Cell Density–Dependent Increase of Constitutive Signal Transducers and Activators of Transcription 3 Activity in Melanoma Cells Is Mediated by Janus Kinases

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Abstract

Signal transducers and activators of transcriptions (STAT) are key mediators of cytokine signaling. Moreover, these transcription factors play a crucial role in oncogenic signaling where inappropriate and sustained activation of STATs, especially STAT3, is a trait of many different cancers and their derived cell lines. Constitutively active STAT3 has been reported to prevent programmed cell death and enhance cell proliferation, whereas the disruption of STAT3 signaling can inhibit tumor growth. The physiologic activation of STAT3 by cytokines has been well established; however, little is known about altered, stimulation-independent STAT3 activation. Here, we show that, in most but not all melanoma cell lines, STAT3 phosphorylation increased substantially with cell density and that this STAT3 was able to bind to DNA and to activate transcription. Inhibitor studies showed that the cell density–dependent STAT3 activation relies on Janus kinases (JAK) rather than Src kinases. Using a specific JAK inhibitor, sustained STAT3 activation was completely abrogated in all tested melanoma lines, whereas inhibition of Src or mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1/2 had no effect on constitutively tyrosine-phosphorylated STAT3 levels. Although STAT3 activation was completely blocked with JAK inhibitor I and to a lesser extent with the common JAK inhibitor AG490, only the latter compound markedly decreased proliferation and induced apoptosis. Taken together, variations in cell density can profoundly modify the extent of JAK-mediated persistent STAT3 phosphorylation; however, STAT3 activation was not sufficient to provide critical growth and survival signals in melanoma cell lines. (Mol Cancer Res 2007;5(12):1331–41)

Introduction

Although melanomas account for only 4% of all dermatologic cancers, they cause 80% of skin cancer–related deaths and only 14% of patients survive more than 5 years once the primary tumor has metastasized (1). In the present study, we investigated a panel of melanoma cell lines with regard to the presence and function of constitutively active signal transducers and activators of transcription (STAT) 3.

Many studies over the past decades have clearly established a central role for STATs in cytokine signaling (see refs. 2-4 for recent reviews). Classic cytokine signaling events require the binding of a ligand (cytokine) to its receptor, an event that activates Janus kinases (JAK) that are transphosphorylated and thereby activated. Subsequently, active JAKs phosphorylate tyrosine residues within the receptor cytoplasmic domain, which form docking sites for STATs. JAKs then phosphorylate receptor-bound STAT factors on a single tyrosine residue so that active STAT dimers can form by intermolecular phosphotyrosine-SH2 domain interactions. Such activated STAT dimers finally translocate to the nucleus where they initiate transcription by binding to response elements in the promoter of target genes (3). STAT3 is one of seven mammalian STAT proteins acting as a signal transducer for many cytokines and growth factors, and thus, it represents an important player in many different biological processes, such as immunoregulation and development (5), induction of acute phase proteins (2, 6), hematopoiesis (2), and proliferation and apoptosis (7). As a consequence of these diverse functions, dysregulation of STAT signaling has been implicated in various inflammatory, autoimmune, and neoplastic diseases (8-10).

STAT3 is constitutively active in many melanoma cell lines and primary tumors, and this has been proposed to protect these as well as other cancer cells from apoptosis by altering expression profiles of genes that are critically involved in cell proliferation and survival (such as $BCL-X_L$, $MCL-1$, $survivin$, Myc, and $p53$; refs. 8, 11-13). In this context, many studies have shown that inhibition of STAT3 by dominant-negative STATs, specific peptide aptamers, small-molecule inhibitors, or antisense oligonucleotides reduces tumor survival and this

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makes persistently activated STAT3 a potentially interesting target for cancer therapy (8, 11, 12, 14-16). On the other hand, STAT3 can also have opposite effects on the survival of cancer cells. Whereas melanocytes and early-stage melanoma cells are growth inhibited by a variety of cytokines, including interleukin-6 (IL-6), oncostatin M (OSM), or IFN-γ, melanoma cells of advanced tumor stages are often found to be multicytokine resistant (17-19). We and others have previously shown that STAT3 plays a key role in the IL-6–mediated and OSM-mediated growth inhibition of A375 melanoma cells (20, 21), whereas STAT1 is crucially involved in the growth inhibition mediated by IFN-γ (22). In line with this, constitutive or inducible expression of JAK/STAT signaling inhibitor SOCS3 was found to confer a growth advantage to melanoma cells (23). Moreover, lack of receptor expression can contribute to cytokine resistance (24), which has been associated with an impaired STAT3 activation (25), and in view of this, it has recently been discovered that melanoma progression coincides with silencing of the *OSMRβ* gene (26). Evidently, further investigations into the conditions that may favor either the antiapoptotic or the proapoptotic properties of STAT3 are necessary to clarify the exact role that STAT3 plays for the survival of cancer cells, *in vitro* and *in vivo*.

Here, we examined in melanoma cell lines whether STAT signals can be altered by stimulation-independent mechanisms, such as cell density, to elucidate the role that cell confluence–induced stress may have on the presence or levels of persistently activated STATs. Cell confluence has been shown to enhance cytokine-independent induction of STAT3 activation in head and neck squamous cell carcinomas and bladder cancer cells (27). Similar observations have recently been made in breast carcinoma cell lines, and moreover, it was shown that augmentation of active STAT3 by increased cell-to-cell contacts in confluent and growth-arrested cultures was mediated by JAK rather than Src kinases (28, 29). Conversely, other studies also using breast carcinoma and melanoma cells have emphasized the role of Src kinases for the maintenance of constitutively active STATs (14, 30). We show here that the levels of persistently active and functional STAT3 (phosphorylated Tyr705) strongly increase on high cell density in most melanoma lines and in two cervix carcinoma cell lines but not in three of four cell lines that were established from healthy tissue. Using several kinase inhibitors, we show that the cell density–dependent increase of phosphorylated STAT3 was mediated by JAKs, whereas inhibition of Src kinases had no influence on the amounts of active STAT3 in confluent melanoma cells. Furthermore, application of the recently described specific JAK inhibitor I (31, 32) to various melanoma cells resulted in complete inhibition of STAT3 activation; however, this inhibitor did not alter the growth characteristics of tested cell lines, whereas inhibition of Src reproducibly derogated cell proliferation and enhanced apoptosis. Interestingly, the widely used but rather unspecific JAK inhibitor AG490 resulted in a profound growth reduction and apoptosis induction, although levels of tyrosine-phosphorylated STAT3 were only marginally affected by this compound. These observations suggest that persistent STAT3 activation is mediated by JAKs but this does not confer a growth advantage to melanoma cells.

Results

Cell Density Influences the STAT3 Phosphorylation Status

Eleven different melanoma cell lines and three cancer cell lines of different origin (cervix carcinomas HeLa and A431 and liver carcinoma HepG2) as well as four noncancer cell lines [primary melanocytes (NHEM), normal human dermal fibroblasts, the keratinocyte cell line HaCaT, and human embryonic kidney cells Hek293] were analyzed with respect to their STAT3 activation status (Table 1). Irrespective of cytokine stimulation, 11 of 18 cell lines showed various levels of constitutively active STAT3 (phosphorylated Tyr705), with the degree of STAT3 activation increasing with cell density in most of these cell lines (Fig. 1, representative selection of six melanoma cell lines). By contrast, levels of activated phosphorylated extracellular signal-regulated kinase (ERK) 1/2 did not change with cell density, although absolute levels differed between cell lines (Fig. 1). The strongest activation of STAT3 in melanoma cells was detected in Wm9 and 1102 cells and signal strength increased with higher cell density 24 h after confluence. At this time point, STAT3 activation was considerably stronger than in subconfluent cells seeded at lower density. Quantification of Western blot signals revealed that the signals were up to 2- to 8-fold higher compared with sparse cells (Fig. 1). 586, HeLa, and HepG2 cells were the only cell lines that had a weak constitutive STAT3 phosphorylation, which, however, did not increase with higher cell densities (Table 1). In addition to HaCaT keratinocytes and the melanoma lines depicted here, we have also tested primary melanocytes (NHEM) and Hek293 cells, where density-dependent STAT3 activation was not detected, whereas a cancer cell line of different origin (A431) clearly showed increased phosphorylated STAT3 signals with higher cell density. Normal human dermal fibroblasts showed a weak but detectable phosphorylated STAT3 signal in unstimulated but dense cells and were thus the only noncancer cells that had density-dependent constitutive STAT3 activation, albeit at a very low level (data not shown).

In agreement with a study by Vultur et al. (29) using breast carcinoma cells, phosphorylated STAT3 activation was most prominent 24 h after 100% confluence had been reached and thereafter slowly declined to a level similar to that in sparse cells, with the exception of A375 cells, where phosphorylated STAT3 remained constant for 3 days (Fig. 2A, lanes 2-4). To control for the influence of soluble factors secreted by the cells, medium of high-density cultures was changed daily for 3 consecutive days. Levels of phosphorylated STAT3 did not change significantly compared with cultures that had been left untreated for 3 days (Fig. 2A, lane 5). Furthermore, culturing melanomas without FCS caused the cells to round up and detach from the culture vessel; however, this did not alter levels of sustained STAT3 activation after 24 h of starvation (Fig. 2B).

Interestingly, Wm9 cells (late-stage metastatic melanoma; refs. 20, 25, 33) showed a pronounced density-dependent STAT3 activation, whereas Wm35, an early-stage sample from the same patient, had little constitutive STAT3 activation (Fig. 1). We confirmed that Wm9 cells produce high amounts of IL-6 (>10 ng/mL supernatant), as was shown previously (33), whereas 1102 was the only other cell line that had much lower but detectable levels of IL-6 in the supernatant of dense cells

Table 1. Summary of Tested Cell Lines with Respect to Constitutively Active STAT3

Cell line	Origin	Constitutive phosphorylated STAT3	Density phosphorylated STAT3
A375	Melanoma	√	√√
586	Melanoma	√	√
729	Melanoma	√	√√
888	Melanoma	No	No
1102	Melanoma	√√	√√√
1286	Melanoma	No	No
MelIm	Melanoma	No	No
MeWo	Melanoma	√	√√
Wm9	Melanoma	√√	√√√
Wm35	Melanoma	No	No
Wm239	Melanoma	√	√√
A431	Cervix carcinoma	√√	√√√
HeLa	Cervix carcinoma	√	√
HepG2	Liver carcinoma	√	√
HaCaT	Keratinocyte	No	No
Hek293	Kidney	No	No
NHDF212	Dermal fibroblast	(√)	(√)
NHEM	Melanocyte	No	No

NOTE: Increase of phosphorylated STAT3 signals as a result of high cell densities is indicated (number of "√" indicates the relative strength of phosphorylated STAT3 signals in Western blot analysis).

(<10 pg/mL). All other tested lines (A375, MelIm, Wm35, 568, 729, 888, 1286, and 1326) did not produce IL-6 (data not shown). Figure 2C illustrates that the supernatant of high-density Wm9 cells, when transferred to sparse cells, causes a profound STAT3 activation. The equivalent experiment done with A375 cells yielded no STAT3 activation in matched low-density cells. Thus, endogenously produced IL-6 certainly contributes to the constitutive activation of STAT3 in Wm9 cells but not in the other tested cell lines.

### STAT3 DNA-Binding Capacity Increases with Cell Density

To establish that the stimulation-independent but cell density–related increase of STAT3 is translated into enhanced DNA-binding capacity, cell lysates of 1102, A375, and MelIm (negative example cell line) were subjected to electrophoretic mobility shift assay. As positive controls, cells were stimulated for 30 min with OSM, a treatment yielding maximum levels of active phosphorylated STAT3. The DNA-binding capacity of the STAT3/3 dimer to the radiolabeled c-*sis*–inducible element probe clearly augments with higher cell density. Quantification of phosphorylated STAT3 signals showed that in A375 cells the portion of active STAT3 is more than double in dense versus sparse cells, whereas 1102 revealed an increase of 25% in signal strength (Fig. 3A). No STAT1 DNA binding was detected with the above samples, except after OSM stimulation, and this was also confirmed by Western blot analysis, where no constitutively active STAT1 could be detected in any of the tested cell lines under either high- or low-density culturing conditions (data not shown). Next and to further support the biological relevance of sustained phosphorylated STAT3, a reporter gene assay was done, where 1102 cells showed the strongest density-dependent increase in STAT-mediated reporter gene activity (Fig. 3B). Taken together, these results indicate that the elevated levels of phosphorylated STAT3 in highly confluent cells are biologically active. Nevertheless, the strength of the constitutive tyrosine-phosphorylated STAT3 signal can vary considerably between cell lines, and hence,

comparison with stimulation-dependent activation of STAT3 is somewhat difficult. In 1102, A375, and MeWo, signal intensities approached those brought about by 2-h stimulation with IL-6 or OSM (20). Although phosphorylated STAT3 levels generally peak at ∼30 min after cytokine stimulation with IL-6 or OSM, the amount of phosphorylated STAT3 detected after 2 h of cytokine stimulation and, accordingly, after 24 h after confluence would certainly be sufficient to contribute to biologically relevant signaling events. Thus, constitutively active STAT3, albeit to various degrees, may contribute to aberrant signaling in cancer or other chronic diseases.

Increased Cell Density–Dependent STAT3 Activation in Melanoma Cells Is Mediated by JAKs

Under most physiologic conditions, STAT3 is activated by JAKs but other transforming nonreceptor kinases, such as Src (34), v-Eyk, v-Fps, Lck (8), and receptor tyrosine kinases such as epidermal growth factor receptor, platelet-derived growth factor receptor, and vascular endothelial growth factor receptor (35, 36), are also known to activate STAT3. To investigate which kinases are involved in the elevated STAT3 activation in postconfluent cells, inhibitor studies were done with selected melanoma cell lines cultured at high and low densities (Fig. 4). For reasons of simplicity, only high-density cells are shown, as results for sparse cells were similar only that signals were weaker.

The JAK inhibitor I reproducibly and efficiently blocked constitutive STAT3 activation, strongly suggesting that this cytokine-independent STAT3 activation requires JAKs (Figs. 4 and 5). Titration of the compound revealed that only 200 nmol/L were sufficient to prevent constitutive STAT3 signaling in A375 and 1102 cells, whereas Wm9 melanoma cells, which produce IL-6, required 500 nmol/L for this effect. Furthermore, this amount completely abrogated STAT activation by 30 to 90 min in all postconfluent cell lines and signals remained undetectable for up to 48 h (Fig. 5, Western blots; data not shown). Strikingly, the less specific JAK inhibitor AG490, which mostly prevents autophosphorylation of
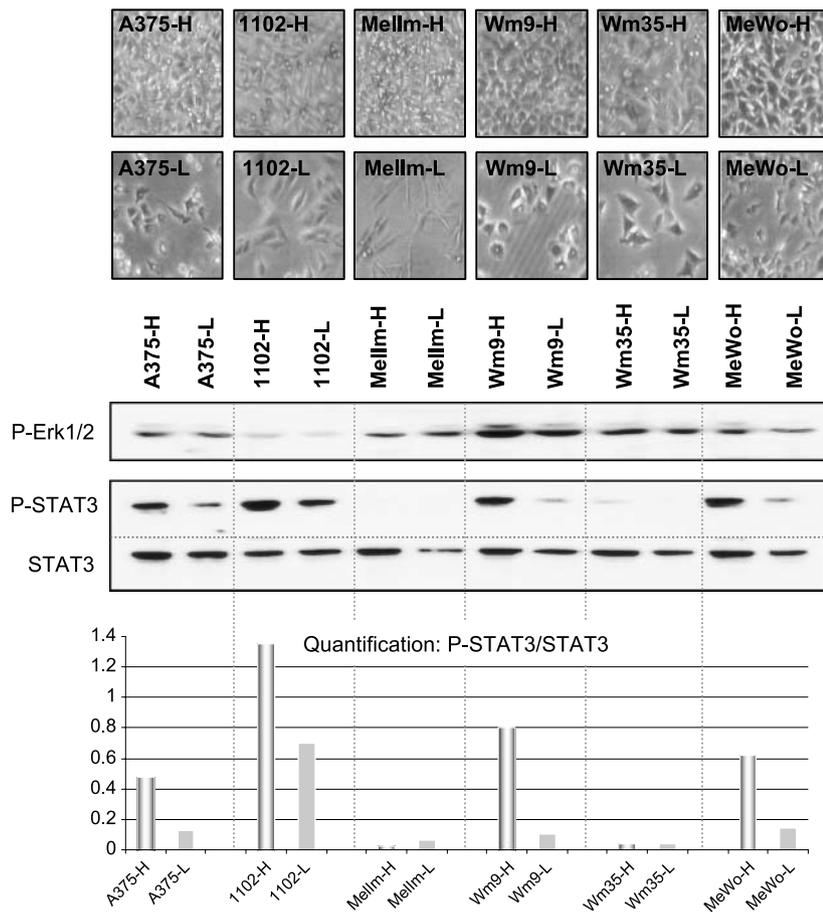
FIGURE 1. Analysis of density-dependent constitutive phosphorylated STAT3 (*P-STAT3*; Tyr[705]) signals in selected melanoma cell lines. High-density cells (*H*; 1×10^6 per well) and low-density cells (*L*; 2×10^5 per well) were seeded in six-well plates, left for 24 h, and then photographed under phase contrast with a ×400 magnification. Subsequently, cells were washed once with PBS followed by direct lysis on ice with 100 or 400 μL of Laemmli buffer for low- and high-density cells, respectively. Phosphorylated ERK1/2 (*P-Erk1/2*) levels remained unchanged between high- and low-density cells, whereas phosphorylated STAT3 signals in A375, 1102, Wm9, and MeWo cells varied considerably. Bottom, a quantification of chemiluminescent phosphorylated STAT3 signals. Representative blot of four independent experiments.

epidermal growth factor receptor kinase and only to a lesser extent activation of JAKs (Calbiochem, Inhibitor Source book), at best resulted in a reduction of phosphorylated STAT3 but could not prevent STAT3 activation even when 10 μmol/L of inhibitor were applied (Fig. 4). Concentrations of 50 μmol/L AG490 were required to completely block phosphorylation of STAT3 in 1102 cells, whereas this concentration had little effect on A375 and Wm9 cells (Fig. 5).

Depending on the cell line, the p38 mitogen-activated protein kinase (MAPK) inhibitor (SB203580) had opposite effects with regard to phosphorylated STAT3 signals: no effect was detected in A375 cells, and it slightly but reproducibly increased stimulation-independent phosphorylated STAT3 levels in 1102 cells, whereas it somewhat reduced the signals in Wm9 cells (Fig. 4, compare *lane 5* with *lane 1*). Blocking MAPK/ERK kinase 1/2 (U0126), however, did not influence phosphorylated STAT3 levels (Fig. 4) and neither did an epidermal growth factor receptor inhibitor (PD153035) reduce constitutive STAT3 phosphorylation (data not shown). The postconfluent cell lines tested here all presented stimulation-independent phosphorylation of ERK1/2 (Fig. 4), common for melanoma cells (1, 37). This was efficiently prevented by blocking MAPK/ERK kinase 1/2 (U0126) and surprisingly by inhibition of p38, implying a possible cross-talk between p38 and ERK in response to, for example, mechanical stress as has recently been reported (23, 38, 39). As expected, phosphorylation of ERK1/2 was influenced neither by the JAK nor by the Src inhibitors.

Interestingly, the Src kinase inhibitor PP2 did not alter density-dependent STAT3 activation, implying that in this experimental setup activation of STAT3 was not mediated by Src kinases. Figure 5 depicts titrations of PP2 and JAK inhibitor I for four selected melanoma lines (24 h after confluence), highlighting that activation of STAT3 in postconfluent melanoma cells is brought about by JAK rather than Src kinases. This is in contrast to a study by Niu et al. (40) where blocking Src with another inhibitor (PD180970) was able to reduce STAT3 DNA binding (electrophoretic mobility shift assay) in two melanoma cell lines in a dose-dependent manner. However, we only detected very low levels of constitutively tyrosine-phosphorylated Src in the tested melanoma lines A375, 1102, and MelIm (Fig. 5), which could explain why inhibition of Src had no influence on phosphorylated STAT3 levels in highly confluent cells. Nevertheless, even in Wm9 cells displaying active Src, PP2 did not reduce phosphorylated STAT3 signals.

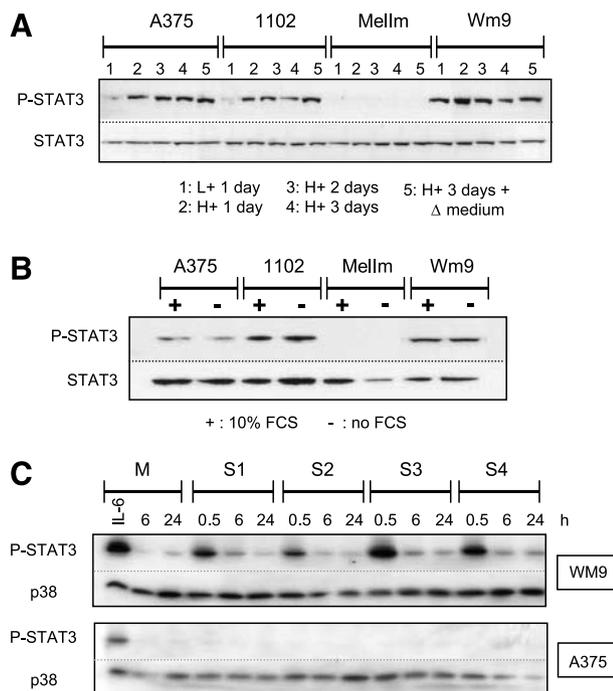### Roles of JAK and Src Kinases for Melanoma Cell Growth

In many cancer cells, constitutively active STAT3 is believed to prevent apoptosis and thus to confer a growth advantage to cells (41). Therefore, we tested whether inhibition of STAT3 or Src leads to increased cell death and concomitantly to reduced

proliferation. Inhibition of Src kinases clearly increased the amount of apoptotic cells in all cell lines (Fig. 6). These findings correlated well with results from proliferation assays, where PP2 dose dependently reduced proliferation of A375, 1102, and Wm9 cells (Fig. 5). Similarly, others have reported that abrogation of Src phosphorylation resulted in cell death and growth inhibition of melanoma and head and neck squamous carcinoma cells that display constitutive STAT3 activation (40, 42). Although only Wm9 cells had detectable levels of phosphorylated Src, PP2 was still able to increase apoptosis and reduce the proliferation of all melanoma cells (except MelIm), indicating that this compound is likely to inhibit additional targets other than Src kinases and that this may contribute to enhanced cell death.

Vice versa, inhibition of JAKs with JAK inhibitor I for 48 h completely abrogated STAT3 activation but did not induce apoptosis or reduce proliferation in tested melanoma cells, indicating that constitutively active STAT3 is not critical for cell survival in this experimental setup. Strikingly, different results were obtained with AG490, which reproducibly brought about an up to 2.5-fold increase of apoptotic cells and a dose-dependent reduction of proliferation (Fig. 5), although

phosphorylation of STAT3 was not prevented by concentrations below 50 μmol/L (Fig. 5, Western blots), implying that AG490-induced inhibition of signaling kinases other than JAKs (such as epidermal growth factor receptor or other unknown targets; ref. 31) drives melanoma cells into apoptosis. 1102 was the only cell line where 50 μmol/L AG490 was sufficient to significantly reduce STAT3 phosphorylation; however, this did not lead to a further decreased proliferation when compared with A375 and Wm9 cells. Interestingly, AG490 clearly enlarged the portion of apoptotic MelIm cells (Fig. 6), which do not show constitutively active phosphorylated STAT3, again emphasizing that the growth-inhibiting action of AG490 is not brought about by selectively blocking STAT3 activity.

Taken together, we tested melanoma cell lines displaying different kinds of STAT3 activation: Wm9 cells, which, by secretion of IL-6, have a high amount of constitutively active STAT3; MelIm cells without persistent STAT3 activation; and other cell lines that have STAT3 activated by unknown mechanisms. Cell growth of all but MelIm cells was impaired by AG490, which, however, did not interrupt STAT3 activation. Vice versa, JAK inhibitor I thoroughly inhibited STAT3 activation, which, in turn, did not result in a reduction of cell viability.

Discussion

In the present article, we document that several melanoma cell lines show phosphorylation of STAT3 independent of cytokine stimulation, which was enhanced in dense cultures, and thus, the STAT3 phosphorylation status was found to be sensitive to culture conditions. Treatment of cells with JAK inhibitor I abrogated STAT3 phosphorylation. However, and in contrast to the inhibitors AG490 and PP2, JAK inhibitor I did not influence cell growth and viability, indicating that the observed STAT3 phosphorylation is not crucially involved in cell survival.

STAT3 can be persistently activated under various circumstances in which cellular transformation is not the ultimate phenotype (41). Apart from classic cytokine stimulation, other mechanisms by which STAT3 can be activated and, importantly, remains activated are not well understood. One such condition, which renders STAT3 constitutively active, seems to be high cellular density. Both, under experimental cell culture conditions as well as in growing solid tumors, increased cell density imposes various challenges on cells: cadherin-mediated contact inhibition (43), nutrient depletion, hypoxia, and mechanical stresses (44). Augmentation of persistent phosphorylated STAT3 levels depending on the state of cell confluence has been described for breast and head and neck squamous carcinoma cells (27-29). Here, we add melanoma cell lines, which respond to high cell density with an increased activation of STAT3 but not ERK1/2 (Fig. 1), Src, or p38 MAPKs (data not shown). Essentially, our results expand for melanoma cells on what has recently been described for breast carcinoma cells (29). In agreement with Niu et al. (14), strong constitutive STAT3 activation was almost exclusively restricted to cancer cells (Table 1), whereas Vultur et al. (29) have observed this phenomenon also in normal breast epithelial cells and rodent fibroblasts. Furthermore, we have not detected an increase of
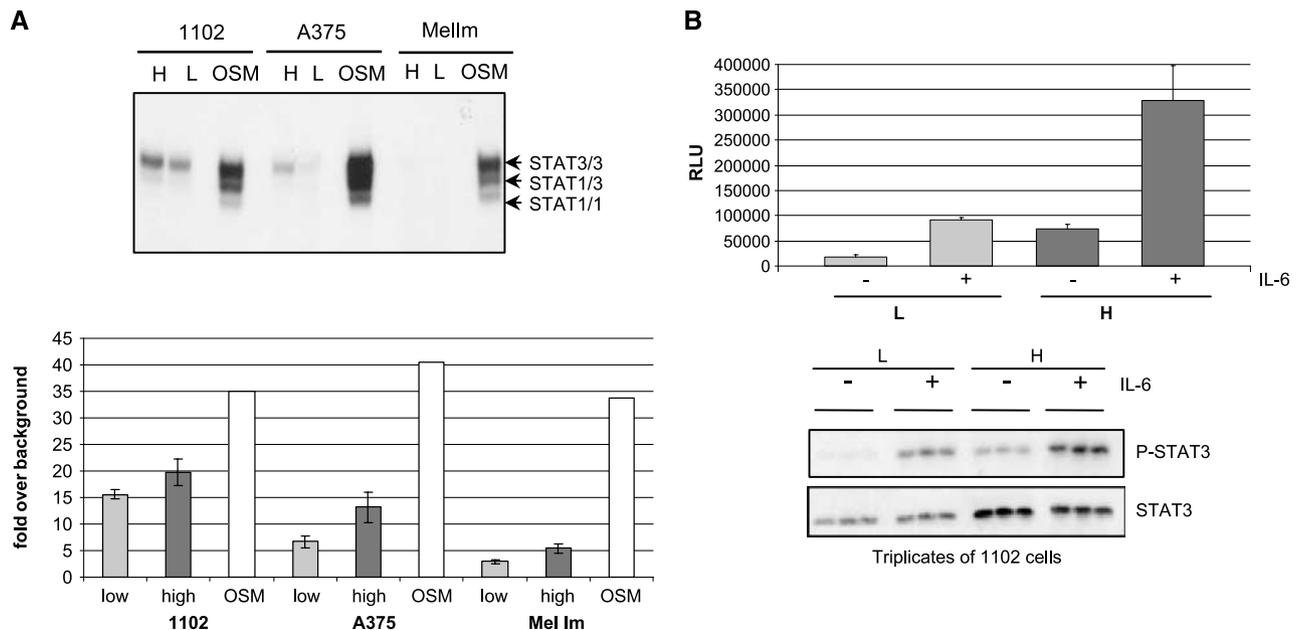

FIGURE 2. **A.** The waning of phosphorylated STAT3 signals was investigated in melanoma cell lines, four of which are shown. Low (*L*) and high (*H*) cell numbers were seeded and left untreated for up to 3 days or medium was changed daily (*lane 5*). Cell lysates were prepared and processed as described in Fig. 1. **B.** Withdrawal of FCS does not influence constitutive STAT3 signals in highly confluent cells. Cells were seeded as in **A** in normal medium with 10% FCS or without FCS and left for 24 h. Lysates were analyzed by Western blot for phosphorylated STAT3 and STAT3. **C.** Wm9 cells, but not A375 cells, secrete bioactive IL-6, which contributes to enhanced STAT3 activation. Supernatants of highly confluent (10^6 cells per well) A375 and Wm9 cells were collected after 1, 2, 3, and 4 days (*S1, S2, S3*, and *S4*) and transferred to low-density cells for different times as described in Materials and Methods. Transfer of plain medium served as a control (*M*). Lane 1, cells treated for 30 min with medium supplemented with 200 units IL-6.

FIGURE 3. Density-dependent constitutive phosphorylated STAT3 is transcriptionally active. **A.** Nuclear extracts (5 μg) of high- and low-density cells were subjected to electrophoretic mobility shift assay with a ^{32}P-labeled optimized STAT3-binding site (m67SIE). Results are shown for three representative cell lines, two of which show density-dependent constitutive STAT3 activation (1102 and A375). MelIm cells were included as a negative example cell line. Arrowheads, STAT dimers on the autoradiograph; bottom, quantification of signals. **B.** 1102 cells were plated at high and low cellular density and grown overnight. Top, stimulated samples were treated for 30 min with IL-6 before reporter gene assays were done as described in Materials and Methods. High-density 1102 cells show a 4.5-fold increase of active phosphorylated STAT3-mediated transcription compared with low-density cells. As a control for the quality of input material, triplicates of high- and low-density cells [with (+) and without (−) IL-6] were lysed in equal volumes of Laemmli buffer and processed by Western blot, and membranes were probed with anti-phosphorylated STAT3 antibody followed by STAT3 detection (bottom).

basal STAT3 levels on higher cell confluence in our experimental settings, although phosphorylated STAT3 can initiate transcription of its own promoter (45). In melanoma cells, different cell densities only influenced the portion of phosphorylated STAT3-Tyr^{705} but not the basal gene expression (Figs. 1 and 2A).

Intriguingly, Wm9 cells (late-stage metastatic melanoma; ref. 18) showed a strong density-dependent increase in constitutive STAT3 phosphorylation, whereas Wm35, an early-stage sample from the same patient, had a normal stimulation-dependent STAT3 activation status. It has previously been shown that Wm9 cells, but not Wm35 cells, are resistant to IL-6–mediated growth inhibition and this correlated with lack of inhibition of cyclin E–associated kinase (25). Of note, Wm9 was the only cell line that, increasing with cell numbers, produced high amounts of IL-6, which certainly contributes to STAT3 activation (Fig. 2C). Interestingly, inhibition of p38 by SB203580 (and also by another p38 inhibitor, SB202190, not shown) was paralleled by a dose-dependent reduction in phosphorylated STAT3 (Fig. 4; data not shown), further hinting at the possibility that in Wm9 cells autocrine production of IL-6, possibly influenced by p38, is involved in the persistent STAT3 activation, as IL-6 expression is positively affected by p38 activity (46, 47).

In 1102 cells, but not in A375 cells, blocking p38 reproducibly increased phosphorylated STAT3 signals (Fig. 4). As p38 activity has been implicated in enhanced expression of the inhibitory protein SOCS3 (48), we analyzed expression of SOCS proteins following p38 inhibition by RNA protection assays. Of all seven SOCS family members (CIS, SOCS1, SOCS2, SOCS3, SOCS5, SOCS6, and SOCS7), only SOCS3 was slightly up-regulated in 1102 cells following treatment with the p38 inhibitor, whereas no effect was seen in A375 or MelIm cells (data not shown). However, this minor augmentation of SOCS3 in 1102 cells did not justify an interpretation of p38, directly or indirectly, regulating SOCS3 expression in the investigated cell lines.

In the available postconfluent melanoma cell lines, most of which did not display constitutive phosphorylated Src, we were not able to establish a role of Src kinases in the activation and maintenance of STAT3 activation. In agreement with our results and although a different inhibitor (PD180970) was used, blocking Src did not cause a substantial reduction of constitutively active STAT3 in highly dense breast cancer cells (29), whereas in another study it had a profound effect on STAT3 activation in sparse cells, again emphasizing the influence of cell densities on the activation of signaling cascades (29, 30). Similarly and also using the PD180970 Src inhibitor, Niu et al. (40) have described that in two of three melanoma lines Src kinases, rather than JAKs, seemed to confer constitutive STAT3 activation. These apparent differences with regard to STAT3-activating kinases might be cell type/line specific and cell density dependent or may result from the use of different inhibitors. Collectively, this suggests that the persistent activation of STAT3 can be mediated by JAK or Src kinases, or a combination of both, as was previously shown for breast cancer cells (30).

Numerous studies have shown that blocking STAT3 by different means (peptide aptamers, inhibitors, transfection of dominant-negative STAT3, or by antisense oligonucleotides)

leads to apoptosis and tumor regression in a variety of cancer settings (16, 42, 49, 50). However, most inhibitor studies to date have used tyrphostin AG490 to block STAT3 activation, but because this compound is not specific to JAKs, concentrations beyond any pharmacologic relevance have to be applied to block STAT3 activation and subsequently to increase apoptosis (51, 52). Conversely, the recently described JAK inhibitor I (pyridone 6; ref. 32) is at least 50-fold more sensitive in preventing STAT3 activation. A recent study on myeloid cell lines has conclusively described the pharmacologic effects of JAK inhibitor I (31) and found that cell lines with constitutively active STAT3 responded with increased apoptosis, to various degrees, following treatment with this compound. Testing JAK inhibitor I, we could not detect any growth-inhibiting effects on a selection of melanoma cell lines even when using concentrations of up to 1 μmol/L over a period of 24 or 48 h, suggesting that active phosphorylated STAT3 alone is not sufficient to confer a growth advantage to the assayed cell lines.

The parallel comparison of JAK inhibitors I and AG490 as well as the Src inhibitor PP2 in inhibition, proliferation, and apoptosis assays under the experimental conditions used here suggests that (*a*) levels of constitutively active STAT3 strongly increase with cell density in many cancer cell lines and this STAT3 activation is mediated by JAKs, (*b*) STAT3 is not sufficient to provide critical growth signals for the melanoma cell lines tested here, (*c*) apoptosis-inducing effects following AG490 treatment are likely due to inhibition of kinases other

than JAKs (or in addition to JAKs), and (*d*) Src kinases do not seem to be involved in the constitutive activation of STAT3 in the investigated cell lines. However, blocking Src slightly increased apoptosis, which might be caused by unspecific inhibition of other Src or unknown kinases. These results highlight that inhibitor studies may lead to entirely diverse conclusions about the role of different kinases and their target proteins (here transcription factors) depending on the specificity and sensitivity of the inhibitors, the experimental settings, and the investigated cellular systems. Finally, enhanced but stimulation-independent activation of STAT3, brought about by high cell density and stress signals resulting from growth arrest, can have profound consequences on the outcome of signaling studies if experimental cell numbers and culturing conditions are not tightly controlled for. On the other hand, highly dense or overgrowing cell cultures, albeit only two dimensional, may in part mimic some of the physiologic stress signals that increase in fast-growing tumors and might therefore contribute to sustained and increased STAT3 activity in cancer cells. To what extent this constitutively active STAT3 contributes to the survival of the selected melanoma cell lines remains to be investigated further.

Materials and Methods
Cell Lines

In total, 10 *Mycoplasma*-free melanoma cell lines were analyzed: A375 (American Type Culture Collection), 586, 729,
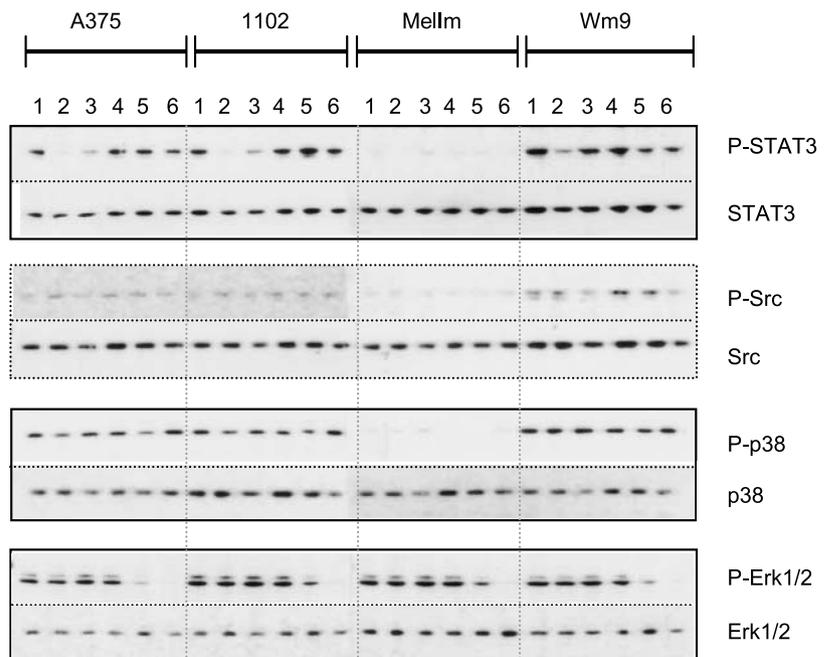
FIGURE 4. Inhibition of JAK but not Src abrogates persistent phosphorylated STAT3 signaling. Four representative melanoma cell lines were seeded at high cell densities in six-well plates and grown for 24 h. Inhibitors, as indicated in the figure, were added for 18 h before cells were harvested by direct lysis on ice. Control wells were treated with DMSO and processed by Western blot as described in Materials and Methods. P-Src, phosphorylated Src; P-p38, phosphorylated p38.

1: vehicle control (DMSO)
2: Jak: Jak Inhibitor I, 200 nM
3: Jak: AG490, 10 μM
4: Src: PP2, 2 μM
5: p38 MAPK: SB203580, 10 μM
6: MEK1/2: U0126, 5 μM
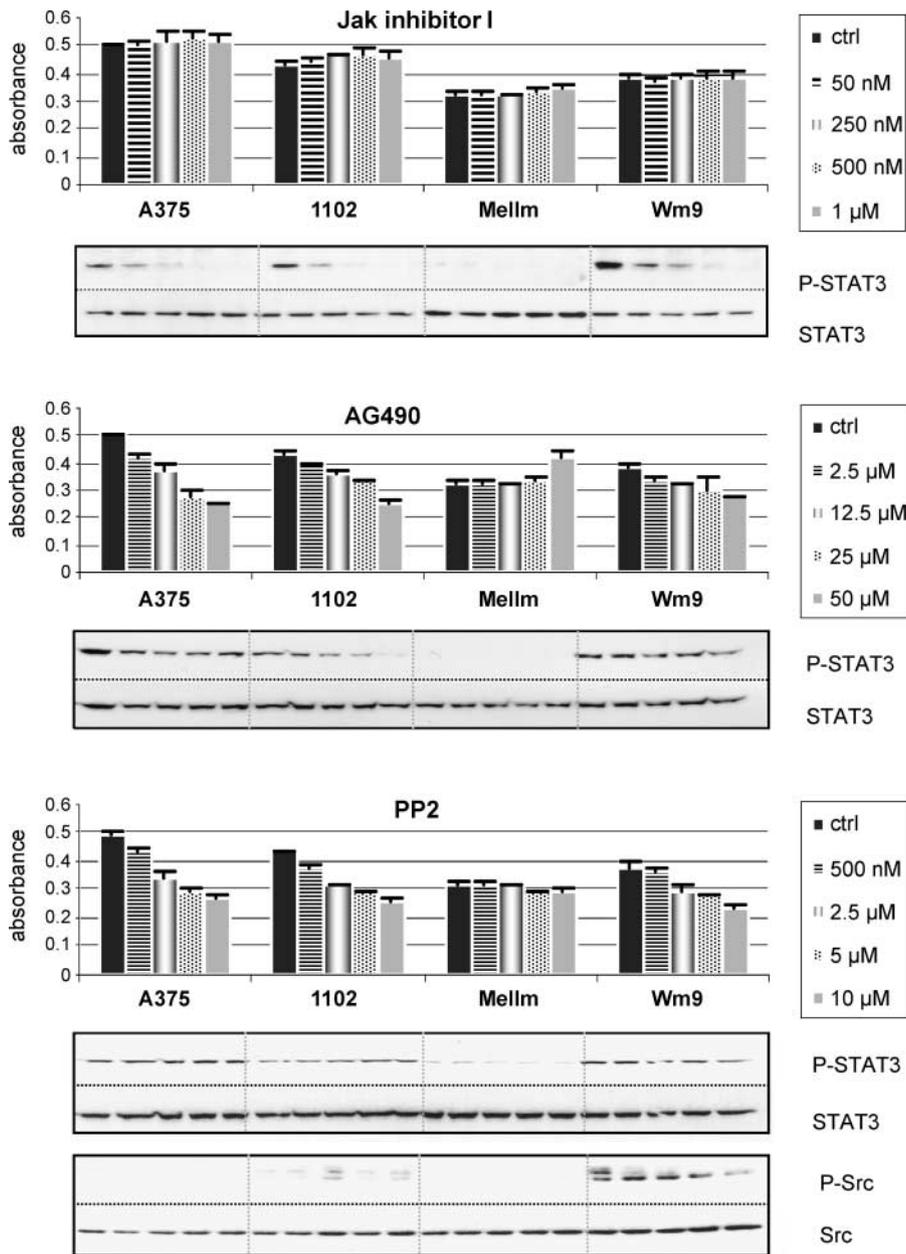
FIGURE 5. Dose-dependent influence of JAK and Src inhibitors on constitutive STAT3 activation. Four melanoma cell lines (A375, 1102, MelIm, and Wm9) were analyzed in proliferation assays in the presence of increasing amounts of JAK inhibitor I (*top*), AG490 (*middle*), and PP2 (*bottom*) for 48 h as described in Materials and Methods. Below each bar diagram, Western blot analyses of correspondingly treated cells (from 24-well plates) are illustrated.

888, 1102, 1286, MelIm, Wm239, Wm9, Wm35, and MeWo. All cell lines were adapted to growth in RPMI 1640 supplemented with 10% FCS, 50 μg/mL penicillin, 100 μg/mL streptomycin, and 0.5 mmol/L L-glutamine in a humidified atmosphere with 5% CO_2. Cervix carcinoma lines A431 and HeLa as well as Hek293 cells were grown in DMEM (supplemented with 10% FCS and antibiotics as above), whereas HepG2 liver carcinoma cells were cultured in DMEM-F12 medium with 10% FCS. Normal human melanocytes (NHEM) were purchased from Clonetics (Cambrex Bio Science) and cultured according to the supplier's instructions in complete MGM-4 medium. Normal human dermal fibroblasts (NHDF212) were prepared and maintained as described previously (53). The keratinocyte cell line HaCaT was grown in DMEM supplemented with 10% FCS, 50 μg/mL penicillin, and 100 μg/mL

streptomycin. Cells were photographed under phase contrast using a ×400 magnification.

Reagents and Antibodies

Where applicable, cells were stimulated for 30 min with 200 units IL-6 or with 20 ng/mL OSM (PeproTech). The following antibodies were used for protein detection in Western blots: STAT3 (BD Transduction Laboratories, BD Biosciences); phosphorylated STAT3-Tyr[705]; phosphorylated ERK1/2 (phosphorylated p44/42 MAPK-Thr[202]/Tyr[204]); ERK1/2 (p44/42 MAPK); and p38 MAPK, active Src (phosphorylated Tyr[416]), and Src antibodies (all from Cell Signaling Technology). Horseradish peroxidase–labeled secondary antibodies were purchased from Dako. The following inhibitors were purchased from Calbiochem (Merck) and used at the indicated concentrations:

JAK inhibitor I, JAK inhibitor AG490, p38 inhibitors SB203580 and SB202190, Src inhibitor PP2, and MAPK/ERK kinase 1/2 inhibitor U0126.

Western Blotting

High-density cells (1×10^6 per well) and low-density cells (2.5×10^5 per well) were seeded in six-well plates, left for 24 h, and washed once with PBS followed by direct lysis on ice with 100 or 400 μL of Laemmli buffer [20% glycerine, 10% β-mercaptoethanol, 4% SDS, 0.125 mol/L Tris-HCl (pH 6.8), 0.002% bromphenol blue] for low- and high-density cells, respectively. Alternatively, cells were lysed in lysis buffer [1% Triton X-100, 20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 10 mmol/L NaF, 1 mmol/L Na_3VO_4, 10 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L benzaminidine, 5 μg/mL aprotinin, 3 μg/mL pepstatin, 5 μg/mL leupeptin] and the concentration of total cell lysates was determined by Bradford assay (Bio-Rad). Total lysates (50 μg) were boiled for 5 min at 95°C and loaded on a 10% SDS-PAGE gel. Proteins were transferred onto a polyvinylidene difluoride membrane and detected with the relevant antibodies. Before reprobing, membranes were stripped for 30 min at 70°C in 62.5 mmol/L Tris, 2% SDS (pH 6.7), and 78 μL β-mercaptoethanol/10 mL buffer. Alternatively, for reprobing with phosphorylated-specific antibodies, blots were stripped for 4 to 5 h at room temperature in 2 mol/L glycine (pH 2.5) with 1 g SDS for each blot, washed extensively in H_2O, blocked for 1 h (TBS + 5% bovine serum albumin), and incubated with the next antibody.

For highly sensitive detection of chemiluminescent signals, the enhanced chemiluminescence solution pCA was used as described (54). Western blot signals were acquired with an Intas ChemiLux Pro Photoimager using the ChemiLux 1400M software (Intas). Quantification of signals was done using the Gel-Pro Express software (version 2.0).

Supernatant Transfer Experiment

Supernatants (3 mL) of highly confluent (10^6 cells per well) A375 and WM9 cells were collected after 1, 2, 3, and 4 days. Medium was separated from cellular debris by centrifugation and stored at 4°C until further use. A low amount (10^5) of A375 and WM9 cells were grown in a six-well plate overnight; the medium was removed and cells were washed once with PBS. Cells were then incubated with 1 mL prewarmed, conditioned medium for 1, 2, 3, and 4 days or with RPMI 1640 for 30 min, 6 h, and 24 h. As a positive control, cells were stimulated for 30 min with 200 units IL-6 per mL RPMI 1640. Cells were harvested through direct lysis with $1\times$ Laemmli buffer. Lysates were analyzed by Western blot using an antibody for the detection of phosphorylated Tyr^{705} STAT3 and p38 antibody as a loading control.

Electrophoretic Mobility Shift Assay

Electrophoretic mobility shift assays were done using 5 μg nuclear extracts of cells grown for 24 h at high or low densities and from control cells stimulated with 20 ng/mL OSM. Detection of the phosphorylated STAT3 interaction with a ^{32}P-labeled optimized STAT3-binding probe (m67SIE) was done as described before (55). Signals were quantified using a Typhoon phosphoimager and the ImageQuant software (Molecular Dynamics, GE Healthcare).

Reporter Gene Assay

Reporter gene assay was done essentially as described previously (56). Briefly, 1102 cells were plated at low (1×10^5 per well) or high (1×10^6 per well) cellular density and grown overnight. The next day, cells were transiently transfected with an m67-SIE-TK-Luc construct (m67, the high-affinity form of the c-sis–inducible element from the human *c-fos* gene) and a cytomegalovirus promoter-driven β-galactosidase construct using Fugene according to the manufacturer's instructions (Roche Biochemicals). Sixteen to 18 h after transfection, medium was changed and cells were incubated for another 12 h. Luciferase activities were determined and the data were normalized according to coexpressed β-galactosidase activities.

Apoptosis and Proliferation Assays

For proliferation assays, 3,000 cells per well were seeded in quadruplicate wells in 96-well plates. Increasing concentrations of DMSO, JAK inhibitor I, AG490, and PP2 were added for 48 h. WST-1 reagent (5 μL/well; Roche Biochemicals) was added for 30 min at 37°C and absorbance was measured at 450 nm.

For apoptosis assays, 750,000 cells were grown in quadruplicate in six-well plates with addition of 1 μmol/L JAK inhibitor I, 50 μmol/L AG490, or 10 μmol/L PP2 for 48 h.
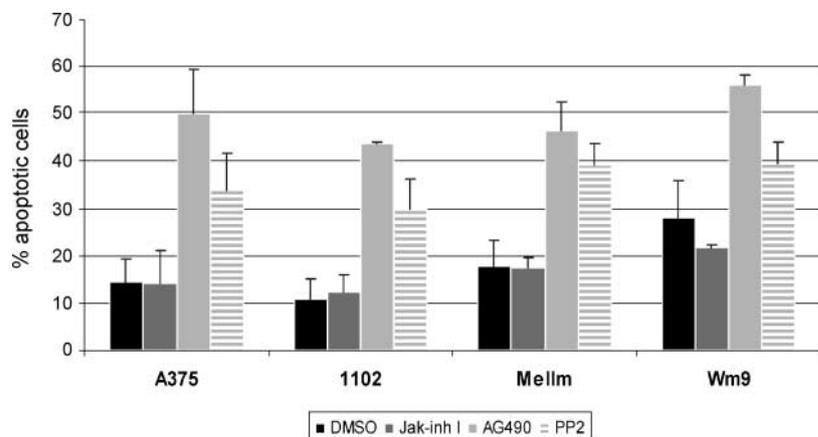
FIGURE 6. Effects of different inhibitors on the apoptosis of melanoma cells. Cells were seeded in six-well plates. DMSO, 1 μmol/L JAK inhibitor I, 50 μmol/L AG490, or 10 μmol/L of PP2 inhibitors were added for 48 h before cells were analyzed for Annexin V-FITC fluorescence by fluorescence-activated cell sorting analysis. Columns, mean percentages of total dead cells (Annexin V and propidium iodide positive) of triplicate samples; bars, SD.

Control cells were treated with DMSO. Cells were harvested and processed as described in the Annexin V-FITC Apoptosis Detection Kit II manual (BD Transduction Laboratories, BD Biosciences). FITC fluorescence was measured using a BD FACSCanto II. One of the four wells was washed with PBS and lysed directly on ice with 1× Laemmli buffer followed by Western blot analysis.

Acknowledgments

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Cell Density–Dependent Increase of Constitutive Signal Transducers and Activators of Transcription 3 Activity in Melanoma Cells Is Mediated by Janus Kinases

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