Down-Regulation of SOX2 Underlies the Inhibitory Effects of the Triphenylmethane Gentian Violet on Melanoma Cell Self-Renewal and Survival

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Human melanomas contain a population of tumor-initiating cells that are able to maintain the growth of the tumor. We previously showed that the embryonic transcription factor SOX2 is essential for self-renewal and tumorigenicity of human melanoma-initiating cells. However, targeting a transcription factor is still challenging. Gentian violet (GV) is a cationic triphenylmethane dye with potent antifungal and antibacterial activity. Recently, a combination therapy of imiquimod and GV has shown an inhibitory effect against melanoma metastases. Whether and how GV affects melanoma cells remains unknown. Here we show that GV represses melanoma stem cell self-renewal through inhibition of SOX2. Mechanistically, GV hinders EGFR activation and inhibits the signal transducer and activator of transcription-3 [(STAT3)/SOX2] axis. Importantly, we show that GV treatment decreases STAT3 phosphorylation at residue tyrosine 705, thus preventing the translocation of STAT3 into the nucleus and its binding to *SOX2* promoter. In addition, GV affects melanoma cell growth by promoting mitochondrial apoptosis and G2 cell cycle arrest. This study shows that in melanoma, GV affects both the stem cell and the tumor bulk compartments, suggesting the potential use of GV in treating human melanoma alone or in combination with targeted therapy and/or immunotherapy.

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INTRODUCTION

Cutaneous melanoma is one of the most aggressive types of skin cancer. Early-stage melanoma can be cured in most patients by surgical excision. On the contrary, once disseminated, melanoma becomes a highly lethal condition. Targeted therapies and immunotherapies have improved progression-free and overall survival in this disease, extending median survival from 9 to over 25 months. However, resistance emerges in most patients treated with targeted therapy, and not all patients respond to immunotherapy; therefore, most patients still die from metastatic disease (Lo and Fisher, 2014).

Accumulating evidence supports the existence of cancer stem cells in human melanomas that possess the ability to self-renew and differentiate into tumor bulk cells. These cells drive tumor initiation, progression, and metastasis, and they might contribute to relapse after therapy (Murphy et al., 2014). Our group has previously shown that the embryonic transcription factor SOX2 is required for self-renewal and tumorigenicity of human melanoma-initiating cells (MICs) (Santini et al., 2014). Although preclinical studies began to evaluate strategies to inhibit SOX2 (Favaro et al., 2014; Narasimhan et al., 2011; Stolzenburg et al., 2012), the possibility to selectively target SOX2 remains a challenge.

The cationic triphenylmethane pharmacophore gentian violet (GV) has been used for a long time as an antimycotic and antibacterial agent (Docampo and Moreno, 1990). Recent studies suggest that GV has also a potent inhibitory activity against reduced nicotinamide adenine dinucleotide phosphate oxidases (NOX) Nox2 and Nox4 (Bhandarkar et al., 2009), whose expression is linked to tumor progression, and that it is able to induce cell death by disrupting the mitochondrial system (Zhang et al., 2011). A recent study reported a case of cutaneous melanoma metastasis successfully treated with a combination of GV and imiquimod. The patient was free of melanoma with no recurrence for 2 years, until death from congestive heart failure at 94 years of age (Arbiser et al., 2012; Bonner and Arbiser, 2014). These findings prompted us to investigate the mechanism of action of GV in melanoma.

Here, we show that GV impairs melanoma stem cell self-renewal and survival through the inhibition of SOX2.

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Abbreviations: 7-AAD, 7-amino actinomycin D; ALDH, aldehyde dehydrogenase; GV, gentian violet; MIC, melanoma initiating cells; NOX, reduced nicotinamide adenine dinucleotide phosphate oxidase; ROS, reactive oxygen species; STAT, signal transducer and activator of transcription

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Mechanistically, GV negatively affects the signal transducer and activator of transcription (STAT) 3/SOX2 axis by reducing STAT3 phosphorylation, with consequent decreased STAT3 translocation into the nucleus and binding to *SOX2* promoter. In addition, we show that GV exerts a potent antiproliferative effect on melanoma cells by promoting mitochondrial apoptosis and G2 cell cycle arrest.

RESULTS

GV inhibits self-renewal and survival of melanoma spheres

Melanomas contain a subpopulation of MICs, which drives melanoma initiation and progression and is responsible for tumor relapse after therapy (Murphy et al., 2014). Hence, we tested whether GV administration might affect the behavior of melanoma spheres, which display self-renewal ability and mimic the original tumor after transplantation into athymic nude mice (Santini et al., 2012). Treatment of M33x, SSM2c, and A375 cells with increasing doses (0.1, 0.25 and 0.5 μmol/L) of GV led to a dose-dependent reduction in the number of primary spheres and a progressive loss of their ability to self-renew as secondary spheres (Figure 1a and b). GV-treated primary and secondary melanoma spheres were reduced in size (Figure 1c), thus suggesting an effect on proliferation and/or death of progenitors or more differentiated cells composing the sphere. Indeed, GV decreased proliferation, as determined by analysis of BrdU incorporation, and increased both early and late apoptosis and the number of cells testing positive for cleaved caspase-3 (Figure 1d–f).

We next assessed whether GV might affect the expression of stemness genes. Quantitative real-time PCR analysis showed that GV treatment consistently reduced *SOX2* mRNA levels in M33x, SSM2c, and A375 melanoma spheres (see Supplementary Figure S1a online). *KLF4* expression was not significantly affected by GV treatment, whereas *OCT4* was slightly decreased in SSM2c and increased in M33x and A375 spheres (see Supplementary Figure S1a), likely because of a compensatory effect. Western blot analysis confirmed the GV-mediated decrease of SOX2 expression also at the protein level in both melanoma cells and their derived melanoma spheres (Figure 1g). Interestingly, SOX2 protein levels correlated with sphere-formation and self-renewal abilities of M33x, SSM2c, and A375 cells treated with increasing doses of GV (Figure 1g vs. Figure 1a). Collectively, these data indicate that GV impairs self-renewal and survival of melanoma spheres by reducing the expression of the embryonic transcription factor SOX2.

GV affects maintenance of putative melanoma stem cells

As an alternative approach to test the effect of GV on melanoma stem cells, we sorted melanoma cells with high aldehyde dehydrogenase ($ALDH^{high}$) activity (Luo et al., 2012; Santini et al., 2012). Cytometric analysis showed a significant reduction in the number of $ALDH^{high}$ M33x, SSM2c, and A375 cells treated with increasing doses of GV (Figure 2a and b). Treatment with GV reduced the expression of SOX2 in $ALDH^{high}$ putative MICs and, to a lesser extent, in $ALDH^{low}$ tumor bulk cells (Figure 2c). Moreover, GV drastically reduced the self-renewal ability of $ALDH^{high}$, and only marginally that of $ALDH^{low}$ spheres (Figure 2d), consistent with lower SOX2 levels in the $ALDH^{low}$ subpopulation (Santini et al., 2014). AnnexinV/7-amino actinomycin D (7-AAD) staining indicated that GV increased apoptosis in both $ALDH^{high}$ and $ALDH^{low}$ subpopulations (Figure 2e), as also shown by the drastic reduction of the anti-apoptotic factor BCL2 (Figure 2c). To determine whether GV-induced cell death in $ALDH^{high}$ cells could be attributable to the ability of GV to lead to a more differentiated status characterized by lower ALDH activity, we sorted both $ALDH^{high}$ and $ALDH^{low}$ fractions after GV treatment (Figure 2f). Western blot analysis showed activation of caspase-3 only in the GV-enriched $ALDH^{low}$ fraction. Notably, no induction of apoptosis was observed in the residual $ALDH^{high}$ subpopulation (Figure 2g). In an effort to provide additional evidence that GV affects melanoma stem cells, we used CD271/$p75^{NTR}$, an established melanoma stem cell marker (Boiko et al., 2010; Civenni et al., 2011). FACS analysis showed that GV treatment reduced the number of $CD271^{+}$ putative melanoma stem cells in a dose-dependent manner in all three cell types (see Supplementary Figure S1b and c), confirming results obtained with Aldefluor (StemCell Technologies, Vancouver, Canada) and sphere assays. Altogether, these results indicate that GV decreases MIC survival and self-renewal by affecting cancer stem cell features.

Enhanced expression of SOX2 partially rescues the inhibitory effect of GV on melanoma stem cell self-renewal

We have previously shown that SOX2 is critical for the maintenance of melanoma stem cells and for the survival and proliferation of melanoma cells that constitute the tumor bulk (Santini et al., 2014). Our data indicate that GV significantly decreases SOX2 expression in melanoma cells and MICs; therefore, we assessed whether SOX2 is involved in mediating the effects of GV on MIC self-renewal and survival. To test this hypothesis, we overexpressed SOX2 in patient-derived melanoma cells SSM2c and M33x, using a replication-incompetent lentivirus expressing SOX2 (LV-SOX2) (Figure 3a). SOX2 overexpression reduced (in M33x spheres) and nearly abolished (in SSM2c spheres) the inhibitory effect of GV on sphere formation (Figure 3b) and self-renewal as secondary spheres (Figure 3c). On the other hand, GV was able to overcome the effect of SOX2 on survival of more differentiated progenitors composing the sphere. Indeed, ectopic SOX2 expression failed to prevent the decrease in sphere size observed after GV treatment (Figure 3d). Consistently, AnnexinV/7-AAD staining confirmed the induction of apoptosis in both control and SOX2-overexpressing spheres (Figure 3e). These results suggest that enhanced SOX2 expression is able to counteract the apoptotic effect of GV on sphere-initiating cells but does not prevent the apoptotic effect of GV on the tumor bulk. Altogether these data indicate that down-regulation of SOX2 mediates, at least in part, the inhibitory effect of GV on melanoma stem cell self-renewal.

GV inhibits melanoma cell viability by promoting mitochondrial apoptosis and G2 cell cycle arrest

Our data indicate that GV acts through two parallel mechanisms: the impairment of self-renewal ability of MICs in a SOX2-dependent manner and the decrease of survival and proliferation of melanoma tumor bulk cells independently of SOX2 (Figure 3). To better define the pro-apoptotic and

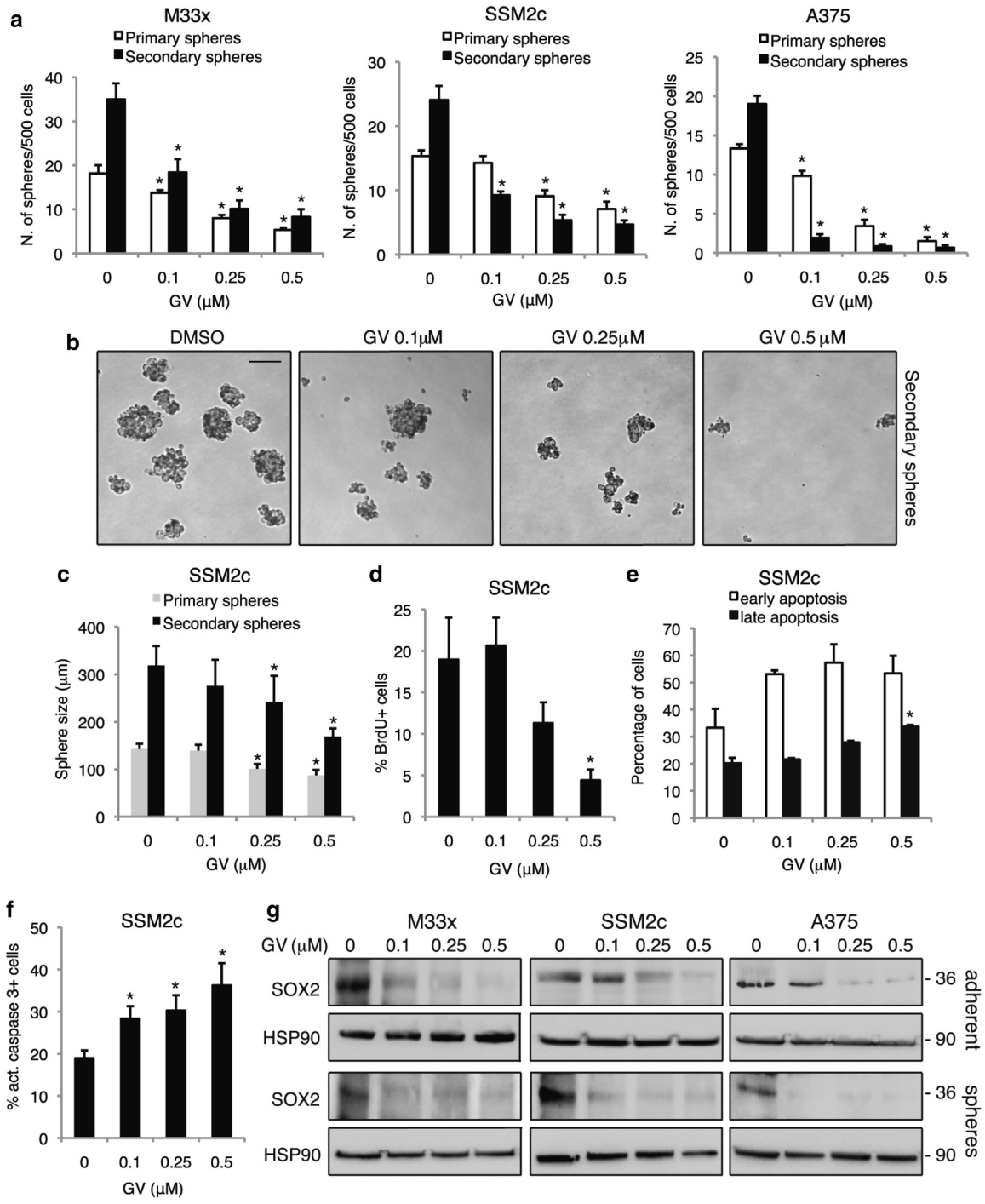

Figure 1. GV inhibits melanoma sphere self-renewal and survival. (**a**) Primary and secondary melanoma spheres. Melanoma cells were treated for 72 hours during primary sphere formation and left untreated to form secondary spheres. (**b**) Representative phase-contrast images of secondary SSM2c spheres as indicated in **a**. Scale bar = 100 μm. (**c**) Size of primary and secondary spheres treated with increasing doses of GV. (**d**) Quantification of BrdU incorporation and (**e**) of early (annexin V^+/7-AAD^-) and late (annexin V^+/7-AAD^+) apoptotic cells in SSM2c primary spheres after GV treatment. (**f**) Percentage of caspase 3-positive melanoma cells after GV treatment. (**g**) Western blot of SOX2 in adherent melanoma cells and spheres after GV treatment. HSP90 was used as a loading control. Data shown are mean ± standard error of the mean. n = 3. $^*P \leq 0.05$ versus control. 7-AAD, 7-amino actinomycin D; GV, gentian violet; N, number.

antiproliferative effects of GV we used adherent M33x, SSM2c, A375, M51, and SK-MEL-28 melanoma cells. GV treatment resulted in a dose-dependent decrease of melanoma cell viability in all cell types (Figure 4a and see Supplementary Figure S2a online). Cell cycle analysis showed that GV induced a dose-dependent increase in the percentage of cells in the G2 phase to the detriment of G0/G1 in SSM2c and M33x cells and, to a lesser extent, in A375 (Figure 4b). Consistently, the expression level of the G2/M checkpoint regulator cyclin B1 was decreased in a dose-dependent manner in all three cell lines (Figure 4c). Conversely, GV failed to induce G2 cell cycle arrest in M51

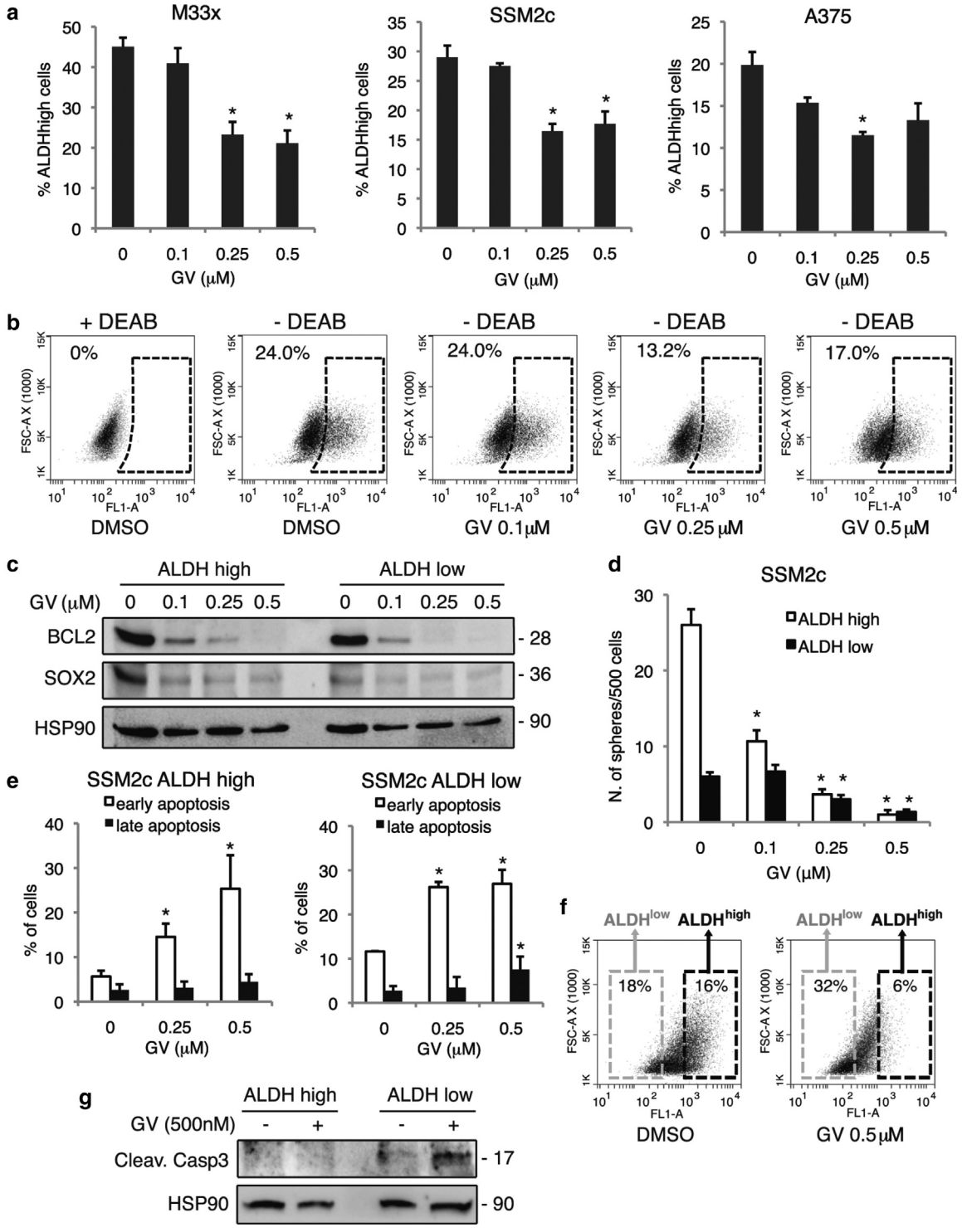

Figure 2. GV reduces the number and self-renewal of ALDH^{high} melanoma stem cells. (**a, b**) Quantification and representative images of ALDH^{high} cells after GV treatment for 24 hours. ALDH inhibitor diethylaminobenzaldehyde was used as negative control. (**c**) Western blot of BCL2 and SOX2 in ALDH^{high} and ALDH^{low} subpopulations after GV treatment. (**d**) Number of secondary spheres in ALDH^{high} and ALDH^{low} SSM2c cells treated with GV. (**e**) Cytometric analysis of apoptotic cells in ALDH^{high} and ALDH^{low} fractions 48 hours after GV treatment. (**f**) Representative images of sorted ALDH^{high} and ALDH^{low} subpopulations. Sorting gates were set at least one logarithm apart from the relative baseline fluorescence (drawn after the incubation with DEAB). (**g**) Western blot of cleaved caspase-3 in ALDH^{low}- and ALDH^{high}-sorted cells, as shown in **f**. HSP90 was used as loading control. Data shown are mean ± standard error of the mean. n = 3. *$P \leq 0.05$ versus control. ALDH, aldehyde dehydrogenase; Cleav, cleaved; DEAB, diethylaminobenzaldehyde; GV, gentian violet; N, number.

and SK-MEL-28 melanoma cells (see Supplementary Figure S2b). AnnexinV/7-AAD staining showed dose-dependent induction of both early (annexin V$^+$/7-AAD$^-$) and late (annexin V$^+$/7-AAD$^+$) apoptosis in all melanoma cells treated with increasing doses of GV (Figure 4d and see Supplementary Figure S2c).

A previous study showed that cationic triphenylmethanes can induce cell death by disrupting the mitochondrial

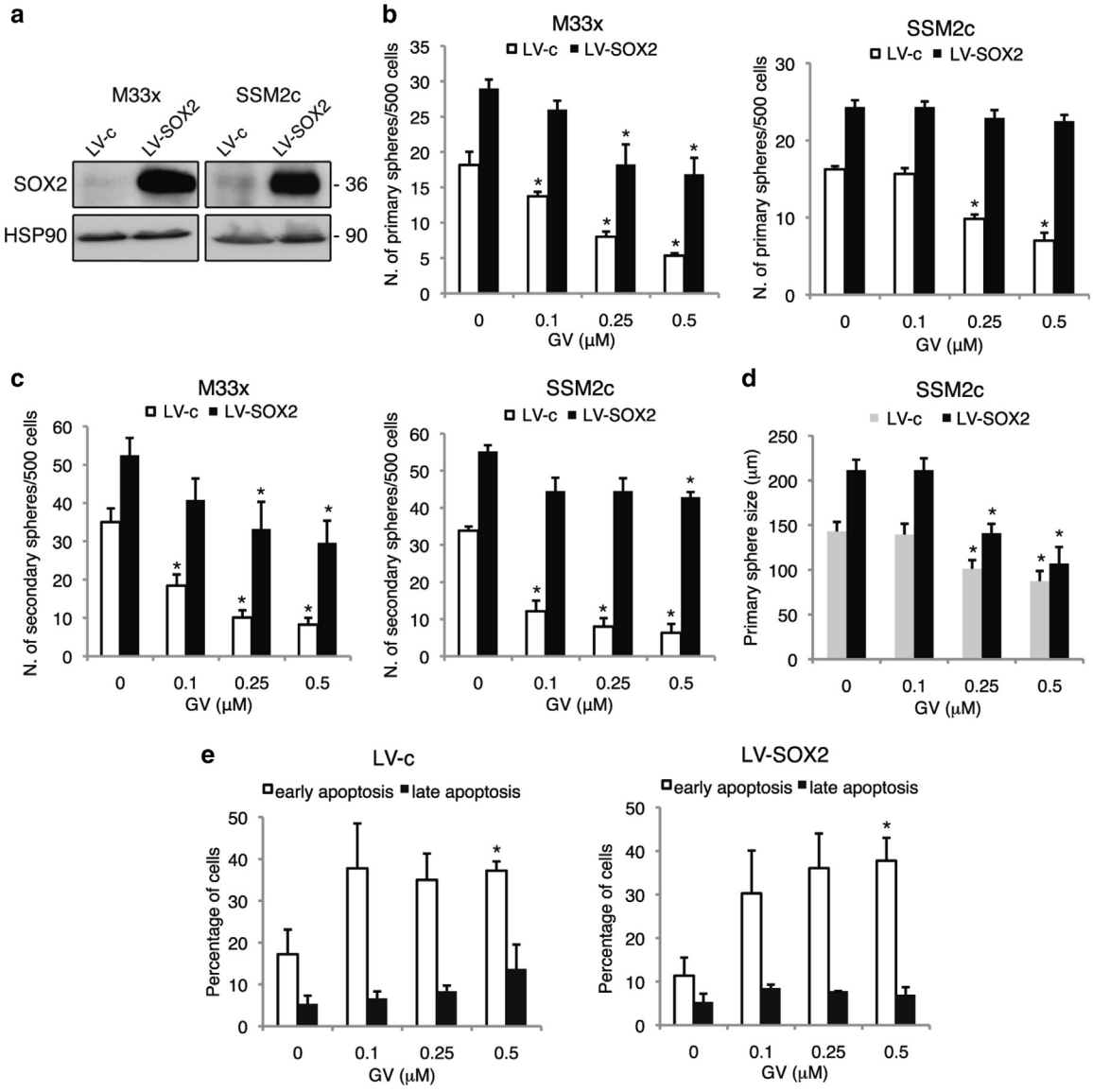

Figure 3. SOX2 mediates the inhibitory effect of GV on melanoma stem cell self-renewal. (**a**) Western blot of SOX2 in M33x and SSM2c spheres transduced with LV-c or LV-SOX2. HSP90 was used as loading control. (**b**) Primary and (**c**) secondary sphere assay shows that ectopic SOX2 counteracts the decrease in the number of spheres induced by GV treatment. (**d**) Size of primary SSM2c spheres transduced with LV-c and LV-SOX2. (**e**) Quantification of the percentage of early (annexin V^+/7-AAD^-) and late (annexin V^+/7-AAD^+) apoptotic cells in SSM2c spheres transduced with LV-c and LV-SOX2. Data shown are mean ± standard error of the mean. n = 3. *$P \leq 0.05$ versus control. GV, gentian violet.

system, followed by release of cytochrome C and apoptosis-inducing factors (Zhang et al., 2011). Indeed, GV treatment increased the BAX/BCL2 ratio, an indicator of susceptibility to mitochondrial apoptosis (Oltvai et al., 1993), and induced cleaved caspase-3 in all cell lines at nanomolar doses (Figure 4c and see Supplementary Figure S2d). In addition, treatment with a high dose of GV (≥ 0.5 μmol/L in SSM2c and ≥0.25 μmol/L in A375) induced an increase of the cytoplasmic fraction of cytochrome C (Figure 4e) and an enhancement of mitochondrial reactive oxygen species (ROS) production (Figure 4f), suggesting that high doses of GV activate the mitochondrial apoptotic pathway in melanoma cells. Inhibition of mitochondrial ROS with 2-(2,2,6,6-tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl) triphenylphosphonium chloride (i.e., mitoTEMPO), a specific scavenger of mitochondrial superoxide (Dikalova et al., 2010), partially prevented late apoptosis without affecting early apoptosis in all melanoma cell types (see Supplementary Figure S3 online).

We next tested whether GV treatment might affect p53 activity (Garufi et al., 2014). Although GV increased total p53 levels in A375 but not in SSM2c and M33x cells (Figure 4c), quantitative real-time PCR analysis showed that GV treatment increased the expression of the p53-target genes *PUMA, PIG3,* and, partially, *NOXA* in all cell types (see Supplementary Figure S4a online). In addition, silencing of p53 in A375 and SSM2c cells decreased basal apoptosis compared with in control cells and reduced sensitivity to GV in terms of early apoptosis (see Supplementary Figure S4b–d), suggesting that GV-induced cell death is partially dependent on p53 function.

Altogether these data indicate that GV inhibits melanoma cell growth with different dose-dependent effects: at low

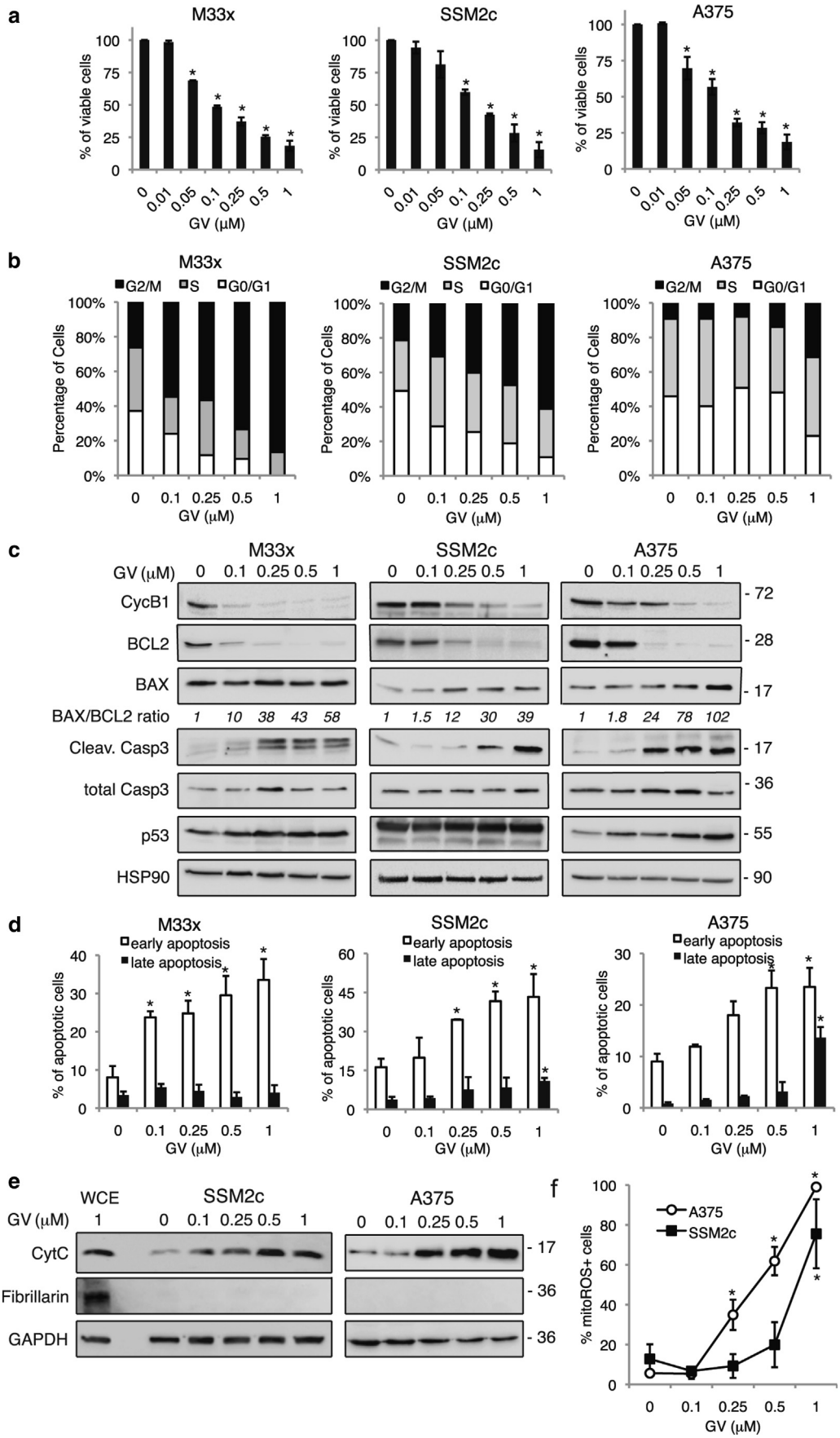

Figure 4. GV impairs melanoma cell viability by promoting mitochondrial apoptosis and G2 cell cycle arrest. (**a**) Cell viability assay 48 hours after GV treatment. Controls (DMSO) were set to 100%. (**b**) Cell cycle analysis after GV treatment (48 hours). (**c**) Western blot of cyclin B1, BCL2, BAX, total and cleaved

doses it induces G2 cell cycle arrest, whereas at high doses it mainly promotes mitochondrial apoptosis.

GV suppresses STAT3 activation through an EGFR-dependent mechanism

Triphenylmethane dyes have been show to inhibit ROS production by targeting Nox4 and Nox2 in different biological contexts (Bhandarkar et al., 2009; Mukawera et al., 2015; Perry et al., 2006). Expression of NOX genes was investigated in six patient-derived primary melanoma cells, in four melanoma cell lines and in normal human epidermal melanocytes. Quantitative real-time PCR showed that *NOX1* and *NOX4* were variably expressed among melanoma cells, whereas *NOX2* was expressed in only two cell lines (see Supplementary Figure S5a online). One possible explanation for lack of G2 cell cycle arrest in M51 and SK-MEL-28 melanoma cells upon GV treatment (see Supplementary Figure S2b) might be the absence of NOX4 expression in these two cell lines (Yamaura et al., 2009). To determine whether GV inhibited ROS production in melanoma cells, we used different assays. Using the oxidant-sensing fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (i.e., DCFH-DA), we failed to observe a decrease of intracellular ROS upon GV treatment in M33x and SSM2c cells. Unexpectedly, we documented an increase in ROS level in A375 cells, which was also confirmed by confocal microscopy using CellRox dye (CellRox, Tel Aviv, Israel) (see Supplementary Figure S5b and c). Consistently, GV treatment decreased the reduced form of Src in A375 cells without affecting phosphorylated Src (see Supplementary Figure S5d and e).

A previous study showed that triphenylmethane analogs exert a strong inhibitory activity against tyrosine-kinase receptors (Antipova et al., 2008). Therefore, we hypothesized that GV might act on EGFR, which is expressed in almost all melanoma cell lines (see Supplementary Figure S6a online). To test whether GV affects EGFR activation, we immunoprecipitated endogenous EGFR and performed Western blot with an anti-phospho-Tyr antibody. The experiment showed that GV treatment reduced EGFR phosphorylation but did not change total levels of EGFR (Figure 5a). Consistently, GV reduced phospho-AKT (Ser473) and phospho−extracellular signal-regulated kinase−1/2 levels (see Supplementary Figure S6b), two downstream mediators of EGFR activation. GV strongly decreased STAT3 phosphorylation at Tyr^{705} and hence its activation both in adherent and melanoma spheres (Figure 5b), thus suggesting the existence of an inhibitory mechanism on STAT3.

To further confirm the involvement of EGFR on STAT3 activation, melanoma cells were treated with GV alone or in the presence of the EGFR inhibitor gefitinib (Patel et al., 2011; Wakeling et al., 2002). Western blot analysis showed that gefitinib down-regulated STAT3 by itself, and that combination of gefitinib and GV prevented the effect of GV on STAT3 activation (see Supplementary Figure S6c). Furthermore, treatment of melanoma cells with ruxolitinib, a specific JAK1/2 inhibitor (Ostojic et al., 2011), completely abrogated STAT3 phosphorylation (see Supplementary Figure S6d). Altogether, these data suggest that GV reduces phospho-STAT3 by inhibiting EGFR activation, likely through a JAK-dependent and ROS/Src-independent mechanism.

The inhibitory effect of GV on EGFR activation might also explain the induction of apoptosis on adherent melanoma cells. PI3K/AKT signaling has been shown to inhibit programmed cell death through a shift from pro- to anti-apoptotic signals (Fulda, 2012) or by inducing Mdm2-mediated p53 degradation (Ogawara et al., 2002). Consistently, our data showed that inhibition of PI3K signaling by LY294002 (Aziz et al., 2009) primed melanoma cells to mitochondrial apoptosis and minimized the ability of GV to further enhance both early and late apoptosis (see Supplementary Figure S7 online).

GV down-regulates SOX2 expression by inhibiting STAT3 in melanoma cells

STAT3 phosphorylation at tyrosine 705 has been shown to mediate STAT3 translocation into the nucleus as a dimer, thus allowing dimeric STAT3 to bind target genes and to promote gene transcription (Darnell, 1997). Western blot analysis of STAT3 confirmed that treatment with GV reduced nuclear STAT3 levels and increased its cytoplasmic fraction in both SSM2c and A375 melanoma cells (Figure 5c).

Because STAT3 positively correlates with SOX2 in several contexts (Foshay and Gallicano, 2008; Zhao et al., 2015), we investigated whether STAT3 directly regulates SOX2 expression in melanoma cells. Bioinformatic analysis identified a putative site possessing the STAT3 canonical binding motif $TTC(N)_{2\text{-}4}GAA$ (Darnell, 1997; Ehret et al., 2001) within the proximal region of the *SOX2* promoter (−447 base pairs [bp]/−426 bp) (Figure 5d). Chromatin immunoprecipitation in melanoma cells showed STAT3 bound to *SOX2* promoter at approximately 400 bp upstream to the SOX2 transcription start site (Figure 5d). Interestingly, treatment with GV prevented STAT3 binding to *SOX2* promoter, consistent with its cytoplasmic accumulation (Figure 5c). To confirm that SOX2 is a direct transcriptional target of STAT3 in melanoma, we co-transfected STAT3 expression construct with the *SOX2* promoter driven by a luciferase reporter (−601bp/+293bp), as previously described (Eberl et al., 2012). Luciferase assay showed that STAT3 increased SOX2 promoter activity by 3-fold and that treatment with GV reverted the effect of STAT3 on the endogenous *SOX2* promoter (Figure 5e), indicating that GV reduces SOX2 expression through inhibition of STAT3 transcriptional activity. To further validate these results, we performed site-directed mutagenesis to mutate the tyrosine in position 705 into a glutamic acid to mimic STAT3

caspase-3, and p53 in melanoma cells treated with GV for 48 hours. HSP90 was used as loading control. Densitometric quantification of BAX/BCL2 ratio is shown. (**d**) Cytometric analysis of apoptotic cells after GV treatment for 48 hours. (**e**) Western blot of cytochrome C in cytosolic extracts 48 hours after GV treatment. Fibrillarin (nuclear) and GAPDH (cytosolic) were used as loading control. (**f**) Quantification of mitochondrial ROS in cells treated with GV, stained with MitoSOX (Thermo Fisher Scientific, Waltham, MA) and analyzed by fluorescence-activated cell sorting. Data shown are mean ± standard error of the mean. n = 3. $*P \leq 0.05$ versus control. Cleav, cleaved; Cytc, cytochrome; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GV, gentian violet; ROS, reactive oxygen species; WCE, whole cell extract.

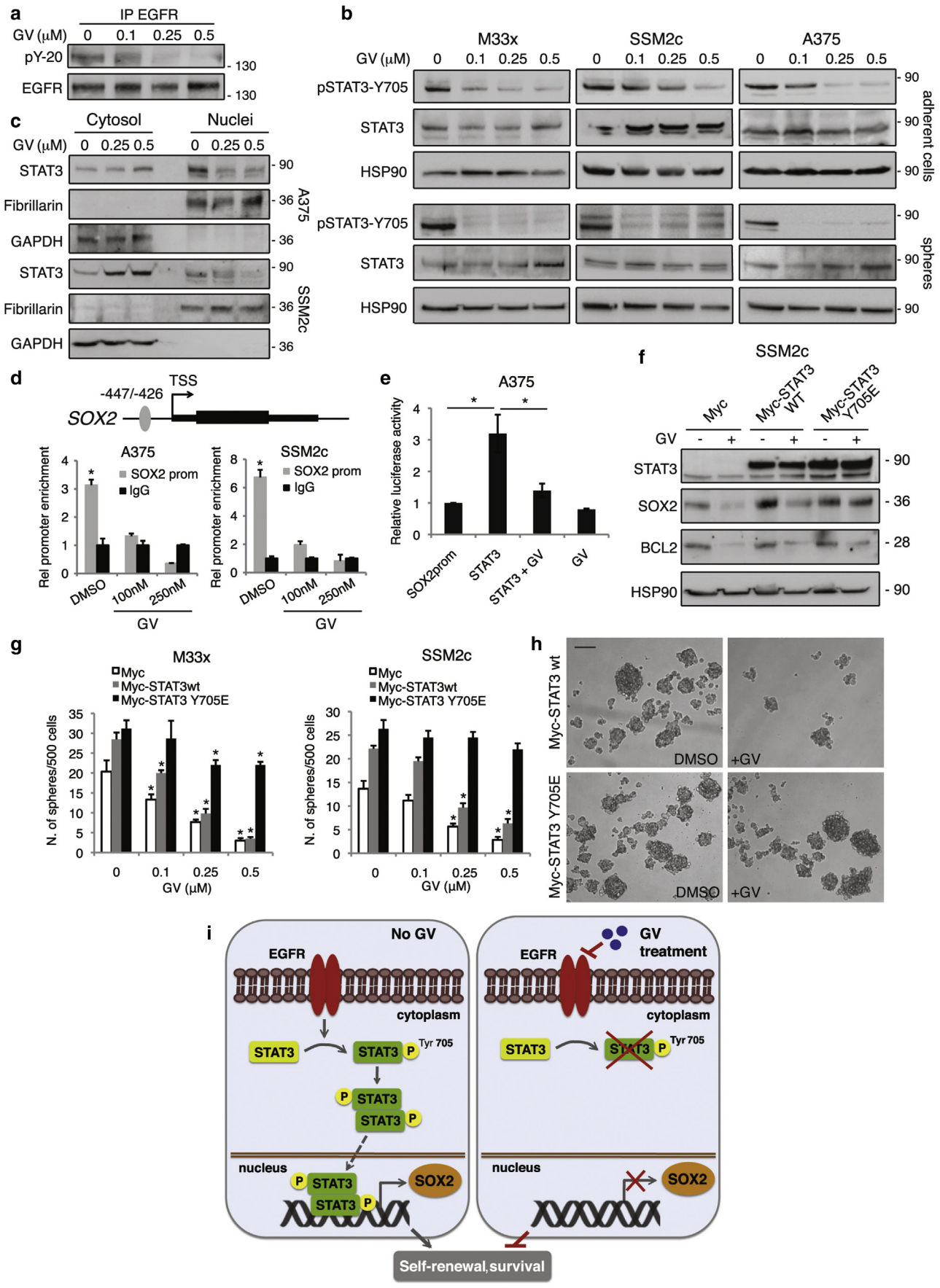

Figure 5. GV inhibits STAT3/SOX2 axis. (**a**) Immunoprecipitation of endogenous EGFR in A375 cells. (**b**) Western blot of pSTAT3^{Y705} and STAT3 in adherent cells and spheres treated with GV, for 24 and 72 hours, respectively. HSP90 was used as loading control. (**c**) STAT3 cellular localization 24 hours after GV

phosphorylation and activation, and we overexpressed both wild-type and STAT3 mutant Tyr705Glu (Y705E). Western blot analysis in SSM2c cells showed that ectopic expression of the constitutive active STAT3 mutant, but not of the wild-type, counteracted the decrease of SOX2 protein levels induced by GV treatment (Figure 5f). Consistently, overexpression of both wild-type STAT3 and STAT3-Y705E increased the number of primary spheres compared with the empty vector, but only STAT3-Y705E prevented the inhibitory effect of GV on sphere formation (Figure 5g and h). Collectively, our results indicate that GV inhibits melanoma stem cell survival and self-renewal by affecting the EGFR/STAT3/SOX2 axis (Figure 5i).

DISCUSSION

Current therapies to treat melanoma induce only temporary benefits because they are predominantly directed toward bulk tumor cells and fail to eradicate MICs, which drive tumor regrowth and originate recurrent tumors characterized by high aggressiveness, fast spreading, and resistance to therapy (Frank et al., 2010; Murphy et al., 2014). Hence, strategies that kill or induce differentiation of MICs may improve prognosis and contribute to curing patients (Schlaak et al., 2012; Mukherjee et al., 2015). Treatment of cutaneous melanoma metastases with a combination of GV and imiquimod has been shown to prevent melanoma recurrence (Arbiser et al., 2012; Bonner and Arbiser, 2014). However, it is not known whether and how GV affects melanoma cell growth and stemness.

In this study, we elucidated two antitumor mechanisms of GV. First, we show that GV inhibits melanoma stem cell self-renewal by down-regulating the transcription factor SOX2 through STAT3 (Figure 5). Second, we show that GV harbors a potent antiproliferative effect on melanoma bulk by promoting mitochondrial apoptosis and G2 cell cycle arrest. Altogether these findings suggest that GV might become an effective compound in the treatment of human melanoma, alone or in combinatory regimen.

The major finding of this study is that GV acts on melanoma stem cells by inhibiting SOX2 through the EGFR/STAT3 signaling axis. Indeed, ectopic SOX2 expression protects MICs from the effects of GV. This result is in agreement with the critical role of SOX2 in MIC maintenance (Santini et al., 2014) and with studies showing the importance of SOX2 in mediating the effects of EGFR activation in other cancer types (Chou et al., 2013; Eberl et al., 2012; Rybak and Tang, 2013). Because SOX2 has been shown to induce de-differentiation and to impart stem-like signatures in several tumors (Weina and Utikal, 2014), inhibition of SOX2 by GV may increase the number of differentiated cells, thus sensitizing them to GV-mediated apoptosis. This hypothesis is consistent with the observed decrease of melanoma cells expressing high ALDH activity and CD271, two melanoma stem cell markers (Boiko et al., 2010; Civenni et al., 2011; Luo et al., 2012; Santini et al., 2012), and with the observation that $ALDH^{high}$ putative MICs become sensitive to apoptotic stimuli induced by GV when they are differentiated.

Although several studies have highlighted potential strategies to inhibit SOX2, to date the ability to selectively target SOX2 remains a challenge. In this respect, a molecule such as GV that is well tolerated in humans and can be applied topically on cutaneous melanoma lesions might be an interesting agent to block SOX2 activity and can be regarded as a therapeutic option in combination with targeted therapy and immunotherapy.

Previous studies indicated that GV affects ROS production by targeting NOX enzymes (Bhandarkar et al., 2009; Mukawera et al., 2015; Perry et al., 2006). Nevertheless, here we show that GV blocks melanoma cell growth in vitro and negatively affects the melanoma stem cell compartment without reducing ROS production. This may depend on the fact that melanoma cells undergo mitochondrial apoptosis that may mask the effect of GV. On the other hand, our data indicate that both NOX4-positive (A375, SSM2c, and M33x) and NOX4-negative (SK-MEL-28 and M51) melanoma cells are equally sensitive to GV-induced apoptotic stimuli, whereas only NOX4-positive cells display G2 cell cycle arrest (Figure 4 and see Supplementary Figure S2), consistent with a previous report (Yamaura et al., 2009).

Our study shows that GV inhibits activation of STAT3 by EGFR in a Src-independent and JAK-dependent manner. This is supported by studies showing that STAT3 activation in melanoma cells relies on JAK or protein-tyrosine phosphatase rather than on Src (Flashner-Abramson et al., 2016; Kreis et al., 2007;). STAT3 has been reported to enhance invasion and metastasis (Fofaria and Srivastava, 2014) and to mediate the acquisition of a stemness phenotype in melanoma cells (Ohanna et al., 2013). In addition, inhibition of STAT3 has been shown to induce a significant regression of melanoma growth in vivo (Yin et al., 2013). Hence, targeting the EGFR-JAK-STAT3 signaling by GV could represent a valid strategy to treat melanoma.

In conclusion, we uncovered an EGFR/STAT3/SOX2 signaling cascade that regulates melanoma stem cell behavior and identified GV as an inhibitor of this axis. GV has been used as an antibacterial, antimycotic, and anti-angiogenic agent. More recently, GV has shown a strong anticancer activity both in vitro and in vivo (Bhandarkar et al., 2009; Cunniff et al., 2015; Mukawera et al., 2015; Perry et al., 2006; Yamaguchi et al., 2015) with minimal adverse effects, thus making it safe for use in humans (Arbiser, 2009). Taken together, our data suggest that GV could be a good candidate for both chemoprevention and therapeutic treatment of human melanoma. Further studies will help give

treatment. GAPDH (cytoplasm) and fibrillarin (nuclear) were used as loading control. (**d**) Chromatin immunoprecipitation on *SOX2* proximal promoter. IgG was used as negative control and set to 1. Data represent mean ± standard error of the mean. n = 3. (**e**) Quantification of dual-luciferase reporter assay. Relative luciferase activities were firefly/*Renilla* ratios, with the level induced by control equated to 1. Data represent mean ± standard error of the mean. n = 4. (**f**) Western blot of SOX2 and BCL2 in SSM2c transfected with Myc-$STAT3^{wt}$, Myc-$STAT3^{Y705E}$, or Myc after 250 nmol/L of GV for 24 hours. (**g, h**) Primary spheres formation and representative images in cells transfected with Myc-$STAT3^{wt}$, Myc-$STAT3^{Y705E}$, or Myc. Scale bar = 100 μm. *$P < 0.05$ versus control. (**i**) Proposed mechanism of action of GV. *$P < 0.05$ versus control. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GV, gentian violet; Rel, relative; STAT3, signal transducer and activator of transcription-3.

insights into the potential use of GV in combination with other drugs targeting signaling pathways involved in melanoma progression.

MATERIALS AND METHODS

Human melanoma samples and cell cultures

Commercial melanoma cell lines used in this study were A375 and SK-MEL-28 (a gift from Laura Poliseno, Istituto Toscano Tumori) (Marranci et al., 2015). Human melanoma samples were obtained after approved protocol by the institutional review board of the hospital, and written informed consent of patients was obtained following the Helsinki protocol. Cells were periodically screened for contamination with *Mycoplasma* species by PCR. For cell viability assay, 15,000 cells/well were plated in 12-well plates and treated with GV at indicated concentrations for 48 hours in 1% fetal bovine serum.

Primary sphere formation and self-renewal assay

For melanoma sphere cultures, cells were seeded in human embryonic stem cell medium supplemented with 4ng/ml basic fibroblast growth factor, as reported (Santini et al., 2012). For primary sphere formation assay, melanoma cells were seeded in 12-well plates at 1cell/μl dilution, and spheres were counted after 72 hours. For self-renewal assay, primary melanoma spheres were dissociated into single cells and plated at 1cell/μl dilution in 12-well plates. After 1 week secondary spheres were counted (Santini et al., 2014).

Aldefluor assay and flow cytometry analysis

Aldefluor assay was performed using the Aldefluor kit (Stemcell Technologies, Vancouver, Canada) as previously reported (Santini et al., 2012). For cell cycle analysis, melanoma cells were resuspended in 50μg/ml propidium iodide, 0.1% Triton X-100, and 0.1% sodium citrate 48 hours after GV treatment. Data were collected on BD Accuri C6 software (BD Biosciences, Franklin Lakes, NJ) and analyzed using ModFit LT software (Verity Software House, Topsham, ME). Apoptosis was measured after 48 (adherent cells) or 72 (spheres) hours of GV treatment using annexin V-phycoerythrin/7-AAD apoptosis kit (BD Biosciences) according to the manufacturer's instructions. The number of apoptotic cells was detected and analyzed using BD Accuri C6 software.

Plasmids, cloning, mutagenesis, and lentiviral vectors

See Supplementary Materials online for details.

Luciferase reporter assays

Luciferase reporters were used in combination with *Renilla* luciferase pRL-TK reporter vector (Promega, Madison, WI) to normalize luciferase activities; pGL3Basic vector (Promega) was used to equal DNA amounts. Luminescence was measured using the Dual-Glo Luciferase Assay System (Promega) and the GloMax 20/20 Luminometer (Promega).

Western blot analysis

Western blot was performed as already described (Pandolfi et al., 2015). See Supplementary Materials for antibodies.

Chromatin immunoprecipitation

Chromatin immunoprecipitation was performed as already described (Pandolfi et al., 2015). Sonicated cell lysates were incubated overnight at 4 °C with 20 μl of protein G magnetic dynabeads (Invitrogen, Carlsbad, CA) coupled to 3 μg of anti-STAT3 antibody (H-190) (Santa Cruz Biotechnology, Santa Cruz, CA) or IgG control. DNA was purified, and quantitative real-time PCR was carried out at 60 °C using FastStart SYBR Green Master (Roche Diagnostics, Basel, Switzerland). Primers are listed in Supplementary Table S1 online.

Statistical analysis

Data represent mean ± standard error of the mean values calculated on at least three independent experiments. *P*-values were calculated using one-way analysis of variance and Bonferroni correction when more than two samples were analyzed and Student *t* test when two samples were compared. A two-tailed value of $P < 0.05$ was considered statistically significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2016.06.610.

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