whether ciglitazone affects the production of autocrine and paracrine factors known to be implicated in melanoma malignancy. In this report, we demonstrate that ciglitazone inhibits the expression of (C-X-C motif) ligand 1 (CXCL1), a chemokine involved in melanoma growth, survival, angiogenesis and metastasis.[8–11] Interestingly, ciglitazone treatment decreases CXCL1 level in various human melanoma cell lines but not in normal human melanocytes. We also show that treatment of melanoma cells with recombinant CXCL1 compensates for the loss of endogenous CXCL1 secretion induced by ciglitazone and abolishes the loss of viability of melanoma cells. Furthermore, CXCL1 inhibition by ciglitazone is mediated by the decrease in microphthalmia-associated transcription factor (MITF) expression. CXCL1 appears to be a new target gene of MITF, the master gene of melanocyte differentiation that was also involved in melanoma development.[12–16] Finally, we show that a dramatic inhibition of melanoma xenograft development in mice in response to ciglitazone is associated with a decrease in MITF gene expression and in circulating CXCL1 level.

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Abbreviations: BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulfoxide; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; IBMX, 3-Isobutyl-1-methylxanthine; NHM, normal human melanocytes; PARP, poly(ADP-ribose) polymerase; PPARγ, peroxysome proliferator-activated receptor-γ; TNFα, tumor necrosis factor-α; TZD, thiazolidinedione
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Our results demonstrate for the first time that ciglitazone-induced apoptosis of melanoma involves an inhibition of a MITF/CXCL1-signaling cascade.

Results

Effect of ciglitazone on CXCL1 expression and secretion. We first determined whether the biological effects of ciglitazone in melanoma cells were mediated by secreted factors. We confirmed that ciglitazone did not affect normal human melanocytes (NHM) viability (Figure 1a).

In contrast, the number of melanocytes was significantly reduced when incubated in the presence of conditioned medium from A375 melanoma cells exposed to ciglitazone compared to conditioned medium from Dimethyl sulfoxide (DMSO)-treated A375 cells (Figure 1b). These results suggest that the inhibition of melanoma cell growth or survival elicited by ciglitazone was at least in part mediated by secreted factors.

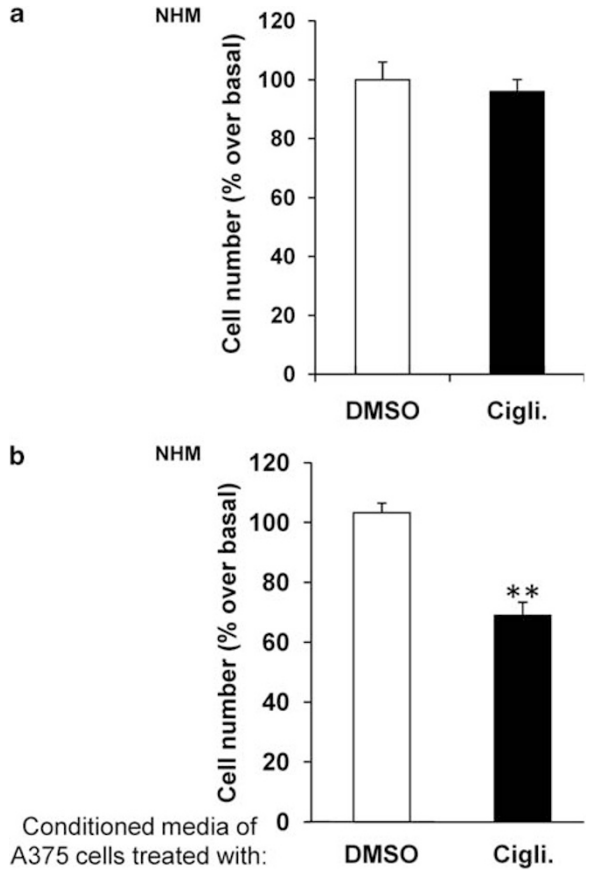


Figure 1 Ciglitazone modifies melanoma cell secretome. (**a**) Starved normal human melanocytes (NHM) were treated or not with 10 μM ciglitazone (Cigli.) for 96 h. Cells were then harvested and counted using trypan blue. Results are expressed in percent of control (100%). (**b**) Starved A375 melanoma cells were treated or not with 10 μM ciglitazone for 24 h. Conditioned media were collected and centrifuged for 5 min at 2000 r.p.m. Supernatants were immediately added to the culture medium of NHM (1:1). After 96 h, NHM were harvested and counted using trypan blue. Results are expressed in percent of control (100%). Data are mean ± S.D. of three independent experiments performed in triplicate, significantly different from the corresponding control $^{**}P<0.01$

To substantiate this observation, we analyzed by quantitative-PCR, the expression of 19 transcripts encoding for proteins known to be secreted by melanoma (Table 1). Among them ciglitazone clearly repressed the expression of *CXCL1*. Owing to the important role of the CXCL1 chemokine in melanoma progression, we decided to investigate its involvement in the effect of ciglitazone on melanoma cells.

RT-QPCR analysis confirmed a dose-dependent down-regulation of *CXCL1* expression in ciglitazone-treated A375 melanoma cells (Figure 2a). Then, we demonstrated a decrease in CXCL1 cytoplasmic protein by immunofluorescence (Figure 2b). TNFα, an activator of NF-κB pathway, significantly increased the level of CXCL1. CXCR2 labeling was not modulated in response to ciglitazone (Figure 2b, lower part).

CXCL1 amount in medium was evaluated by ELISA. TNFα increased CXCL1 level, whereas ciglitazone induced a dose-dependent reduction of CXCL1 in both basal and TNFα conditions (Figure 2c). Western blot of the secreted matricellular SPARC protein was used as loading control.

Relationship between CXCL1 downregulation and cell viability inhibition induced by ciglitazone. One could think that the inhibition of CXCL1 is the consequence of the decrease in cell viability induced by ciglitazone. However, reduction of CXCL1 was detectable after 2 h and therefore could not result from the decrease in viability of cells that was observed after 12 h (Figure 3a). In contrast, the slight decrease in CXCL1 level observed after 12 h to 48 h of staurosporine treatment appeared to be the consequence of cell death.

Next, other TZD, pioglitazone and rosiglitazone had no significant effect on CXCL1 secretion (Figure 3b). In contrast, troglitazone showed a dose-dependent decrease in CXCL1 level comparable to that observed with ciglitazone. Moreover, the decrease in CXCL1 level in response to ciglitazone treatment was still observable when PPARγ expression was abrogated by siRNA silencing, indicating that ciglitazone acts independently of PPARγ to repress CXCL1 expression (Supplementary Figure S1).

Interestingly, no CXCL1 was detectable in NHM (Figure 3c). However, treatment of melanocytes by TNFα induced an increase in the level of CXCL1 that was not modulated by ciglitazone.

Finally, we tested ciglitazone on different tumor cell lines. SK-Mel-28, WM793 and 1205 Lu melanoma cells had basal CXCL1 levels comparable to that found in A375, whereas MeWo had very low CXCL1 level. In all melanoma cells, TNFα induced a significant increase in CXCL1 level and ciglitazone strongly decreased the chemokine level. In contrast to neuroblastoma cells SH-SY5Y, prostate tumor cells PC-3 presented a high CXCL1 basal level. As in melanoma cells, TNFα significantly increased the cytokine level in both cell lines, whereas ciglitazone strongly reduced the level of CXCL1(Figure 3d). The effects of ciglitazone on growth and survival in these cell lines were tested (Figure 3e and Supplementary Figure S2). Ciglitazone treatment decreased the cell number in all tumor cell lines tested so far. However, the most striking effect was observed in melanoma cell lines with a high basal CXCL1 level.

Table 1 Genes differentially expressed in melanoma cells stimulated or not with ciglitazone

Gene name	Symbol	Gene bank ID	Fold change
Growth factors			
Epidermal growth factor	EGF	NM_001963	ND
Transforming growth factor-β2	TGFB2	NM_001135599	0.8
Vascular endothelial growth factor A	VEGFA	NM_001025366	0.9
Platelet-derived growth factor α	PDGFA	NM_002607	1.0
Hepatocyte growth factor	HGF	NM_000601	ND
Insulin-like growth factor 1	IGF1	NM_001111283	ND
Colony-stimulating factor 2 (granulocyte-macrophage)	CSF2	NM_000758	1.0
Cytokines			
Chemokine (C-X-C motif) ligand 1 (melanoma growth-stimulating activity, α)	CXCL1	NM_001511	0.4
Chemokine (C-X-C motif) ligand 2/3	CXCL2/CXCL3	NM_002089/90	0.8
Chemokine (C-X-C motif) ligand 5	CXCL5	NM_002994	1.4
Chemokine (C-C motif) ligand 2	CCL2	NM_002982	1.0
Interleukin 1-β	IL1B	NM_000576	1.1
Interleukin 6	IL6	NM_000600	1.0
Interleukin 8	IL8	NM_000584	0.8
Other melanoma-secreted proteins			
Insulin-like growth factor-binding protein 7	IGFBP7	NM_001553	0.9
Endothelin 1	EDN1	NM_001955	0.9
Endothelin 2	EDN2	NM_001956	ND
Endothelin 3	EDN3	NM_000114	0.9
Wingless-type MMTV integration site family, member 3A	WNT3A	NM_033131	1.0

The expression level of 19 genes from proteins secreted by melanomas was evaluated by real-time quantitative PCR analysis. Total RNA was extracted from A375 cells stimulated or not with ciglitazone for 24 h and then subjected to real-time quantitative PCR analysis as described in Materials and methods section. Data are expressed in arbitrary units as fold change between DMSO-treated control cells and ciglitatone-treated cells and are a mean of two independent amplifications performed in duplicate.

Effect of recombinant CXCL1 on the decrease in cell viability induced by ciglitazone. To determine whether CXCL1 inhibition had an important role in ciglitazone-induced loss of melanoma cell viability, we compensated the loss of endogenous CXCL1 by adding human recombinant CXCL1 (rCXCL1). As expected, ciglitazone (10 μM) induced a loss of 65% of cell viability (Figure 4a). Addition of rCXCL1 prevented in a dose-dependent manner the loss of cell viability. Similarly, the reduction of cell viability induced by ciglitazone was reversed by TNFα (Figure 4b).

Interestingly, rCXCL1 did not prevent cell viability reduction mediated by two apoptosis inductors, TRAIL and staurosporine (Figure 4c). The protective effect of rCXCL1 thus seems specific of the ciglitazone action.

We have previously established that low concentrations of ciglitazone induced a G0/G1 cell cycle arrest of A375 melanoma cells. Our results demonstrated that rCXCL1 was able to reverse ciglitazone-induced cell cycle arrest (Supplementary Figure S3).

We have also shown that high concentrations of ciglitazone induced melanoma cells apoptosis. We observed that 10 μM ciglitazone induced the disappearance of the zymogenic form of caspases 3, 8 and 9 as well as a cleavage of poly(ADP-ribose) polymerase (PARP). Consistently with the protective effect of rCXCL1, cleavage of caspases and PARP evoked by 10 μM ciglitazone were not detected in the presence of rCXCL1 (Figure 5a). These results were confirmed by caspase 3, 8 and 9 activation assays (Figure 5b). Interestingly, rCXCL1 was not able to prevent the activation of these three caspases induced by staurosporine. Furthermore, flow cytometry analysis showed that rCXCL1 blocked the accumulation of the cell in sub-G1 (Supplementary Figure S3) and prevented the increase in annexin-V labeling (Figure 5c), both induced by ciglitazone. In conclusion, CXCL1 protects melanoma cells from apoptosis induced by ciglitazone.

Our observations prompted us to check whether CXCL1 inhibition was sufficient to reduce melanoma cell viability. Transfection of A375 cells with increasing concentrations of si-CXCL1 induced a strong and dose-dependent reduction of the CXCL1 protein level (Figure 6a). Next, we observed that A375 cells transfected with si-CXCL1 showed a dose-dependent reduction of cell viability that was impaired by rCXCL1 (Figure 6b). Then, we observed a dose-dependent reduction of A375 cell viability in the presence of anti-CXCL1 neutralizing antibodies (Figure 6c).

We next showed that CXCL1 silencing induced the activation of caspases 9, 8 and 3 (Figure 6d) as well as an increase in annexin-V labeling (Figure 6e). Taken together, these results indicate that the inhibition of CXCL1 production is sufficient to induce apoptosis in melanoma cells. In the same way, inhibition of CXCR2 signaling by neutralizing antibody or pharmacological inhibitor (SB225002) mimics the effects of ciglitazone on growth inhibition (Supplementary Figure S4).

Involvement of MITF in the regulation of CXCL1 expression by ciglitazone. To further investigate the mechanism responsible for ciglitazone-induced CXCL1 downregulation, we focused our attention on MITF that was recently reported to be regulated by some PPARγ agonists.[17,18] Ciglitazone induced a dose-dependent reduction of MITF expression in both A375 and SK-Mel-28 melanoma cells (Figure 7a). We demonstrated that MITF inhibition by ciglitazone was not due to its cleavage during

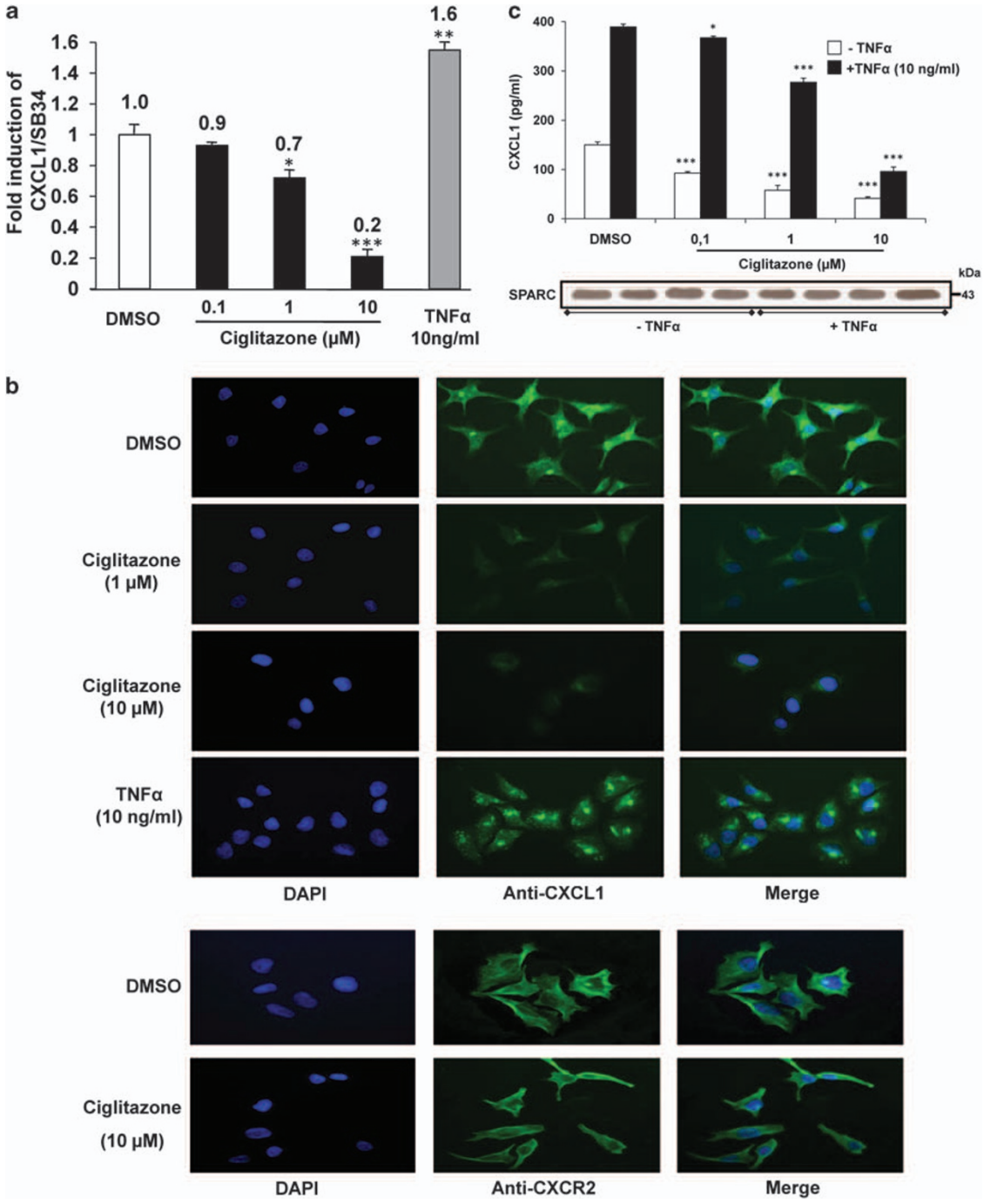


Figure 2 Ciglitazone decreases CXCL1 level in A375 melanoma cells. Starved A375 melanoma cells were treated for 24 h with various concentrations of ciglitazone or TNFα (10 ng/ml). (**a**) Total RNA was extracted and analyzed by real-time quantitative PCR using *CXCL1* primers. mRNA expression was normalized using *SB34* RNA level. Results are expressed as mean ± S.D. from three independent experiments. Significantly different from the corresponding control $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$. (**b**) A375 cells were fixed and stained for CXCL1 or CXCR2 (green) and with DAPI (blue). DAPI staining was used to identify cell nucleus. Right panels show merger of DAPI and CXCL1 or CXCR2 staining. Slides were examined with a Zeiss Axiophot fluorescence microscope and pictures were taken at × 200 magnification. Representative field of three different experiments are shown. (**c**) ELISA of CXCL1 was performed on supernatants from starved A375 melanoma cells treated as indicated. Data are mean ± S.D. of three independent experiments performed in triplicate. SPARC western blotting on those supernatants was used as loading control. Significantly different from the corresponding control $^{*}P<0.05$; $^{***}P<0.001$

apoptosis because the MITF decrease was observed earlier than PARP cleavage. To confirm this result we showed that the pan-caspase inhibitor Z-VAD-FMK prevented melanoma cell death induced by ciglitazone treatment (Supplementary Figure S5) by preventing apoptosis induction monitored by PARP processing (Figure 7a, right panel). However, MITF inhibition evoked by ciglitazone was still observed in the presence of Z-VAD-FMK. Finally, using RT-QPCR, we also

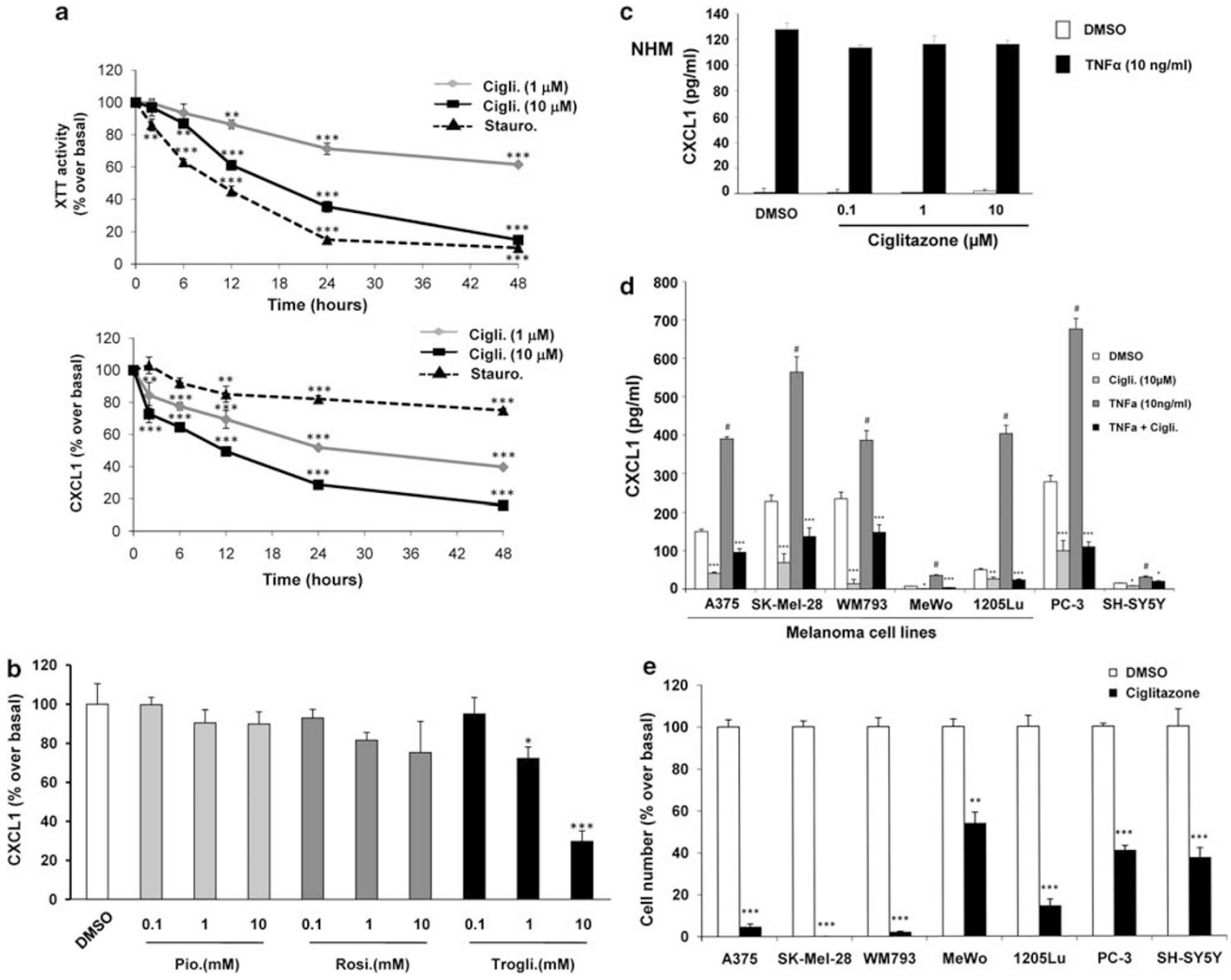


Figure 3 CXCL1 expression in response to various TZD and in different cell lines. (**a**) Time course of XTT activity (upper panel) and ELISA assay for CXCL1 (lower panel) were performed on starved A375 melanoma cells treated for different times with 1 or 10 μM ciglitazone or with staurosporine. Results are expressed in percent of control (100%) for each time. Data are mean ± S.D. of three independent experiments performed in triplicate. (**b**) ELISA of CXCL1 was performed on supernatants from starved A375 melanoma cells treated for 24 h with different TZD: pioglitazone (Pio.), rosiglitazone (Rosi.), troglitazone (Trogli.). Results are expressed in percent of control (100%) for each time. Data are mean ± S.D. of three independent experiments performed in triplicate. (**c**) ELISA of CXCL1 was performed on supernatants from starved normal human melanocytes (NHM) treated or not for 24 h with various concentrations of ciglitazone added or not with TNFα 10 ng/ml. Data are mean ± S.D. of three independent experiments performed in triplicate. (**d**) ELISA of CXCL1 was performed on supernatants from different cell lines treated or not for 24 h with 10 μM of ciglitazone added or not with TNFα 10 ng/ml. Data are mean ± S.D. of three independent experiments performed in triplicate. Significantly different from the corresponding control $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$; $^{\#}P<0.05$. (**e**) Starved cells from various cell lines were treated or not with 10 μM ciglitazone for 60 h. Cells were then harvested and counted using trypan blue. Results are expressed in percent of control (100%). Data are mean ± S.D. of three independent experiments performed in triplicate

found a dose-dependent decrease of *MITF* expression confirming that ciglitazone inhibited MITF expression at the transcriptional level (Figure 7b).

We next checked whether MITF might control CXCL1 expression. MITF silencing by specific si-RNA induced a strong reduction of the *MITF* transcript and a concomitant reduction of *CXCL1* gene expression (Figure 7c). Melanoma cells transfected with a vector encoding for wild-type MITF revealed an increased in CXCL1 labeling by immunofluorescence (Figure 7d). Consistently, forskolin or forskolin/IBMX that increased endogenous MITF level showed a significant increase in CXCL1 (Figure 7e).

To determine whether MITF controlled the CXCL1 promoter activity, we performed luciferase assays on melanoma cells using either tyrosinase or CXCL1 promoter upstream the luciferase coding sequence. Transfection with a dominant negative form of MITF (DN MITF) led to a decrease in both tyrosinase and CXCL1 promoter activity. Conversely, transfection with the wild-type form of MITF (MITF) led to an upregulation of tyrosinase and CXCL1 promoter activity (Figure 7f).

Then, to verify the binding of MITF to the CXCL1 promoter in intact cells, we performed chromatin immunoprecipitation (ChIP) assays. Direct PCR amplification with specific primers spanning the CXCL1 promoter between -410 and $+77$, on total DNA extract or after immunoprecipitation with antibody to polymerase II as a positive control amplified a 500-bp fragment (Figure 7g). After immunoprecipitation with anti-MITF,

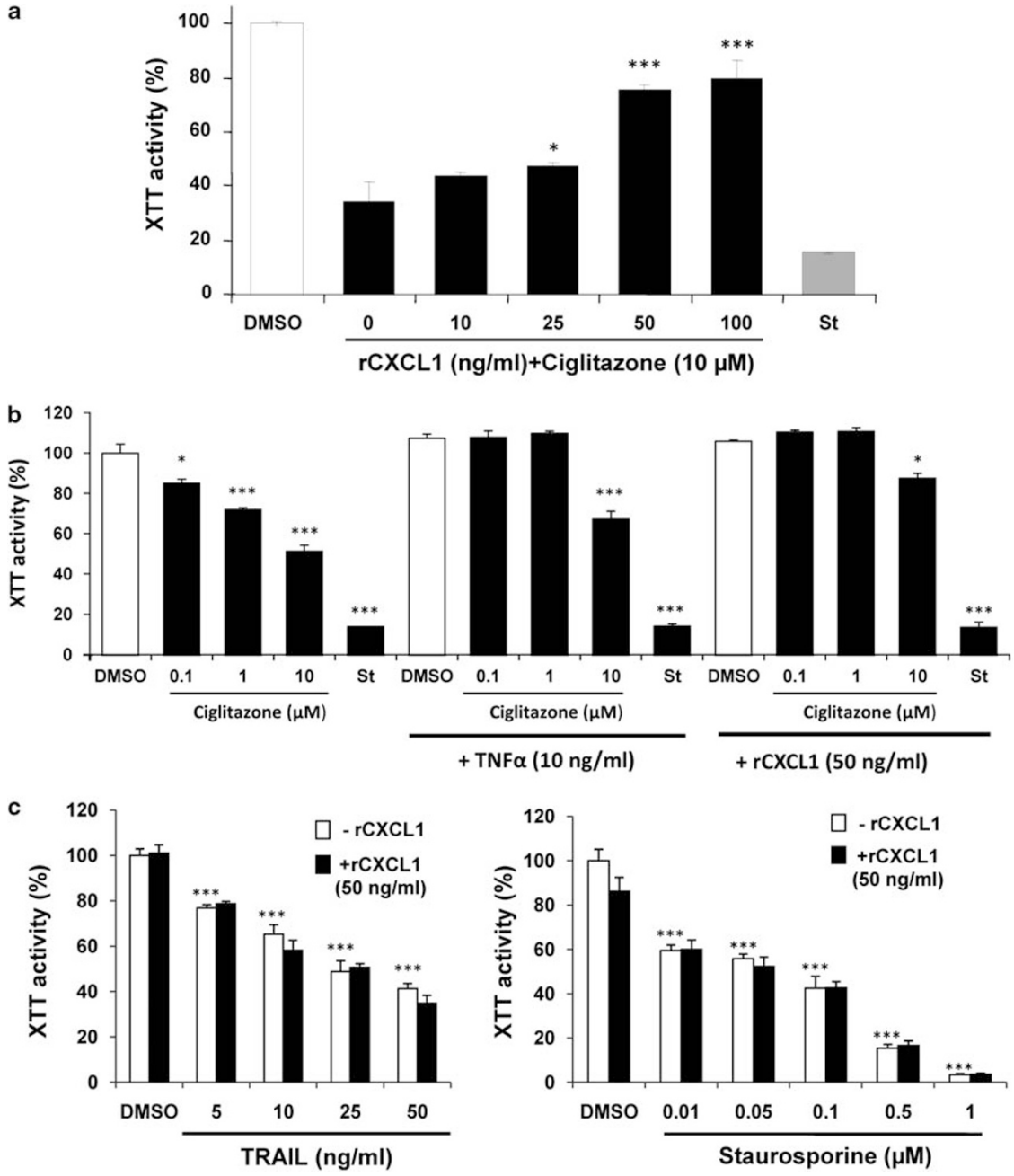


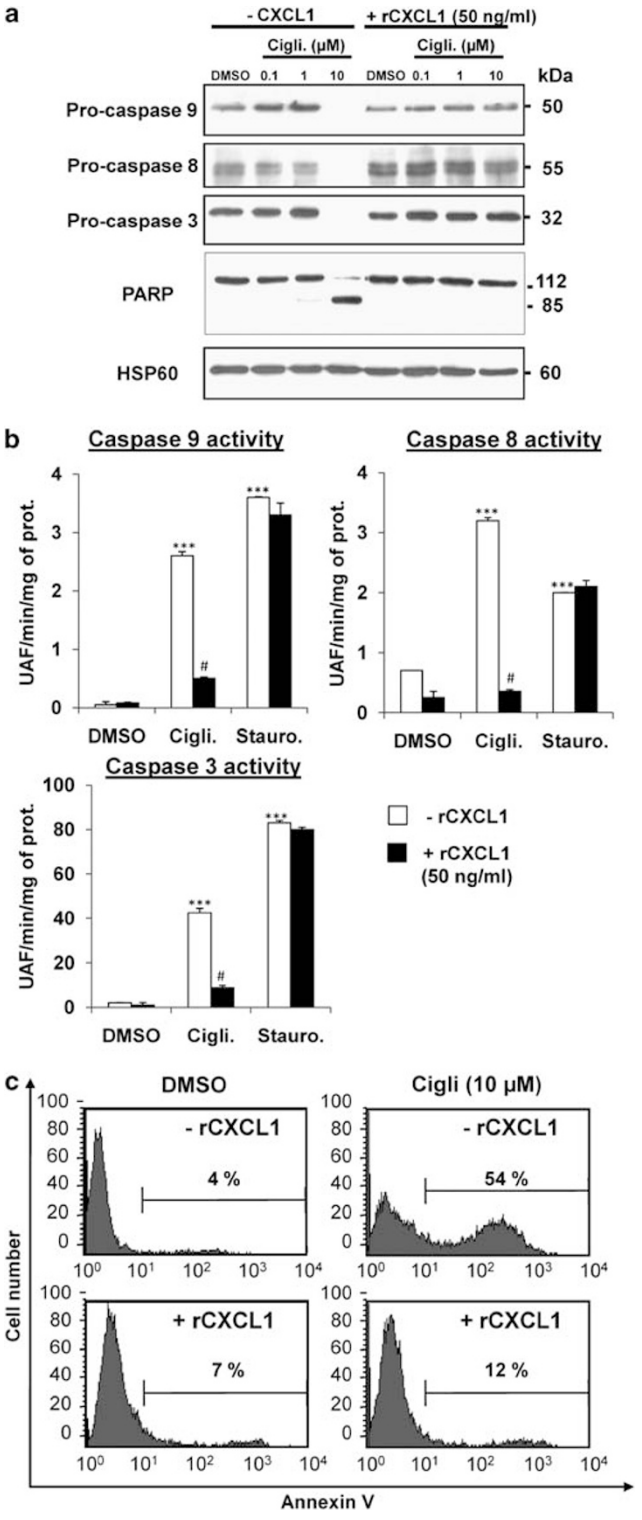
Figure 4 Recombinant CXCL1 is sufficient to abrogate decrease of cell viability induced by ciglitazone. XTT activity was performed on starved A375 melanoma cells treated with ciglitazone 10 μM added or not with various concentrations of recombinant CXCL1 (**a**), 10 ng/ml TNFα (**b**) or treated with various concentrations of TRAIL or staurosporine added with 50 ng/ml of recombinant CXCL1 (**c**). Results are expressed in percent of control (100%) for each time. Data are mean $\pm$ S.D. of three independent experiments performed in triplicate. Significantly different from the corresponding control $^{*}P<0.05$; $^{***}P<0.001$

we amplified a band at 500 bp corresponding to the CXCL1 promoter. The intensity of this band was clearly decreased in extracts from cells exposed to ciglitazone, but not in extracts from cells exposed to forskolin (Fsk). Conversely, using specific GAPDH promoter primers, we amplified 160-bp fragment corresponding to the GAPDH promoter in both the total extract and the anti-Pol II precipitates, but not in the anti-MITF precipitates. These results show that MITF binds to the CXCL1 promoter in intact cells and that this interaction is abolished by ciglitazone treatment.

Effect of ciglitazone on circulating CXCL1 and melanoma tumor xenografts development in mice. Then, to determine the effect of ciglitazone on circulating CXCL1 and on tumor development *in vivo*, human A375 melanoma cells were injected subcutaneously in athymic nude mice. Nineteen days post injection, mice were treated with vehicle or ciglitazone (50 mg/kg/day) for 11 days.

Tumors from untreated mice dramatically grew throughout the time course of the study (Figure 8a). In contrast, treatment

of mice with ciglitazone markedly attenuated the capacity of cells to develop visible tumors. Indeed, 3 out of 6 ciglitazone-treated mice did not have a measurable tumor at the end of the treatment, and tumors of other mice were significantly smaller compared with untreated control mice.



After killing mice, RT-QPCR were performed on RNA extracted from tumors. Interestingly, MITF and CXCL1 gene expression were significantly lower in tumors of ciglitazone-treated mice (Figure 8b). Further, no significant CXCL1 level was detected in ciglitazone-treated mice in comparison with vehicle-treated mice (Figure 8c). When we expressed the quantity of serum CXCL1 as a function of tumor volume, we observed a 3.5-fold significant reduction in the level of circulating CXCL1 in ciglitazone-treated mice. Therefore, the decrease in CXCL1 serum level observed in ciglitazone-treated mice was not due to a decrease in tumor volume, but was likely a consequence of CXCL1 inhibition induced by ciglitazone. Finally, to unequivocally demonstrate the importance of CXCL1 downregulation in the *in vivo* effects of ciglitazone, we evaluated the effects of rCXCL1 peritumoral injections (200 ng/tumor/day for 14 days) on the development of tumors in mice treated or not with ciglitazone. As expected, treatment of mice with ciglitazone markedly impaired tumor development (Figure 8d). However, rCXCL1 treatment dramatically favored tumor development and abrogated anti-tumoral effects of ciglitazone. Injection of human recombinant CXCL1 therefore compensates for the decrease in endogenous CXCL1 level mediated by ciglitazone treatment and promotes tumor growth.

Discussion

Because metastatic melanoma are resistant to all currently used treatments, the discovery of new therapeutic drugs is a very important challenge. We and others have previously shown that ciglitazone, a molecule of the TZD family, is able to induce apoptosis independently of PPARγ activation and to inhibit proliferation of melanoma cells.[7,19–21] In this report, we have investigated the molecular mechanism by which ciglitazone exerts its anti-melanoma activity.

Our results showed that conditioned media from ciglitazone-treated melanoma cells inhibit normal human melanocyte growth indicating that the effects of ciglitazone on melanoma cells are mediated by secreted factors. RT-QPCR screening demonstrating an inhibition of CXCL1 chemokine upon ciglitazone treatment prompted us to focus our attention on this cytokine. CXCL1 also named melanoma growth stimulating activity or growth-regulated oncogene α (GROα) belongs to the CXC family. CXCL1 is essential for the

Figure 5 Recombinant CXCL1 is sufficient to abrogate apoptosis induced by ciglitazone. Starved A375 melanoma cells were treated for 24 h with various concentrations of ciglitazone added or not with 50 ng/ml of recombinant CXCL1 (rCXCL1). (**a**) Western blot was performed on cell lysates (30 ig total protein per lane). Proteins were separated by 10% SDS-PAGE and analyzed by western blot using the indicated antibody. HSP60 was used as loading control. One representative experiment of three is shown. (**b**) Caspase 3, 8 and 9 activities were performed on cell lysates (30 μg per condition). Lysate from cells treated for 5 h with 1 μM staurosporine was used as positive control of caspases activation. Results are expressed in relative fluorescence units per minute and per mg of protein (UAF/min/mg of prot.). Data are mean ± S.D. of three independent experiments performed in triplicate. Significantly different from the corresponding control $^{***}P<0.001$; $^{\#}P<0.05$. (**c**) Cells were detached and stained with Annexin-V-Fluorescein before being analyzed by flow cytometry. Data are representative of three independent experiments performed in triplicate

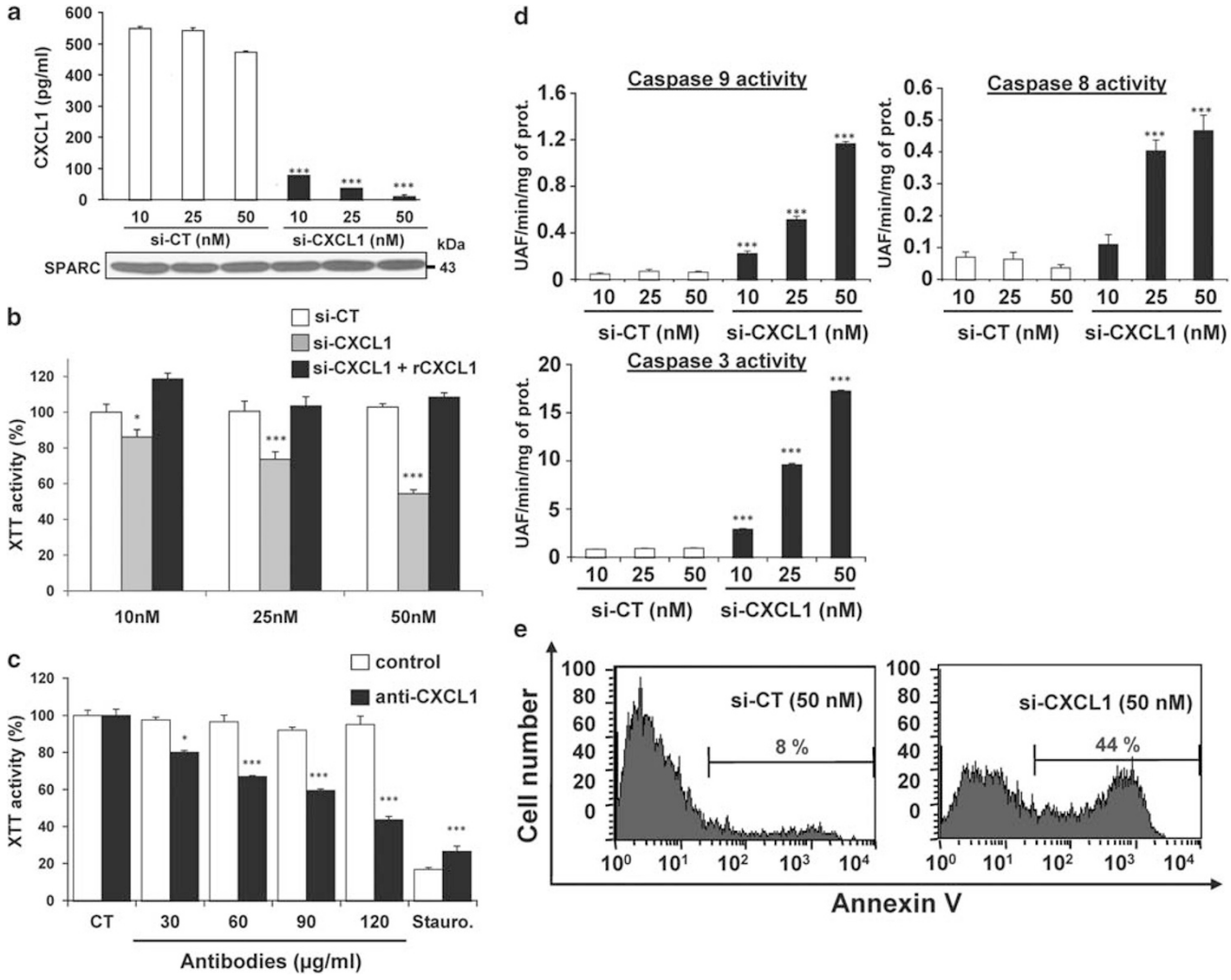


Figure 6 Inhibition of CXCL1 decreases cell viability. (**a**) Starved A375 cells were transfected for 48 h with various concentrations of siRNA-targeting CXCL1 (si-CXCL1) or a scramble sequence (si-CT). ELISA for CXCL1 was performed on supernatants from transfected cells. Results are expressed in percent of control (100%). Data are mean ± S.D. of three independent experiments performed in triplicate. SPARC western blotting on those supernatants was used as loading control. Significantly different from the corresponding control ***$P<0.001$. (**b**) XTT activity was performed on starved A375 transfected for 48 h with various concentrations of siRNA targeting CXCL1 and added or not with rCXCL1 (50 ng/ml). Results are expressed in percent of cells transfected with the control siRNA (100%) for each concentration. Data are mean ± S.D. of three independent experiments performed in triplicate. Significantly different from the corresponding control *$P<0.05$; ***$P<0.001$. (**c**) XTT activity was performed on starved A375 treated for 24 h with various concentrations of blocking antibody targeting CXCL1. Results are expressed in percent of control (100%). Data are mean ± S.D. of three independent experiments performed in triplicate. Significantly different from the corresponding control *$P<0.05$; ***$P<0.001$. (**d**) Caspase 3, 8 and 9 activities were performed on cell lysates (30 μg per condition) from starved A375 transfected for 48 h with various concentrations of control (CT) or CXCL1 siRNA. Results are expressed in relative fluorescence units per minute and per mg of protein (UAF/min/mg of prot.). Data are mean ± S.D. of three independent experiments performed in triplicate. Significantly different from the corresponding control ***$P<0.001$. (**e**) Starved A375 cells were transfected for 48 h with 50 nM of siRNA targeting CXCL1 (si-CXCL1) or a scramble sequence (si-CT). Cells were detached and stained with Annexin-V-Fluorescein before being analyzed by flow cytometry. Data are representative of three independent experiments performed in triplicate

establishment and the maintenance of the tumoral potential of melanoma.[22,23] CXCL1 bind with high affinity to CXCR2. Overexpression of CXCL1 in INK4a/ARF$^{-/-}$ mice favors melanoma development[24] and promotes malignant growth of murine squamous cell carcinoma by a CXCR2-dependent pathway.[25] Blocking antibodies to either CXCL1 or CXCR2 inhibit melanoma cell growth showing the key role of the CXCL1/CXCR2-signaling pathway in melanoma development.[26,27] In addition to its autocrine role, CXCL1 has been shown to have an important paracrine role by regulating angiogenesis during tumor development including melanoma.[28,29]

First, we show that ciglitazone negatively regulates mRNA and protein CXCL1 levels. This inhibition is accompanied by a reduction of CXCL1 in the medium. The inhibition of CXCL1 in response to ciglitazone precedes the decrease in cell viability, indicating that the decrease in CXCL1 expression induced by ciglitazone is not a consequence of cell death. A similar decrease in CXCL1 is also observed in response to troglitazone whereas rosiglitazone and pioglitazone have no significant effect on CXCL1 secretion. This result is in agreement with our previous report showing that both rosiglitazone and pioglitazone have only marginal

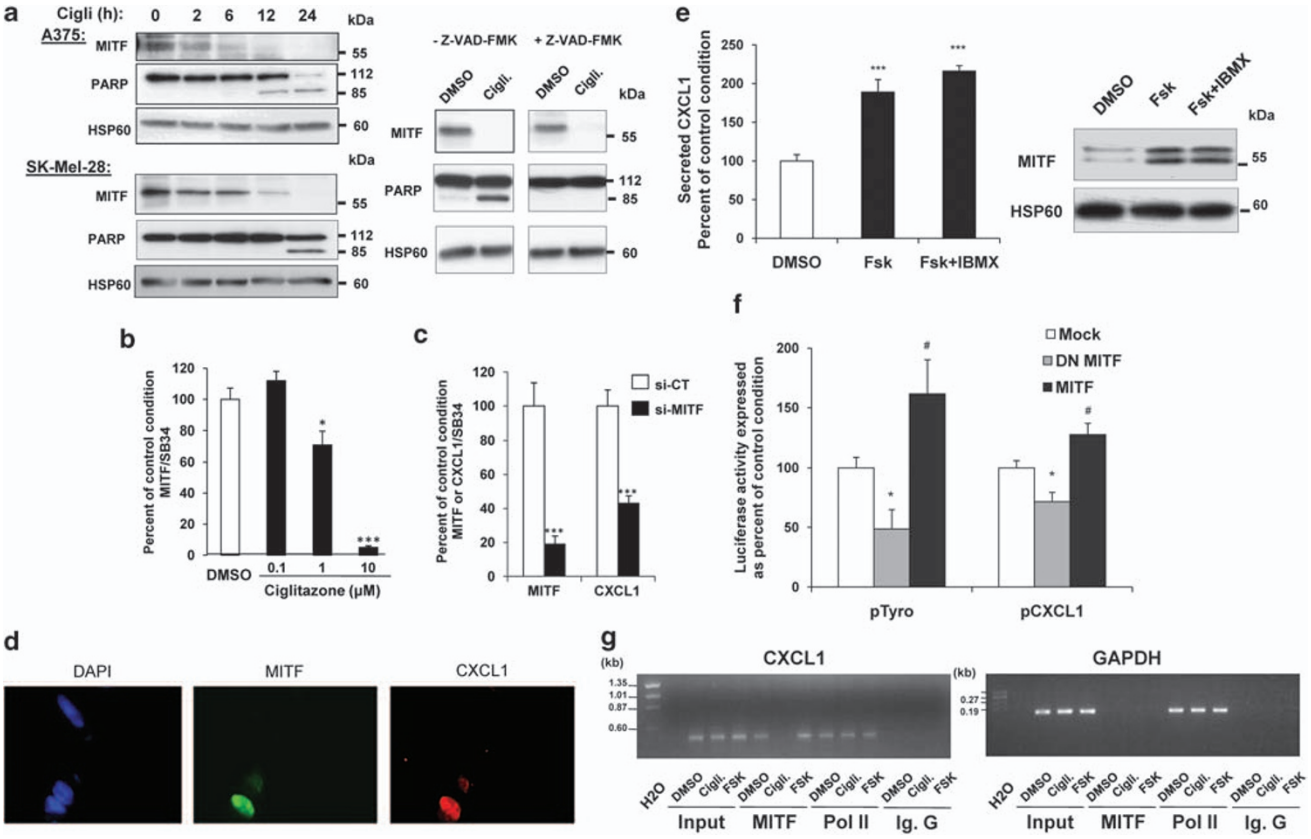


Figure 7 Decrease of CXCL1 mediated by ciglitazone involves MITF transcription factor. (**a**) On left panel, starved A375 or SK-Mel-28 melanoma cells were treated or not with 10 μM ciglitazone (Cigli.) at different times. On right panel, starved SK-Mel-28 were treated for 24 h with DMSO or ciglitazone added or not with 100 μM Z-VAD-FMK. Proteins were separated by 12% SDS-PAGE and analyzed by western blot using the indicated antibody. HSP60 was used as loading control. One representative experiment of three is shown. (**b**) Total RNA from starved A375 cells treated for 24 h with various concentrations of ciglitazone was extracted and analyzed by real-time quantitative PCR using *MITF* primers. mRNA expression was normalized using *SB34* RNA levels. Results are expressed as mean ± S.D. from three independent experiments. Significantly different from the corresponding control $^{*}P<0.05$; $^{***}P<0.001$. (**c**) Total RNA from starved A375 cells transfected for 48 h with siRNA-targeting MITF (si-MITF) or its scramble sequence (si-CT) was extracted and analyzed by real-time quantitative PCR using *MITF* and *CXCL1* primers. mRNA expression was normalized using *SB34* RNA level. Results are expressed as mean ± S.D. from three independent experiments. Significantly different from the corresponding control $^{***}P<0.001$. (**d**) A375 cells transfected with the wild type form of MITF were fixed and stained for MITF (green), CXCL1 (red) and with DAPI (blue). DAPI staining was used to identify cell nucleus. Slides were examined with a Zeiss Axiophot fluorescence microscope and pictures were taken at × 200 magnification. Representative field of three different experiments are shown. (**e**) Starved A375 melanoma cells were treated or not with 20 μM forskolin (Fsk) added or not with 100 μM IBMX for 7 h. ELISA of CXCL1 was performed on supernatants from starved A375 melanoma cells treated as described. Results are expressed in percent of control (100%). Corresponding proteins were separated by 12% SDS-PAGE and analyzed by western blot using the indicated antibody. HSP60 was used as loading control. One representative experiment of three is shown. Significantly different from the corresponding control $^{***}P<0.001$. (**f**) A375 cells were transfected with vector encoding the basal luciferase construct (Mock), wild-type MITF (WT MITF) or its dominant negative form (DN MITF) and with pTyro or pCXCL1 luciferase reporters. Measurement of luciferase activity was carried out 36 h after transfection. Variability of transfection was normalized with βGal activity and results were expressed in percent of control (100%). Data are mean ± S.D. of three independent experiments performed in triplicate. Significantly different from the corresponding control $^{*}P<0.05$; $^{\#}P<0.05$. (**g**) Chromatin immunoprecipitation assays were performed on extracts of cells treated for 24 h with DMSO, 10 μM ciglitazone (Cigli.) or with 20 μM forskolin (Fsk) for 7 h. Immunoprecipitations were performed using specific anti-MITF or anti-polymerase II (*Pol II*) antibody, and rabbit IgG (IgG) as control. Primers spanning the *CXCL1* promoter region were used for the PCR amplification. A control of PCR amplification was performed on non-immunoprecipitated extracts (*Input*). Another control was performed using a primer pair to the human GAPDH promoter

PPARγ-dependent effect on viability of melanoma cells.[7] Consistent with this finding, PPARγ silencing by siRNA does not abrogate the effects of ciglitazone on CXCL1 expression, indicating that the regulation of CXCL1 is PPARγ independent.

In normal human melanocytes, there is not basal CXCL1 production. Treatment of normal human melanocytes with TNFα stimulation increases CXCL1 secretion that is not affected by ciglitazone. Conversely, melanoma cells generally have a constitutive basal CXCL1 expression mainly due to the NF-κB pathway constitutive activation.[22] Independently of the mutational status, melanoma development stage or CXCL1 basal level, we found that ciglitazone inhibits CXCL1 production in all tested melanoma cell lines. Moreover, there is a positive correlation between the level of basal CXCL1 and the decrease in cell viability mediated by ciglitazone, suggesting that melanoma cells producing CXCL1 are addict to these cytokines.

In the same way, in other cancer cell lines such as prostate carcinoma or neuroblastoma, ciglitazone also decreases CXCL1 level and cell viability. These results support the idea that inhibition of CXCL1 cytokine could have a general role in the anti-tumoral effects of ciglitazone.

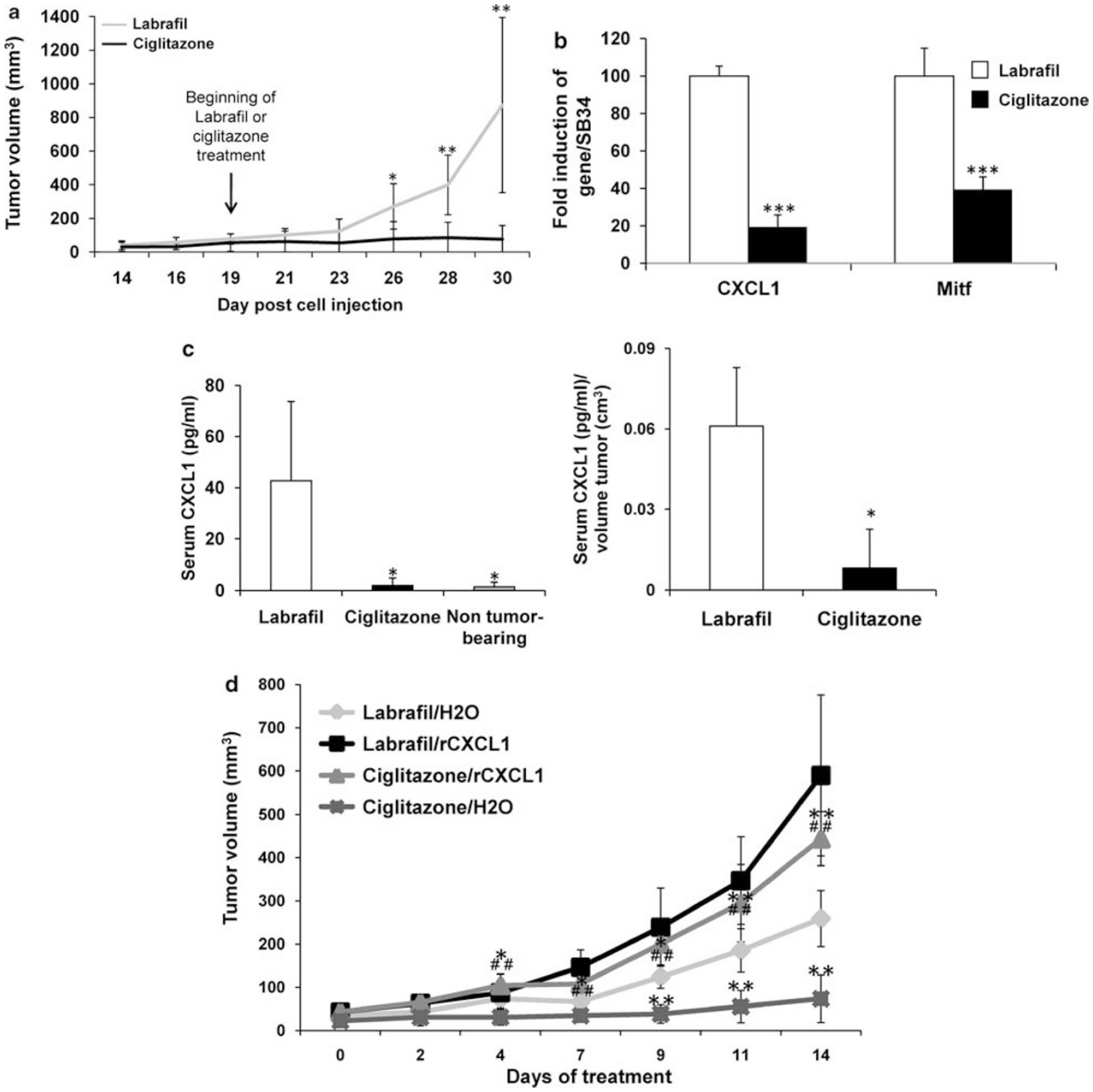


Figure 8 *In vivo* antineoplastic effects of ciglitazone correlate with decrease of MITF and CXCL1 expression. (**a**) Mice were inoculated subcutaneously with A375 melanoma cells (2.5×10^6), and after 19 days animals ($n = 6$ in each group) were treated with ciglitazone (50 mg/kg/day) or labrafil for 11 days. Growth tumor curves were determined by measuring the tumor volume using the equation $V = (L \times W^2)/2$. Significantly different from the corresponding control $^*P < 0.05$; $^{**}P < 0.01$. (**b**) Total RNA was extracted from mice tumors and analyzed by real-time quantitative PCR using *CXCL1* primers. mRNA expression was normalized using *SB34* RNA level. Significantly different from the corresponding control $^{***}P < 0.001$. (**c**) ELISA for CXCL1 was performed on mice sera from bleeding after 11 days of ciglitazone or labrafil treatment. Sera from non tumor-bearing animals were used as negative control. Data are mean ± S.D. of six samples collected in each group. Significantly different from the corresponding control $^*P < 0.05$. Ratio serum CXCL1/tumor volume (right panel). (**d**) Mice were inoculated subcutaneously with A375 melanoma cells (2.5×10^6), and after 12 days animals ($n = 6$ in each group) received an intraperitoneal injection of ciglitazone (50 mg/kg/day) or labrafil and subcutaneous peritumoral injections of human recombinant CXCL1 (200 ng/tumor/day) or water for 14 days. Growth tumor curves were determined by measuring the tumor volume using the equation $V = (L \times W^2)/2$. Significantly different from Labrafil/H2O $^*P < 0.05$; $^{**}P < 0.01$. Significant difference between Ciglitazone/H2O and Ciglitazone/CXCL1 $^{\#\#}P < 0.01$

It has been proposed that some PPARγ agonists lead to inhibition of NF-κB activation,[30–32] but in our system, the inhibition of CXCL1 by ciglitazone is not mediated by the downregulation of NF-κB activity (data not shown). Interestingly, a recent paper showed that ciglitazone led to a decrease in MITF expression[17] and *in silico* analysis of the CXCL1 promoter showed four potential E boxes that match with the consensus binding site for MITF transcription factor (data not shown), thereby suggesting the possible involvement of MITF in the control of *CXCL1* expression. These two observations

prompted us to investigate the role of MITF in the regulation of CXCL1 by ciglitazone. First, ChIP experiments and luciferase assays show that MITF binds to and regulates the *CXCL1* promoter. MITF silencing decreases *CXCL1* messengers and inhibits CXCL1 protein secretion. Additional studies suggest that MITF interacts with the *CXCL1* promoter through a responsive element (CAGGTG) at –375.

Furthermore, we have previously found that MITF is cleaved in response to the apoptosis inductor, TRAIL.[33] MITF diminution that we observed in response to ciglitazone is not due to a protein cleavage by caspases because MITF decrease is seen before apoptosis induction and is also seen on mRNA level and in the presence of pan-caspase inhibitor Z-VAD-FMK. These interesting results highlight a new specific pathway mediated by ciglitazone in melanoma cells. We have demonstrated that the negative regulation of MITF by ciglitazone is a PPARγ-independent event. However, the precise mechanism responsible for this negative regulation of MITF is not known. One possible mechanism might involve downregulation of the Wnt/β-catenin pathway because this pathway is inhibited by some PPARγ agonists and might mediate the inhibition of MITF expression.[17,18] Thus, considering all our results we show for the first time that the control of MITF expression by ciglitazone is involved in the inhibition of CXCL1 expression and secretion. This new signaling pathway involves MITF in the regulation of CXCL1 and strengthens the importance of this transcription factor in melanoma tumorigenicity.

We have previously shown that ciglitazone has cytostatic and cytotoxic effects.[7] We found that both cell cycle arrest and apoptosis evoked by ciglitazone are reversed by addition of rCXCL1 or TNFα. These data suggest that ciglitazone effects on cell viability are mediated mainly by a decrease in CXCL1 expression and secretion. In addition, rCXCL1 does not protect cells from apoptosis induced by TRAIL and staurosporine, suggesting a potential specificity of rCXCL1 on ciglitazone effects. The depletion of CXCL1 with siRNA is sufficient to mimic the biological effects of ciglitazone on A375 cells. Consequently, these results reinforce the hypothesis that inhibition of CXCL1 synthesis by ciglitazone is implied in the biological effects of this TZD. A second approach by CXCL1-neutralizing antibodies confirmed these results. Therefore, the reduction of secreted form of CXCL1 is involved in ciglitazone biological effects.

Finally, we have evaluated the correlation between potential anti-melanoma activity of ciglitazone and CXCL1 secretion in a mouse model of melanoma xenografts. We have previously demonstrated that ciglitazone prevents tumor growth development.[7] Importantly, our present results show that the short-term administration of ciglitazone not only prevents tumor formation but also reduces the volume of already established melanoma tumors. In addition, ciglitazone decreases *MITF* and *CXCL1* mRNA level in tumor xenograft, reinforcing the hypothesis that *in vivo*, MITF is also involved in the control of CXCL1 mediated by ciglitazone. In parallel, we observe a drastic reduction of serum CXCL1 level in mice. This decrease is not due to the reduction of tumor growth, because when we expressed the quantity of CXCL1 as a function of tumor size, we still found a reduction of circulating CXCL1 in ciglitazone-treated mice. Moreover, injection of CXCL1 in ciglitazone-treated mice compensates for the loss of endogenous CXCL1, impairing the effects of ciglitazone and preventing the decrease in tumor volume. From these observations, we can propose that the decrease in CXCL1 secretion mediated by ciglitazone might be responsible for the antineoplastic effects of this TZD *in vivo*.

In summary, we demonstrate for the first time that ciglitazone inhibits tumor growth through a negative regulation of the MITF/CXCL1/CXCR2 axis-signaling pathway. This demonstration brings new and additional clues to the mechanism of ciglitazone-induced melanoma cell death. Finally, taking into account the drastic effect of ciglitazone on melanoma cell growth, survival and anti-melanoma xenograft development, it might be worth evaluating ciglitazone treatment in patients with metastatic melanoma. Our reports also highlight the pivotal role of tumor cell-produced CXCL1 in melanoma cell proliferation and support the idea that CXCL1 might be used as a new progression marker in the follow-up of the metastatic melanomas. Because the overexpression of CXCL1 and the CXCL1 regulation by ciglitazone are not specific to the melanomas, our study, besides its interest in melanoma pathology, contributes to better understand the general anti-cancer effects of ciglitazone.

Materials and methods

Materials. Ciglitazone, Troglitazone and Z-VAD-FMK were purchased from Biomol (Tebu, Le Perray en Yvelines, France). Pioglitazone and Rosiglitazone were from Cayman Chemical Company (Ann Arbor, MI, USA). DMSO, staurosporine, forskolin, 3-Isobutyl-1-methylxanthine (IBMX), hydrocortisone, insulin, phorbol-12 myristate 13-acetate, MCDB 153 medium, sodium fluoride, dimethylacetamide, Hoechst 33258, Tween 80, sodium orthovanadate, 4-(2-aminoethyl)-benzenesulfonyl fluoride, aprotinin and leupeptin were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). Labrafil M 1944 Cs was purchased from Gattefossé (Saint-Priest, France). The caspase substrates and the caspase inhibitors were from MERCK Eurolab (Fontenay-sous-Bois, France). Trypan blue, Dulbecco's modified Eagle's medium (DMEM), penicillin/streptomycin and trypsin were from Invitrogen (Pontoise, France); Fetal Calf Serum (FCS) from Hyclone (Brevieres, France). TRAIL was obtained from R&D Systems (Lille, France). Recombinant human TNFα and recombinant human CXCL1 were purchased from Peprotech (Neuilly-sur-Seine, France).

Cell cultures. Normal human melanocytes were prepared and maintained as described.[34] Human A375 (CRL-1619), SK-Mel-28 (HTB-72) and MeWo (HTB-65) melanoma cells, prostate cancer PC-3 (CRL-1435) and neuroblastoma SH-SY5Y (CRL-2266) cell lines were purchased from American Tissue Culture Collection (Molsheim, France) and grown in DMEM medium supplemented with 10% FCS and penicillin/streptomycin (100 U/ml/50 μg/ml). Human WM793 vertical growth phase melanoma cell line and 1205 Lu metastatic melanoma cell line were generously provided by Dr M Herlyn (Wistar Institute, Philadelphia, PA, USA) and maintained as described.[35] For each experiment, cells were starved in appropriate medium without FCS during 14 hours before drug stimulation.

Antibodies. HSP60 was from Santa Cruz Biotechnology (TEBU; Le Perray en Yvelines, France). The polyclonal pro-caspase 9, monoclonal pro-caspase 8, monoclonal pro-caspase 3 and polyclonal PARP antibodies were from Cell Signaling Technology (Ozyme, Saint-Quentin-en-Yveline, France). Monoclonal anti-SPARC was purchased from Haematological Technologies Inc. (Essex Junction, VT, USA). Monoclonal MITF was purchased from Spring Bioscience (Fremont, CA, USA). Monoclonal CXCL1 and CXCR2 were purchased from R&D Systems (Lille, France).

Real-time quantitative PCR (Q-PCR). For gene array analysis, total RNA was isolated using TRIZOL (Invitrogen). After treatment with Dnase I, 2 μg of RNA were reverse transcribed using the high capacity cDNA archive random priming Kit (Applied Biosystems). The expression level of 19 genes related to melanoma

secretome was evaluated using an ABI Biosystems 7900HT Sequence Detector System and the SYBR Green dye detection protocol as outlined by the manufacturer (Applied Biosystems). Gene-specific primers were designed using the Primer Express software (Applied Biosystems). Relative expression level of target genes was normalized for RNA concentrations of four different housekeeping genes (GAPDH, β-actin, HPRT and ubiquitin). For each sample, C_t values for the housekeeping genes were determined for normalization purposes, and delta C_t (ΔC_t) between the mean of housekeeping-gene values and target-gene values was calculated. Relative expression level of target genes mRNA between DMSO control cells (DMSO) and ciglitazone-treated cells (cigli.) was calculated using the formula ΔC_tCigli.—ΔCTDMSO and expressed as fold over control ($2\Delta\Delta$ C_t). Values represent the mean of duplicates and are representative of two independent experiments. Other Q-PCR were performed exactly as described.[36]

Cell viability test. Cell viability was assessed using the *Cell Proliferation Kit II* (XTT; Roche Diagnostics, Meylan, France) according to the manufacturer's protocol. Cell viability is expressed as the percentage of the value in DMSO-treated cells.

ELISA. CXCL1 ELISA was performed using *Quantikine Human CXCL1/GROα* from R&D Systems (Lille, France) according to the manufacturer's protocol.

Immunoflurescence microscopy. Monolayers prepared for fluorescent staining were grown on glass coverslips. Immunofluorescence experiments were carried out as described.[7]

Western blot assays. Western blot analyses were performed as described.[7]

Caspase activity. Caspase activities were carried out exactly as described.[7]

Flow cytometry analysis. All flow cytometry analyses were performed using the FL2 channel of a FACScan (Becton Dickinson; Cowley, UK) and data were analyzed with CellQuest software as previously described.[7] Annexin-V staining was performed using *Annexin-V-FLUOS Staining Kit* (Roche Diagnostics, Meylan, France) according to the manufacturer's protocol.

Small interfering RNA. Small interfering RNA (siRNA) experiments were carried out exactly as described.[7] siRNA-targeting CXCL1 was obtained from Santa Cruz Biotechnology (sc-43816). siRNA-targeting MITF was described previously.[33] As nonspecific control, a scramble sequence for CXCL1 or MITF siRNA was used.

Construction of the CXCL1 reporter plasmid. A 1.5-kb fragment 5′ of the transcriptional site of the CXCL1 gene was amplified by PCR and isolated by Topo Cloning Kit (Invitrogen) from genomic DNA. The 1.5-kb *Xho*I/*Sac*I fragment was then subcloned using Rapid DNA Ligation Kit (Roche) into the unique *Xho*I/*Sac*I restriction site of the Promega pGL3 basic vector (pGL3b) upstream of the luciferase coding sequence (pCXCL1; $-1477/+77$).

Transfections and luciferase assays. Transfections and luciferase assays were performed as described.[7] The reporter plasmid containing the 2.2-kb fragment of the mouse tyrosinase promoter (pTyro; $-2{,}236/+59$) was described elsewhere.[37] Plasmids coding for microphthalmia-coding sequence (WT Mitf) or its dominant-negative form containing an inframe deletion of the NH_2-terminal domain (DN Mitf) in pCDNA$_3$ expression vector are described elsewhere.[13]

Chromatin immunoprecipitation assay. SK-Mel-28 cells were cultured in 100-cm^2 culture dishes, stimulated or not with ciglitazone or forskolin for 24 h, and then treated with 1% formaldehyde for 10 min at room temperature. Next, the cells were harvested, centrifuged ($700 \times g$, 5 min at 4°C), and resuspended in SDS lysis buffer (EZ-ChIP Chromatin Immunoprecipitation Kit; Upstate). After sonication the sheared chromatin was immunoprecipitated using indicated antibody. Then, protein/DNA cross-linking complexes were reversed by heat treatment (65°C overnight) and proteinase K digestion. The genomic captured fragments were purified using spin columns. Identification of the captured DNA fragments was performed by PCR analysis using the CXCL1 or GAPDH promoter primers. Thirty-two cycles of PCR were performed, and the amplified products were analyzed on a 2.5% agarose gel.

***In vivo* murine cancer model.** Animal experiments were carried out in accordance with the Declaration of Helsinki and were approved by a local ethical committee. Female immune-deficient BALB/c *nu/nu* (nude) mice were obtained at 6 weeks of age from Harlan Laboratory.

Mice were inoculated subcutaneously with A375 melanoma cells (2.5×10^6 cells/mouse). After 19 days, animals received intraperitoneal injection of ciglitazone (50 mg/kg/day) dissolved in a mixture of Labrafil M 1944 Cs (an amphiphilic oil, Oleic Macrogol-6 Glyceride), dimethylacetamide, and Tween 80 (90:9:1%, vol/vol/vol) as previously described.[38]

The growth tumor curves were determined by measuring the tumor volume using the equation $V = (L \times W^2)/2$. After 11 days of treatment, mice were bled and sera were analyzed using ELISA of CXCL1. At the end of the experiment, mice were killed by CO_2 inhalation and tumors were taken for RNA extraction.

Statistical analysis. Data presented are mean ± S.D. of three independent experiments performed in triplicate. Statistical significance was assessed using the Student's *t*-test except for *in vivo* experiments, in which statistical significance was assessed using two-tailed Wilcoxon's rank sum test. A value of $P<0.05$ was accepted as statistically significant.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)