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Abstract

Proliferative resistance to transforming growth factor beta (TGF-beta) is regarded as a critical turning point in the malignant progression of many cancer types. In melanoma this resistance is associated with more aggressive metastatic behaviour. A recent study by our group identified proliferative and invasive subtypes of melanoma cultures and found that these are, respectively, susceptible and resistant to TGF-beta suppression of proliferation. Here, using previously characterised proliferative and invasive phenotype melanoma cultures, we explored molecular responses involved in modulating susceptibility to TGF-beta-mediated inhibition of proliferation. The Id2 gene was identified as being expressed more strongly in invasive phenotype cells less susceptible to TGF-beta repression than in proliferative phenotype cells. We correlated TGF-beta repression of Id2 gene expression in proliferative phenotype cells with p15(Ink4b) induction and cell cycle arrest. Furthermore, ectopic Id2 expression in proliferative phenotype cells counteracted p15(Ink4b) induction and consequently protected them from TGF-beta-mediated inhibition of proliferation. We conclude that transition to increased aggressiveness in melanoma cells requires Id2 upregulation to suppress TGF-beta induction of p15(Ink4b) and thus help to circumvent TGF-beta-mediated inhibition of proliferation.

Id2 suppression of p15 counters TGF-β-mediated growth inhibition of melanoma cells

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Summary

Proliferative resistance to transforming growth factor β (TGF-β) is regarded as a critical turning point in the malignant progression of many cancer types. In melanoma this resistance is associated with more aggressive metastatic behaviour. A recent study by our group identified proliferative and invasive subtypes of melanoma cultures and found that these are, respectively, susceptible and resistant to TGF-β suppression of proliferation. Here, using previously characterised proliferative and invasive phenotype melanoma cultures, we explored molecular responses involved in modulating susceptibility to TGF-β-mediated inhibition of proliferation. The *Id2* gene was identified as being expressed more strongly in invasive phenotype cells less susceptible to TGF-β repression than in proliferative phenotype cells. We correlated TGF-β repression of *Id2* gene expression in proliferative phenotype cells with $p15^{Ink4b}$ induction and cell cycle arrest. Furthermore, ectopic Id2 expression in proliferative phenotype cells counteracted $p15^{Ink4b}$ induction and consequently protected them from TGF-β-mediated inhibition of proliferation. We conclude that transition to increased aggressiveness in melanoma cells requires Id2 upregulation to suppress TGF-β induction of $p15^{Ink4b}$ and thus help to circumvent TGF-β-mediated inhibition of proliferation.

Introduction

Transforming growth factor β (TGF-β) has been shown to have both tumour suppressing and promoting activities. Tumour suppressing activity is demonstrated by its role as a negative regulator of proliferation for most early phase cancer cells. Evasion from this cytostatic response to TGF-β is important in the progression of melanoma and other cancers (Jennings and Pietenpol, 1998). Accordingly, reduced susceptibility to the growth inhibitory effects of TGF-β has been associated with increased invasive and metastatic properties of melanoma cells (Heredia et al., 1996; Krasagakis et al., 1999; Rodeck et al., 1994).

Significance

It has long been considered that the transition of melanoma from lesser to greater malignancy is a critical step in its progression to metastatic disease. One characteristic of melanoma cells undergoing this transition is the change from being susceptible to the growth-inhibitory effects of TGF-β to being resistant. The mechanism of acquired resistance to TGF-β-mediated suppression of proliferation is unknown. We describe here how in susceptible cells TGF-β suppression of *Id2* gene expression allows upregulation of $p15^{Ink4b}$ to induce cell cycle arrest, and how in resistant cells increased levels of *Id2* expression helps to mask the inhibitory properties of TGF-β.

TGF-β signalling remains intact in melanoma as few mutations have been shown to interrupt Smad-dependent signalling and it is therefore believed that desensitisation of melanoma cells to the growth inhibitory effects of TGF-β is highly specific to cell cycle progression (Javelaud et al., 2008). TGF-β regulates genes involved throughout the cell cycle, but its cytostatic effects have been primarily attributed to the regulation of factors targeting G1 events (Flores et al., 1996; Massague and Gomis, 2006). TGF-β induces the expression of cyclin-dependent kinase (CDK) inhibitors (Massague and Gomis, 2006) and represses growth-promoting transcription factors such as c-Myc (Pietenpol et al., 1990) and inhibitor of DNA binding (Id) proteins (Kowanetz et al., 2004; Ling et al., 2002).

Id proteins are positive regulators of cell growth and play a critical role in promoting G1/S cell cycle progression. Their role in the regulation of cell proliferation is thought to be driven by two mechanisms. First, they interfere with bHLH, ETS and Pax transcription factors and consequently regulate the expression of target genes such as the CDK inhibitors (Alani et al., 2001; Lyden et al., 1999; Mori et al., 2000; Ohtani et al., 2001; Prabhu et al., 1997; Rothschild et al., 2006). Secondly, Id2 binds tumour suppressor proteins of the Rb family and, when in large excess, abolishes their growth-suppressing activity by causing the release of E2F transcription factors required for cell cycle progression (Iavarone et al., 1994; Lasorella et al., 1996). In normal cells, Id2 is itself a downstream target of pRb and its family members, which inhibit its function against natural targets. However, Id2 overexpression by tumour cells may saturate the Rb pathway and deprive the cell of a critical cell cycle checkpoint (Lasorella et al., 1996). Rb pathway inhibition is believed to be achieved directly through the binding of Id2 and indirectly through Id2-mediated downregulation of CDK inhibitor expression (Ohtani et al., 2001).

In melanoma, Id levels have been reported primarily for Id1 with few publications examining Id2. For example, Polsky et al. (2001) have demonstrated in early melanoma a correlation between *Id1* expression and loss of $p16^{Ink4a}$ expression. Using tissue microarrays, Straume and Akslen (2005) evaluated the expression of Id1 in 119 cases of nodular melanoma and showed that strong Id1 expression was significantly associated with increased tumour thickness and reduced survival (Straume and Akslen, 2005). Id2 was first associated with melanoma when it was identified as the product of a down-regulated gene in melanomas with homozygous deletion of the CDKN2A locus genes in a global gene expression study (Bloethner et al., 2006). In a microarray gene expression analysis of uveal melanoma, which generated two subgroups representing tumours with low and high risk of metastatic death, *Id2* was one of the top class-discriminating genes (Onken et al., 2006).

Bittner et al. (2000) first suggested that there may be specific transcriptional signatures delineating melanoma cell subgroups. We recently characterised two distinct transcription signatures for melanoma cell cultures which, based on known functions of the genes involved, suggested that their respective contributions to metastatic potential were either proliferative or invasive (Hoek et al., 2006). We hypothesised that melanoma cells may be able to switch back and forth between these phenotypes and thereby drive disease progression. Subsequently, we demonstrated phenotype switching in vivo using an immunocompromised mouse model (Hoek et al., 2008). Many of the genes expressed by invasive phenotype melanoma cells had previously been characterised as being positively responsive to TGF-β-like signalling in other systems. Furthermore, TGF-β-mediated growth inhibition, which has been shown to be absent or less pronounced in aggressively invasive melanoma cells, was also shown to be a discriminating phenotype for the phenotypes we characterised (Hoek et al., 2006). Here, we identify *Id2* as a TGF-β regulated gene involved in the TGF-β growth inhibitory response and identify $p15^{Ink4b}$ as an Id2 regulated gene.

Results

Differential Id2 regulation and expression in human melanoma cultures

In previous DNA microarray experiments we identified two in vitro transcription phenotypes, proliferative and invasive, which we hypothesise together drive melanoma progression (Hoek et al., 2006, 2008). We performed supervised hierarchical clustering of cultured melanoma samples using normalised signal intensity data from 105 genes shown to be tightly linked to signature (Figure 1A). Earlier experiments had shown that proliferative signature cells were significantly more susceptible to TGF-β-mediated growth inhibition than invasive signature cells (Hoek et al., 2006). Selecting pairs of proliferative and invasive phenotype melanoma cell cultures, we performed TGF-β-susceptibility assays to establish that these cultures were appropriately susceptible or resistant to TGF-β-mediated growth inhibition, with at least 50% growth inhibition observed in susceptible proliferative phenotype cells while no inhibition was seen in resistant invasive phenotype cells (Figure 1B). We then looked for differential endogenous expression of Id2 mRNA and protein in our representative cell cultures. Using real-time RT-PCR and western blotting we showed that Id2 expression was diminished in susceptible cells when compared to resistant cells (Figure 1C, D). As Id proteins have been shown to be regulated by TGF-β in epithelial cells (Kowanetz et al., 2004; Ling et al., 2002), we used real-time RT-PCR to further investigate TGF-β modulation of *Id2* in susceptible and resistant cells. Real-time RT-PCR and western blotting show that TGF-β downregulates Id2 expression in susceptible (M010817; M000921) but not in resistant (M990115; M010119) cell cultures (Figure 2).

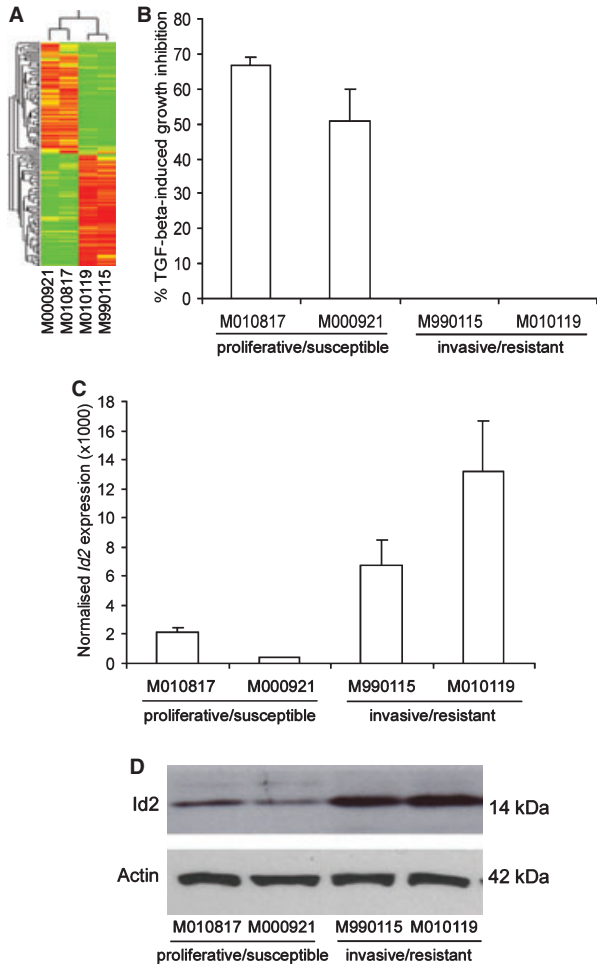

Figure 1. Melanoma cell phenotype characteristics and endogenous Id2 expression levels. (A) A gene expression heatmap, generated by clustering samples based on the normalised expression of 105 metastatic potential genes (Data S1). This highlights phenotype-specific signatures of the four cell cultures where M000921 and M010817 (proliferative phenotype) are clearly distinguishable from M990115 and M010119 (invasive phenotype). (B) Ninety-six hours after the addition of 5 ng/ml TGF-β1, cell proliferation was assessed by a colorimetric assay. The data is expressed as a percentage of TGF-β-induced growth inhibition and shows that proliferative phenotype cells are susceptible to the growth inhibitory effects of TGF-β while invasive phenotype cells are not. (C) *Id2* RNA expression in untreated cells as measured by real-time RT-PCR and (D) western blot analysis of Id2 protein expression in proliferative (M000921; M010817) and invasive (M990115; M010119) cell cultures. For real-time RT-PCR *Id2* mRNA levels are expressed as ratios to GAPDH expression, and for western blotting beta-actin protein expression is shown as the loading control. Proliferation assays were performed three times in quadruplicate and real-time RT-PCR experiments were performed three times in triplicate. Error bars represent standard deviations.

Id2 overexpression protects proliferative cells from the growth inhibitory effects of TGF-β

Given that Id2 is differentially expressed and regulated across melanoma cell phenotypes and that Id proteins are known to influence cell cycle processes, we hypothesised that Id2 overexpression may counteract the growth-inhibitory effect of TGF-β in susceptible cells. We used a myc-tagged wild-type Id2 adenoviral construct (Ad-Id2) (Gleichmann et al., 2002; Kowanetz et al., 2004; Toma et al., 2000) to transduce susceptible cells prior to treatment with TGF-β. Overexpression of Id2 protein was confirmed by western blot analysis (Figure 3A). We found that transduced susceptible cells were less growth inhibited by TGF-β compared with cells transduced with a control construct (Figure 3B). We therefore conclude that experimental overexpression of Id2 in susceptible cells protects them from the growth inhibitory effects of TGF-β by opposing TGF-β-induced downregulation of its endogenous expression. However, as the growth inhibitory effects induced by TGF-β could only be partially reversed, it suggests that Id2 is not solely responsible for this effect. To exclude the possibility that the observed phenotype was not specific to the effect of TGF-β but solely the consequence of Id2-mediated disturbance of cell growth, we compared cell growth of luciferase- and Id2-overexpressing proliferative and invasive cells. As shown in Figure 3C, Id2 overexpression does not affect cell growth and we therefore exclude the hypothesis that the effect of Id2 is dependent on this parameter. We then explored the possibility of mimicking the growth inhibitory effects of TGF-β by suppressing endogenous Id2 expression using an *Id2*-specific siRNA. Using transfectable susceptible cells we showed that TGF-β treatment and Id2 knock-down induced similar growth inhibition levels (Figure 3D).

Id2 regulates TGF-β-induced G1 cell cycle arrest

TGF-β-induced growth inhibition has been attributed to G1 cell cycle arrest in a number of cell types including melanoma (Flores et al., 1996; Massague and Gomis, 2006). To investigate whether Id2 modulation directly influences TGF-β-induced G1 cell cycle arrest, we analysed the cell cycle distribution of TGF-β-treated susceptible cells transduced with Id2-overexpressing or control adenoviruses. Twenty-four hours after infecting M010817 cells we treated them with TGF-β for 48 h before staining the cells with propidium iodide to analyse DNA content using flow cytometry. We detected a TGF-β-induced reduction of control cells in S phase and increase in G1 (Figure 4A). This G1 arrest was reversed when cells were first transduced with an overexpressing Id2 construct.

Id2 restricts TGF-β-induced upregulation of $p15^{Ink4b}$

Having shown that Id2 regulates TGF-β-induced G1 arrest, we sought to identify the mechanism of regulation. G1 cell cycle arrest induced by TGF-β has been partially attributed to its role in driving the expression of CDK inhibitors, which are negative regulators of the cell cycle (Massague and Gomis, 2006). Contrasting this, Ids have been shown to positively regulate cell cycle

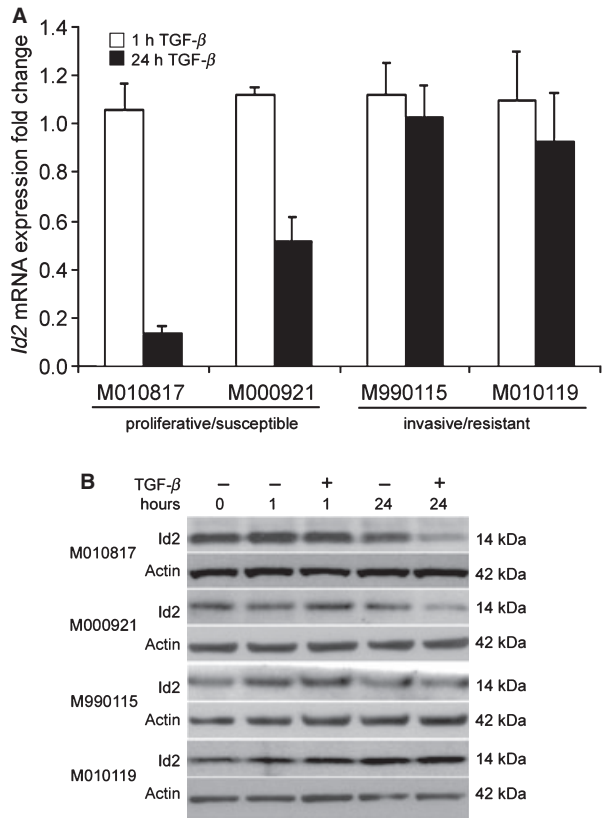

Figure 2. TGF-β suppresses Id2 expression only in TGF-β-susceptible melanoma cells. (A) *Id2* expression was measured by real-time RT-PCR as a ratio to GAPDH expression and expressed in terms of TGF-β-treated cells relative to untreated cells at 1 and 24 h normalised against data acquired at 0 h. This shows that *Id2* levels are more susceptible to TGF-β suppression in TGF-β-susceptible than in the TGF-β-resistant cells. Data represent averages of three independent experiments performed in triplicate with standard deviations (error bars). (B) Western blot analysis of Id2 protein expression after the addition of TGF-β; beta-actin protein expression is shown as a loading control.

progression by inhibiting CDK inhibitor expression (Alani et al., 2001; Lyden et al., 1999; Mori et al., 2000; Ohtani et al., 2001; Prabhu et al., 1997; Rothschild et al., 2006). We therefore hypothesised that Id2 suppressed the induction of CDK inhibitors by TGF-β. To verify this, we transduced susceptible cells with Ad-Id2 or Ad-luciferase, followed by TGF-β treatment and analysed the mRNA levels of CDK inhibitors after 1, 8 and 24 h of treatment. Contrasting with previously published melanoma reports (Florenes et al., 1996; Reed et al., 2001), $p21^{Cip1}$ expression was not induced by TGF-β treatment in our melanoma cells (Figure 4B). Similarly, we did not detect significant induction of $p27^{Kip1}$ or $p57^{Kip2}$. However, we did find a significant increase in $p15^{Ink4b}$ expression by TGF-β treatment. Interestingly, Id2 overexpressing cells showed significantly reduced levels of $p15^{Ink4b}$, which were not rescuable above control levels by TGF-β treatment. From this data, we conclude that Id2 opposes TGF-β-induced upregulation of $p15^{Ink4b}$ and consequent G1 arrest. To confirm that $p15^{Ink4b}$ is a target of Id2, we silenced Id2 expression using an *Id2*-specific siRNA and measured $p15^{Ink4b}$ expression. Both by real-time RT-PCR and western blotting, we could show that $p15^{Ink4b}$ expression was induced by Id2 downregulation (Figure 5A, B). From Id2 overexpressing and silencing assays, we conclude that Id2 is a negative regulator of $p15^{Ink4b}$.

Discussion

In this study, we show that TGF-β represses Id2 expression in TGF-β-susceptible proliferative phenotype melanoma cells. In contrast, invasive phenotype melanoma cells, which are resistant to TGF-β, express higher levels of Id2 and are not repressed by TGF-β (Figure 1). Although a number of reports have demonstrated the modulation of Id proteins by TGF-β in epithelial cells (Di et al., 2006; Kang et al., 2003; Kondo et al., 2004; Kowanetz et al., 2004), we show differential modulation of Id2 expression between melanoma cell phenotypes (Figure 2). Moreover, elevated expression of Id mRNA and protein have been reported for many different human tumours, including carcinomas, neural tumours, leukaemia, as well as melanoma, and in some cases high levels were associated with increased disease severity and poor prognosis (Perk et al., 2005). Complementing those findings, we saw that Id2 expression correlated with the two different transcription signatures we previously identified (Hoek et al., 2006), where Id2 expression is increased in invasive phenotype cells compared with proliferative phenotype cells.

We next demonstrated that we could protect susceptible cells from TGF-β-mediated growth inhibition by overexpressing Id2 to circumvent TGF-β-driven Id2 repression (Figure 3B). To complement this, we mimicked TGF-β-induced repression of Id2 with human *Id2*-specific siRNA in a transfectable susceptible cell culture. We found that silencing Id2 induced levels of growth inhibition similar to TGF-β treatment (Figure 3D). This suggests that Id2 downregulation by TGF-β is partially responsible for the growth inhibitory effect observed. However, as it is possible to further increase the growth inhibitory effect by treating Id2 knock-down cells with TGF-β (data not shown), we have to conclude that Id2 downregulation is not solely responsible for the observed TGF-β-induced suppression of growth. Rather, this complex response is likely to involve the modulation of multiple genes. Furthermore, partial knockdown of Id2 in TGF-β resistant cells (efficiency of siRNA transfection was unfortunately very limited in slow-growing resistant cells) only decreased the minor growth induced by the addition of TGF-β and did not render the cells susceptible to the growth inhibitory effect of the cytokine (data not shown). This strengthens the above hypothesis that the response to TGF-β is complex and

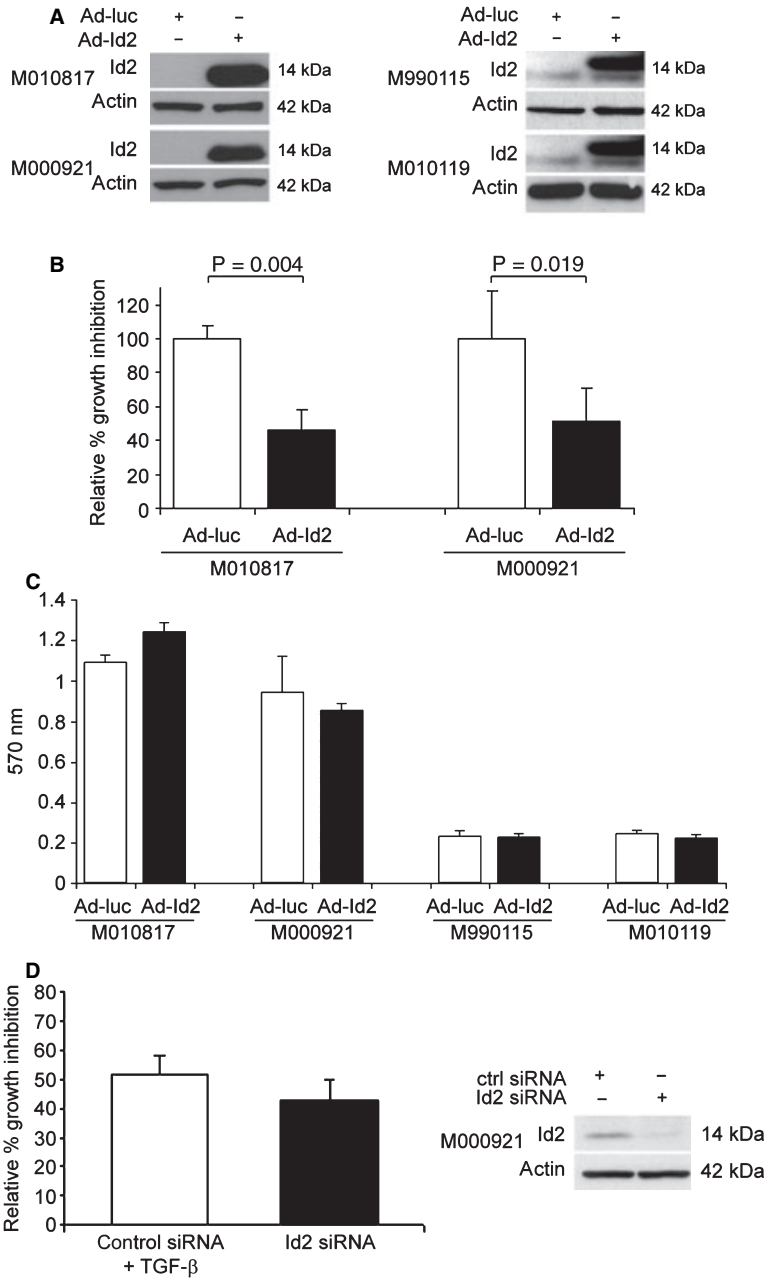

Figure 3. Id2 modulates TGF-β growth inhibition in melanoma. (A) Melanoma cells were infected with an Id2 overexpressing adenovirus or a control adenovirus Ad-luciferase at an MOI of 3. Id2 protein levels in transfected cells were determined by western blot analysis. (B) Twenty-four hours post-infection proliferative phenotype cells were treated with 5 ng/ml recombinant TGF-β1 and cell proliferation was evaluated 50 h later. Id2 overexpression resulted in reduced TGF-β-mediated inhibition of cell growth. (C) After infecting cells as described in (A) for 74 h, cell proliferation was assessed by a colorimetric assay. Cell proliferation was not affected by Id2 overexpression. (D) TGF-β-susceptible cells were transfected with siRNA duplexes targeting Id2 or non-targeting negative control. Id2 protein levels in transfected cells were determined by western blot analysis. Twenty-four hours post-transfection control transfected cells were treated with 5 ng/ml recombinant TGF-β1 and cell proliferation was evaluated 50 h later. Id2 knock-down resulted in reduced growth comparable to TGF-β treatment. Growth inhibition was determined by colorimetric assay and expressed as growth inhibition relative to untreated cells infected with the respective adenovirus or transfected with the respective siRNA. For each cell culture, data represents the results of four independent experiments performed in quadruplicate with standard deviations (error bars). Student's paired t-test was used to calculate significance.

involves multiple genes. We could however exclude the possibility that the decrease in TGF-β-induced growth inhibition resulting from Id2 overexpression is solely a consequence of Id2-induced growth deregulation as Id2 overexpression does not modulate proliferation in susceptible or resistant cells.

The high levels of Id2 expression and the inability of TGF-β to modulate it in TGF-β-resistant invasive phenotype cells, correlate well with the idea that aggressive cancer cells lose their susceptibility to the growth inhibitory effects of TGF-β while retaining their responses to tumour-promoting TGF-β-induced effects such as invasion, evasion of immune surveillance and metastasis. Adding to this, we hypothesise that the loss of Id2 modulation favours an invasive behaviour in melanoma as Id proteins have been shown to play a role in a number of cancer promoting processes, such as proliferation, angiogenesis, invasion and migration (Lasorella et al., 2001).

While TGF-β is a negative regulator of the cell cycle, Id proteins are positive regulators of cell growth and play a critical role in promoting G1/S cell cycle progression. TGF-β-induced growth suppression has been associated with increased expression of CDK inhibitors and a concomitant G1 cell cycle arrest (Massague and Gomis, 2006). In various cell types, TGF-β has been

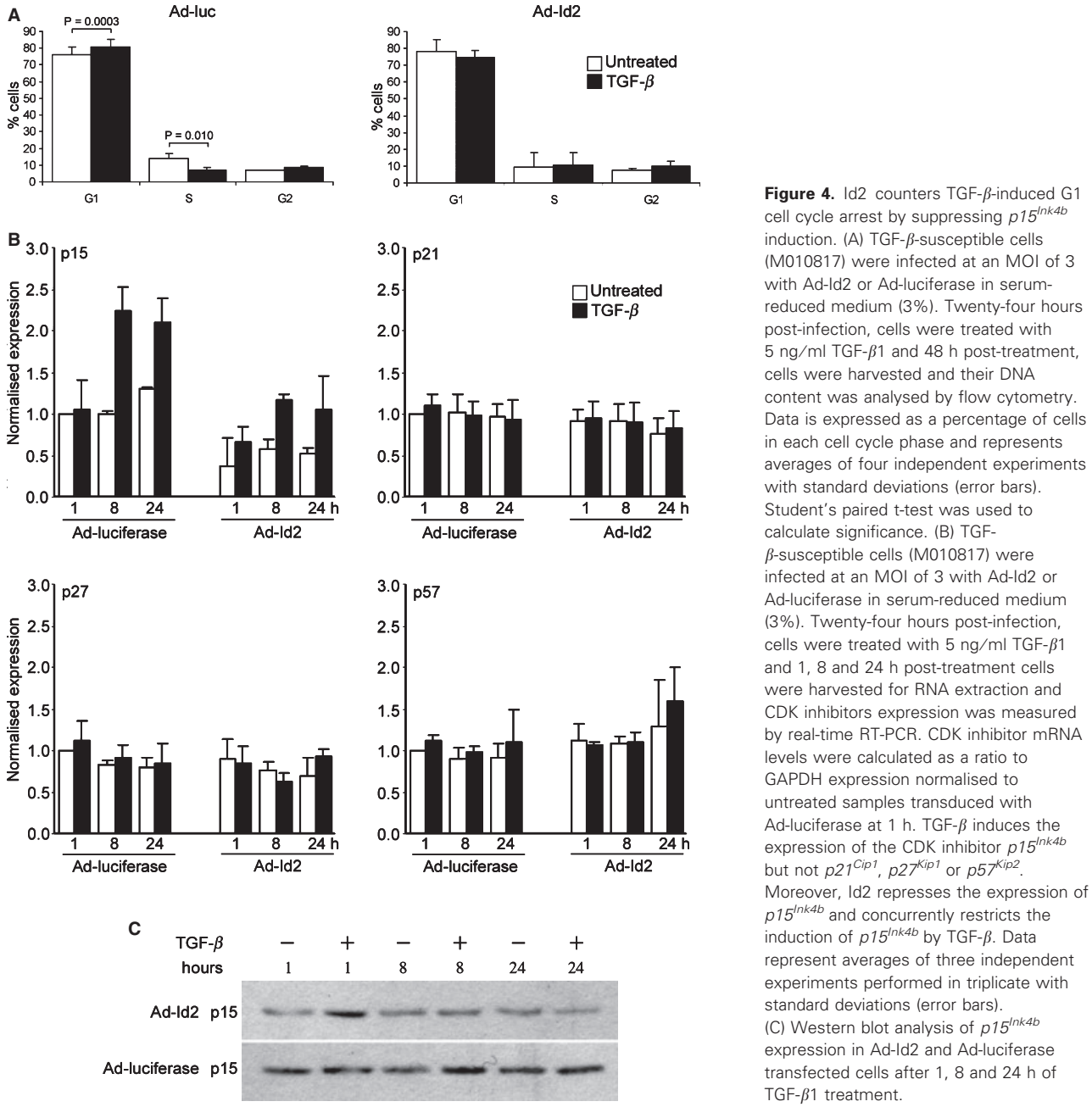

Figure 4. Id2 counters TGF-β-induced G1 cell cycle arrest by suppressing *p15*^{Ink4b} induction. (A) TGF-β-susceptible cells (M010817) were infected at an MOI of 3 with Ad-Id2 or Ad-luciferase in serum-reduced medium (3%). Twenty-four hours post-infection, cells were treated with 5 ng/ml TGF-β1 and 48 h post-treatment, cells were harvested and their DNA content was analysed by flow cytometry. Data is expressed as a percentage of cells in each cell cycle phase and represents averages of four independent experiments with standard deviations (error bars). Student's paired t-test was used to calculate significance. (B) TGF-β-susceptible cells (M010817) were infected at an MOI of 3 with Ad-Id2 or Ad-luciferase in serum-reduced medium (3%). Twenty-four hours post-infection, cells were treated with 5 ng/ml TGF-β1 and 1, 8 and 24 h post-treatment cells were harvested for RNA extraction and CDK inhibitors expression was measured by real-time RT-PCR. CDK inhibitor mRNA levels were calculated as a ratio to GAPDH expression normalised to untreated samples transduced with Ad-luciferase at 1 h. TGF-β induces the expression of the CDK inhibitor *p15*^{Ink4b} but not *p21*^{Cip1}, *p27*^{Kip1} or *p57*^{Kip2}. Moreover, Id2 represses the expression of *p15*^{Ink4b} and concurrently restricts the induction of *p15*^{Ink4b} by TGF-β. Data represent averages of three independent experiments performed in triplicate with standard deviations (error bars). (C) Western blot analysis of *p15*^{Ink4b} expression in Ad-Id2 and Ad-luciferase transfected cells after 1, 8 and 24 h of TGF-β1 treatment.

shown to transcriptionally induce *p21*^{Cip1} (Datto et al., 1995), *p27*^{Kip1} (Kamesaki et al., 1998) *p57*^{Kip2} (Scandura et al., 2004) and *p15*^{Ink4b} (Hannon and Beach, 1994) expression. In contrast, Id proteins have been shown to regulate the expression of CDK inhibitors, for example *p16*^{Ink4a} (Alani et al., 2001; Ohtani et al., 2001), *p21*^{Cip1} (Prabhu et al., 1997), *p57*^{Kip2} (Rothschild et al., 2006) *p27*^{Kip1} (Lyden et al., 1999; Mori et al., 2000). Using proliferative signature cell types, which were significantly growth inhibited by TGF-β, we could show that TGF-β induced the expression of the gene coding for p15^{Ink4b} but not p21^{Cip1}, p27^{Kip1} or p57^{Kip2}. Furthermore, reinforcing the link between TGF-β-induced growth inhibition and TGF-β-induced downregulation of Id2, we could show that only *p15*^{Ink4b} was downregulated by Id2 overexpression. We therefore conclude from these results that Id2 subdues TGF-β-induced upregulation of *p15*^{Ink4b}, leading to attenuated TGF-β-induced growth-inhibition response in our cell cultures.

Although analysis of the promoter region of *p15*^{Ink4b} reveals the presence of E-boxes, which renders this gene competent for Id-mediated repression (Pagliuca et al., 2000), no previous report has shown a direct regulatory relationship. Here, we show that Id2 overexpression

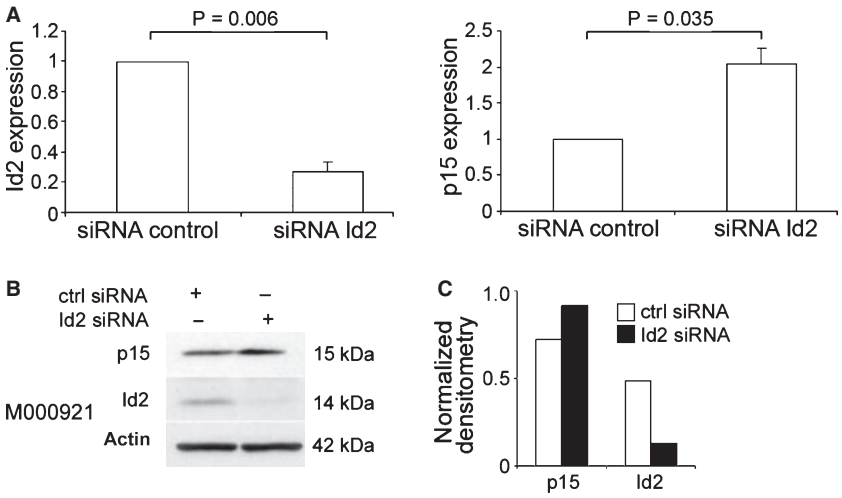

Figure 5. Id2 regulates $p15^{Ink4b}$ expression. (A) TGF-β-susceptible cells were transfected with siRNA duplexes targeting *Id2* or non-targeting negative control. 48 h post-transfection cells were harvested for RNA extraction and $p15^{Ink4b}$ expression was measured by real-time RT-PCR. $p15^{Ink4b}$ mRNA levels were calculated as a ratio to GAPDH expression normalised to negative control samples. Id2 induces the expression of $p15^{Ink4b}$. Data represent averages of four independent experiments performed in triplicate with standard deviations (error bars). Student's paired t-test was used to calculate significance. (B) As shown in Figure 3C, proliferative phenotype cells were transfected with siRNA duplexes targeting Id2 or non-targeting negative control. In addition to Id2 protein levels, $p15^{Ink4b}$ protein levels were determined by western blot analysis. Beta-actin protein expression is shown as a loading control. (C) Densitometric analysis of the Western blot results. Control siRNA data in white, Id2 siRNA data in black.

decreases and Id2 knock-down increases $p15^{Ink4b}$ expression. We also highlight the importance of $p15^{Ink4b}$ as a TGF-β target gene involved in the cytostatic effects observed in melanoma and reveal that upregulation of $p21^{Cip1}$ is not, as shown by others, necessary for this response (Florenes et al., 1996; Reed et al., 2001).

Because others have shown a clinical link between Id1 expression and survival in melanoma patients (Straume and Akslen, 2005), we looked for a similar link to Id2. However, evaluating Id2 expression in cell culture and tissue arrays, we could find no association between Id2 expression and survival in melanoma (data not shown). We observed Id2 expression heterogeneity in most tissue samples and believe that tumour heterogeneity may account for the absence of correlation as tissue array spots or cell cultures derived from tissue are not representative of whole lesions. Equally, our study may be underpowered by the limited number of samples.

TGF-β interference of cell cycle progression is central to its growth inhibitory activity. We showed here that Id2, a positive regulator of cell growth, is differently expressed in proliferative and invasive melanoma cells. More importantly, we showed that Id2 is regulated by TGF-β only in proliferative cells which are responsive to its growth inhibitory activity although other TGF-β target genes are regulated in both cell phenotypes. We also identified $p15^{Ink4b}$ as a mediator of TGF-β-induced G1 block and demonstrated that this CDK inhibitor is a target of Id2. Together, we conclude that the loss of TGF-β-mediated Id2 regulation is important in the desensitisation of melanoma cells to the growth inhibitory effect of TGF-β and that $p15^{Ink4b}$ is a mediator of this response.

Materials and methods

Cell culture and adenoviruses

Melanoma cell cultures were established from surplus material from cutaneous melanoma metastases removed by surgery after having obtained written informed consent of the patient. Clinical diagnosis was confirmed by histology and immunohistochemistry. Melanoma cells were released from tissue sections and grown as previously described (Geertsen et al., 1998). Cell cultures were chosen according to their transcription pattern signatures as previously described (Hoek et al., 2006). Two proliferative signature (M000921, M010817) and two invasive signature (M990115, M010119) melanoma cultures were used. Myc-tagged Ad-Id2 was a generous gift from F.D. Miller, Toronto, Canada. Ad-luciferase and Ad-Id2 were amplified and titrated in 293 cells as described previously (Hemmi et al., 1998).

RNA extraction, cDNA synthesis and real-time RT-PCR

Total RNA was extracted from melanoma cell cultures using TRIzol according to manufacturer instructions (Invitrogen, Carlsbad, CA, USA). One microgram total RNA was used for cDNA synthesis using Promega's Reverse Transcription System according to the manufacturer instructions (Promega, Madison, WI, USA). Real-time PCR was performed on 1 μg template cDNA using Roche's LightCycler DNA Master SYBR Green kit (Roche, Basel, Switzerland). Primers were 5′-CTGCCCAAGCTCTACCTTCC-3′ and 5′-CAGGTCCACATGGTCTTCCT-3′ ($p21^{Cip1}$); 5′-CGTGCGAGTGTCTAACGGGAGC-3′ and 5′-TGCGTGTCCTCAGAGTTAGCC-3′ ($p27^{Kip1}$); 5′-GCGGCGATCAAGAAGCTGTC-3′ and 5′-CCGGTTGCTGCTACATGAAC-3′ ($p57^{Kip2}$). Primers for $p15^{Ink4b}$ were purchased at Qiagen, QT00203147 (Qiagen, Hombrechtikon, Switzerland).

Western blot analysis

Cells were washed twice with cold phosphate-buffered saline (PBS) and lysed at 4°C in lysis buffer containing 20 mM Tris–HCl (pH 7.5), 1% Triton X-100 (Sigma-Aldrich), 137 mM NaCl, 10% glycerol and protease inhibitors (Roche). Proteins were separated by SDS–PAGE under reducing conditions and transferred onto nitrocellulose membranes (Invitrogen, Basel, Switzerland). Membranes were probed with a rabbit anti-Id2 monoclonal antibody (Zymed Laboratories, Invitrogen, San Francisco, CA, USA) or a goat anti-actin polyclonal antibody (SantaCruz, Biotechnology, La Jolla, CA, USA) followed by horseradish peroxidase-conjugated goat anti-rabbit or rabbit anti-goat secondary antibodies (Bio-Rad, Reinach, Switzerland and SantaCruz, respectively). Bound antibodies were detected by chemiluminescence (ECL, GE Healthcare, Buckinghamshire, UK).

Growth inhibition assays

Cells were seeded in 24-well microplates and left to settle down overnight. Cells were then infected at an MOI of 3 with Ad-Id2 or Ad-luciferase in serum-reduced medium (3%). For siRNA treatment, cells were transfected with siRNA duplexes targeting Id2 (sense: GGUGGAGCGUGAAUACCAGtt and antisense: CUGGUAUUCACGCUCCACCtt) or non-targeting negative control (scrambled sequence) using INTERFERin (PolyPlus, Illkirch, France) according to manufacturer instructions. Twenty-four hours post-infection or 48-h post-transfection cells were treated with 5 ng/ml recombinant TGF-β1 (BioSource, Camarillo, CA, USA) and 50 h post-treatment cell metabolic activity was determined with a standard colorimetric assay measuring 3-(4,5-dimethyldiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT; Sigma-Aldrich, Buchs, Switzerland) reactivity and used as an approximation of cell proliferation. Growth inhibition was expressed as a percentage of growth inhibition compared against growth in the absence of TGF-β1.

Cell cycle FACS analysis

Cells were seeded in 24-well microplates and left to settle overnight. Cells were then infected at an MOI of 3 with Ad-Id2 or Ad-luciferase in serum-reduced medium (3%). Twenty-four hours post-infection, cells were treated with 5 ng/ml recombinant TGF-β1 (BioSource) and 48 h post-treatment, cells were harvested by trypsinisation and fixed in 70% ethanol at 4°C overnight. Cells were then washed with PBS, followed by incubation in 300 μl of 0.1% Triton X-100 (Sigma-Aldrich), 50 μg/ml propidium iodide (Fluka BioChemica, Switzerland) and 200 μg/ml DNase-free RNase A (Sigma-Aldrich) for 30 min before analysis on FACSCalibur (Becton-Dickinson, Switzerland).

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Data S1. Metastatic potential genes.

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