Cytoplasmic p21 expression levels determine cisplatin resistance in human testicular cancer

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Platinum-based chemotherapies such as cisplatin are used as first-line treatment for many cancers. Although there is often a high initial responsiveness, the majority of patients eventually relapse with platinum-resistant disease. For example, a subset of testicular cancer patients still die even though testicular cancer is considered a paradigm of cisplatin-sensitive solid tumors, but the mechanisms of chemoresistance remain elusive. Here, we have shown that one key determinant of cisplatin-resistance in testicular embryonal carcinoma (EC) is high cytoplasmic expression of the cyclin-dependent kinase (CDK) inhibitor p21. The EC component of the majority of refractory testicular cancer patients exhibited high cytoplasmic p21 expression, which protected EC cell lines against cisplatin-induced apoptosis via CDK2 inhibition. Localization of p21 in the cytoplasm was critical for cisplatin resistance, since relocalization of p21 to the nucleus by Akt inhibition sensitized EC cell lines to cisplatin. We also demonstrated in EC cell lines and human tumor tissue that high cytoplasmic p21 expression and cisplatin resistance of EC were inversely associated with the expression of Oct4 and miR-106b seed family members. Thus, targeting cytoplasmic p21, including by modulation of the Oct4/miR-106b/p21 pathway, may offer new strategies for the treatment of chemoresistant testicular and other types of cancer.

Introduction

In testicular cancer (TC), even in cases of extensive metastatic disease, an enormous decrease in mortality has been observed with the introduction and use of highly effective cisplatin-containing chemotherapy schemes (1, 2). Therefore, TC is considered the paradigm for curative disease. Despite the overall treatment success, about 20%–50% of the TC patients with extensive metastatic spread belonging to the intermediate or poor risk group will not achieve a durable complete remission after initial treatment and will eventually die from this disease (3). The molecular basis for resistance, however, remains obscure.

A major role in the response to chemotherapeutic drugs and the execution of apoptosis has been ascribed to wild-type p53 (4). In only a small proportion of TCs, mutations in *p53* affect the downstream apoptotic pathway and lead to resistance (5), in particular, as wild-type p53 is expressed at high levels in the majority of TCs (6–9). p53 is a tumor suppressor protein with a dual role in stress response by transactivation of genes that induce apoptosis, such as *FAS*, as well as genes that induce cell-cycle arrest, such as *CDKN1A* (encoding $p21^{cip1/waf1}$). Interestingly, the cyclin-dependent kinase (CDK) inhibitor $p21^{cip1/waf1}$ (p21) has been shown to inhibit apoptosis (10–13). Remarkably, several studies have demonstrated that p21 protein and *CDKN1A* mRNA expression are abundantly expressed in more differentiated TCs, such as mature teratoma (14, 15). Worthy of note, these teratomas are resistant to cisplatin-based chemotherapy (8, 15–17). In contrast, p21 is almost not detectable in seminomas and embryonal carcinomas (EC) that are predominantly sensitive to cisplatin (9, 14, 18).

In human TC/EC cell lines, similar to patients, cisplatin proved to be an extremely cytotoxic drug, inducing massive apoptosis (19–22). Cisplatin treatment of EC cells resulted in enhanced levels of p53 and Mdm2, activation of the Fas apoptotic pathway, and induction of apoptosis, while the expression levels of p21 were almost not affected (7, 20). In contrast, γ irradiation induced p53 and Mdm2 levels and a massive induction of cytoplasmic p21 without inducing apoptosis or cell-cycle arrest in EC cells (7). These results suggested an important role for cytoplasmic p21 in preventing DNA damage–induced apoptosis in EC cells.

Results

High cytoplasmic p21 expression is associated with cisplatin resistance in EC cells. A panel of cisplatin-sensitive and -resistant EC cell lines was used in this study to compare cisplatin responses (Figure 1A and Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI41939DS1) with the levels of (induced) p21 (Figure 1, B and C). Resistance to cisplatin was predominantly associated with high p21 expression levels, since the intrinsically resistant EC cell lines Scha and 2102EP showed higher basal and cisplatin-induced p21 and *CDKN1A* levels compared with the cisplatin-sensitive EC cell lines Tera and 833KE (Figure 1, A–C). Low levels of p21 were not associated with cisplatin response in Tera-CP, a subline with acquired cisplatin resistance due to p21 unrelated mechanisms (23).

The subcellular localization of p21 was investigated with immunofluorescence microscopy (IF) and Western blot analysis (WB) using fractionated protein samples. We stained the EC cell lines Tera, Tera-CP, 833KE, Scha, and 2102EP for basal levels of p21 and p21 levels after treatment with cisplatin (or γ irradiation). All untreated EC cell lines showed a predominant cytoplasmic localization of p21, albeit with large differences in p21 intensity (Figure 1, D and E). Cytoplasmic localization was not affected by cisplatin treatment (Figure 1, D and E, and Supplemental Figure 1B). γ irradiation was used as positive control for p21 induction in EC cells (7). Despite almost a similar p53 increase, γ irradiation, in

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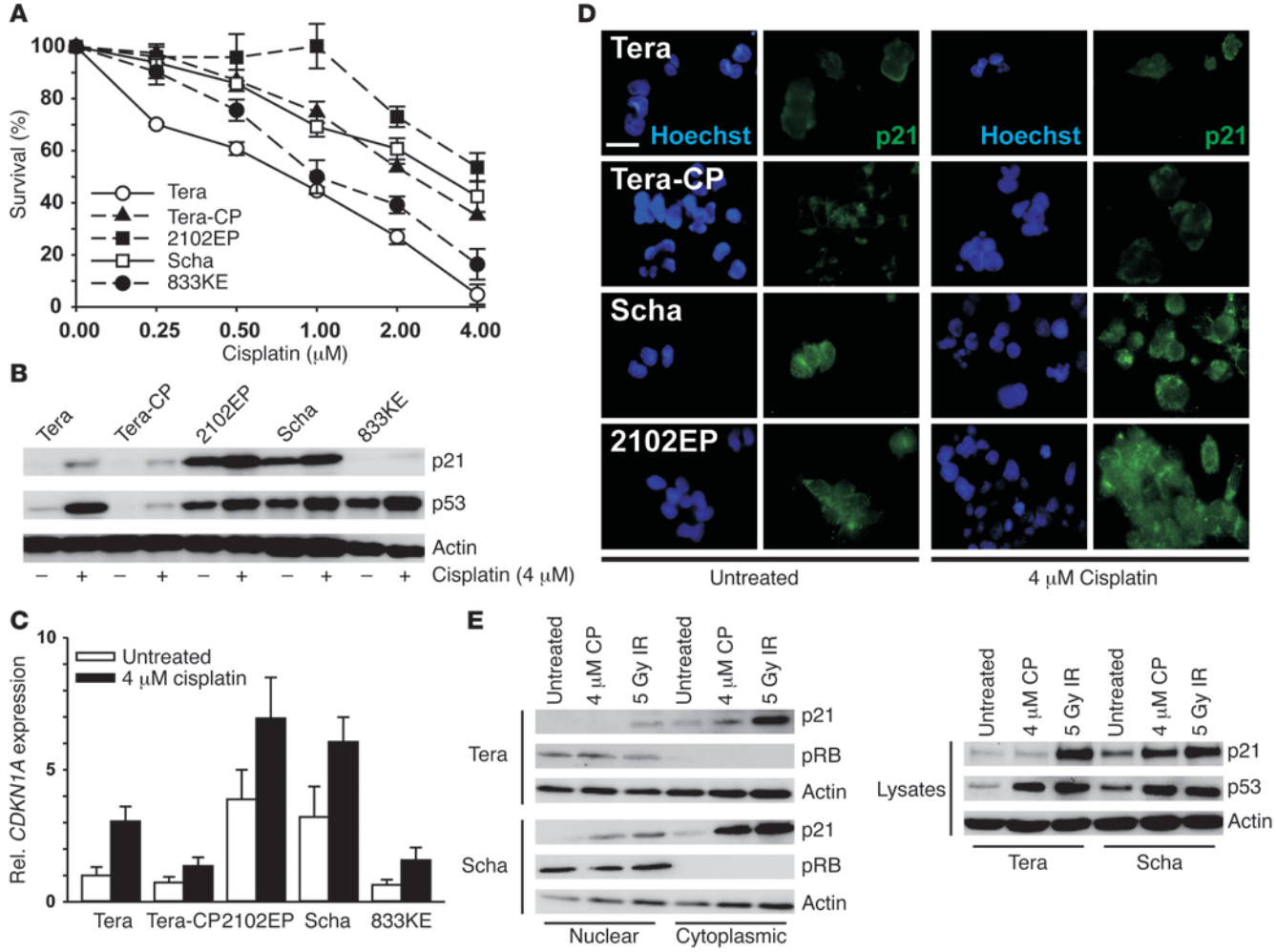

Figure 1

High cytoplasmic p21 expression is associated with cisplatin resistance in EC cell lines. (**A**) Survival of EC cells after 96 hours of continuous cisplatin treatment as indicated. (**B**) Differences in p21 and p53 levels of the EC cell lines treated and untreated with cisplatin for 24 hours as indicated. A representative example of 3 independent experiments is shown. (**C**) Differences in *CDKN1A* expression levels of the EC cell lines, treated and untreated. Note that the intrinsic cisplatin-resistant EC cell lines (2102EP and Scha) have higher basal and cisplatin-induced p21/*CDKN1A* levels compared with cisplatin-sensitive EC cell lines (Tera and 833KE). (**D**) p21 is localized in the cytoplasm in EC cells when untreated and after 24 hours of cisplatin treatment. Hoechst staining was used to visualize nuclei (blue). Scale bar: 30 μm. (**E**) 24 hours after cisplatin (CP) treatment or γ irradiation (IR), nuclear and cytoplasmic proteins were isolated and analyzed by WB for expression of p21 using retinoblastoma protein (pRB) as nuclear control, whereas β-actin is shown as a loading control. WB of total lysates shows that, despite an almost similar p53 increase, γ irradiation in contrast with cisplatin treatment strongly induced accumulation of p21 both in cisplatin-resistant Scha and cisplatin-sensitive Tera cells. Representative examples of 3 independent experiments are shown. Data are represented as mean ± SD.

contrast with cisplatin treatment, strongly induced accumulation of p21 in the cytoplasm in both cisplatin-resistant Scha and cisplatin-sensitive Tera cells (ref. 7, Figure 1E, and Supplemental Figure 1C). We next hypothesized that high levels of cytoplasmic p21 have a causal role in cisplatin resistance in EC cell lines.

Cytoplasmic p21 protects EC cells against cisplatin-induced apoptosis. To investigate the importance of high levels of cytoplasmic p21, both cisplatin-sensitive and cisplatin-resistant EC cell lines were depleted of p21, using siRNA. Downregulation of p21 led to an increase in apoptosis upon cisplatin treatment in the intrinsically resistant cell lines Scha and 2102EP as compared with cells transfected with scrambled siRNA (Figure 2A). The loss of p21 in Scha and 2102EP resulted in enhanced PARP cleavage and higher caspase 3 activation compared with control cells following cisplatin treatment (Figure 2, B and C). In the cisplatin-sensitive 833KE and Tera cells and the acquired cisplatin-resistance Tera-CP cells, all expressing low endogenous cytoplasmic p21 levels, no significant effect of p21 downregulation on apoptosis levels was observed (Supplemental Figure 2, A–C).

Next, we stably transduced the cisplatin-sensitive cell line Tera with a retroviral construct containing both p21-ΔNLS and GFP, or GFP only (Tera-mock) to study more extensively whether enhanced levels of cytoplasmic localized p21 are involved in suppressing apoptosis. The p21-ΔNLS protein lacks its bipartite nuclear localization signal and is therefore maintained in the cytoplasm (Supplemental Figure 2D). Treatment with cisplatin resulted in less apoptosis and reduced caspase 3 activity in Tera-p21-ΔNLS compared with Tera-mock (Supplemental Figure 2, E and F). We downregulated p21-ΔNLS in Tera-p21-ΔNLS cells using p21 siRNA, and we showed that these cells

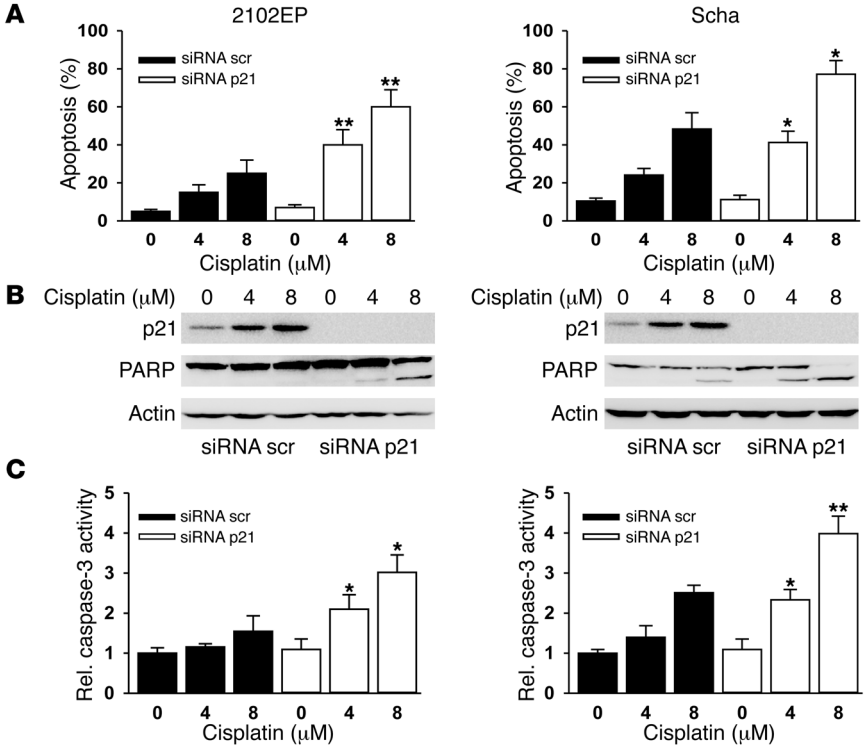

Figure 2
Increased cisplatin sensitivity after p21 downregulation in intrinsically resistant EC cell lines. (**A**) Downregulation of p21 sensitizes intrinsically resistant EC cells for cisplatin-induced apoptosis. Cells were treated with scrambled siRNA (siRNA scr) or with p21 siRNA (siRNA p21) for 24 hours. After cisplatin treatment for 24 hours, apoptosis induction was analyzed by fluorescence microscopy on acridine orange–stained cells for 2102EP and Scha. (**B**) Successful downregulation of p21, using siRNA against p21, and enhanced cleavage of PARP in cisplatin-treated 2102EP and Scha; a representative example of 3 independent experiments is shown. (**C**) Increased caspase 3 activity after treatment with siRNA against p21 in cisplatin-treated 2102EP and Scha. $^{*}P < 0.05$; $^{**}P < 0.01$ compared with matching siRNA scrambled (scr) control. Data are represented as mean ± SD.

became as sensitive as Tera-mock cells to cisplatin. No effect of p21 siRNA on cisplatin-induced apoptosis was observed in Tera-mock cells (Figure 3, A and B, and Supplemental Figure 2, G and H).

Cytoplasmic p21 forms a complex with proapoptotic CDK2 in cisplatin-resistant EC cells. It has been described that caspase 3–mediated cleavage of p21 activates the proapoptotic role of CDK2 (24–26). Therefore, we analyzed the presence of CDK2 in p21 IP. In Tera, Tera-p21-ΔNLS, and Scha, CDK2 coprecipitated with p21, indicating that CDK2 is in complex with cytoplasmic p21 in untreated cells and in cells treated with cisplatin or γ irradiation (Figure 3C). In addition, we stained for the presence of apoptosis signaling kinase 1 (ASK1), since cytoplasmic p21 can bind to ASK1, thereby inhibiting ASK1-mediated apoptosis via the intrinsic mitochondrial apoptotic pathway (10–13). In fact, more CDK2 and ASK1 was coprecipitated with p21 in untreated and cisplatin-treated Scha and Tera-p21-ΔNLS, which have higher p21 levels compared with Tera. In irradiated Tera and Scha expressing equal p21 levels, high levels of p21 were precipitated, leading to coprecipitation of almost similar levels of CDK2 and ASK1 (Figure 3C). We further established the important proapoptotic role of CDK2 by downregulating CDK2 with siRNA in cisplatin-sensitive cell lines, which led to a decrease in cisplatin-induced apoptosis and PARP cleavage compared with scrambled siRNA in Tera and 833KE (Figure 3D and Supplemental Figure 3A). Next, we investigated the proapoptotic role of CDK2 in the high p21-expressing 2102EP and Scha. Therefore, we sensitized these cells to cisplatin by suppression of p21 and, additionally, transfected these cells with CDK2 siRNA. The observed increase in apoptosis in p21-suppressed 2102EP and Scha cells upon cisplatin treatment could be dramatically reduced by cotransfection with CDK2 siRNA (Supplemental Figure 3B).

Interfering in p-Akt–mediated cytoplasmic localization of p21 sensitizes cisplatin-resistant EC cells. The cytoplasmic localization of p21 can be caused by phosphorylation of Thr145 located in the NLS of p21 (11, 27). Upon phosphorylation of the NLS domain by p-Akt, phosphorylated p21 (p-p21) becomes more stable and dynamically shuttles between the nucleus and the cytoplasm (11, 27, 28). In untreated and cisplatin-treated EC cells, p-p21 was detectable with WB (Supplemental Figure 4A). IF indicated that p-p21 was also localized in the cytoplasm (Figure 4A). In order to find out whether phosphorylation of Thr145 could occur via p-Akt, we stained the EC cells for p-Akt and its natural inhibitor phosphatase and tensin homolog (PTEN). In Tera, Scha, and 2102EP, p-Akt is present (Figure 4B and Supplemental Figure 4B), whereas PTEN is not expressed in these cells (data not shown) (29).

Treatment with the PI3K inhibitor LY294002 or with the specific Akt inhibitor triciribine (currently in phase I trial) resulted in dephosphorylation of Akt in Tera, Scha, and 2102EP and concomitant dephosphorylation of p21 (Thr145), which is consistent with a more pronounced nuclear localization of p21 (Figure 4, A and B, and Supplemental Figure 4, B–E). In the cisplatin-sensitive cell line Tera, either LY294002 or triciribine did not affect cisplatin sensitivity (Supplemental Figure 5A), though shuttling of the weakly detectable cytoplasmic p21 to the nucleus was observed (Supplemental Figure 4, C and D). Interestingly, a strong sensitization to cisplatin was observed in Scha and 2102EP with the combined treatment for 24 hours with cisplatin and either LY294002 or triciribine (Figure 4C and Supplemental Figure 5A). Shuttling of p21 to the nucleus following LY294002 treatment resulted in a loss of CDK2 in complex with p21 in Scha cells (Supplemental Figure 5B). Release of CDK2 was instrumental in the enhanced induction of apoptosis, since the sensitization to cisplatin combined with LY294002 in 2102EP and Scha cells was almost completely blocked following suppression of CDK2 (Supplemental Figure 5C). Downregulation of Akt using siRNA targeting Akt1–3 reduced the overall levels of p-p21 and resulted in a more pronounced nuclear local-

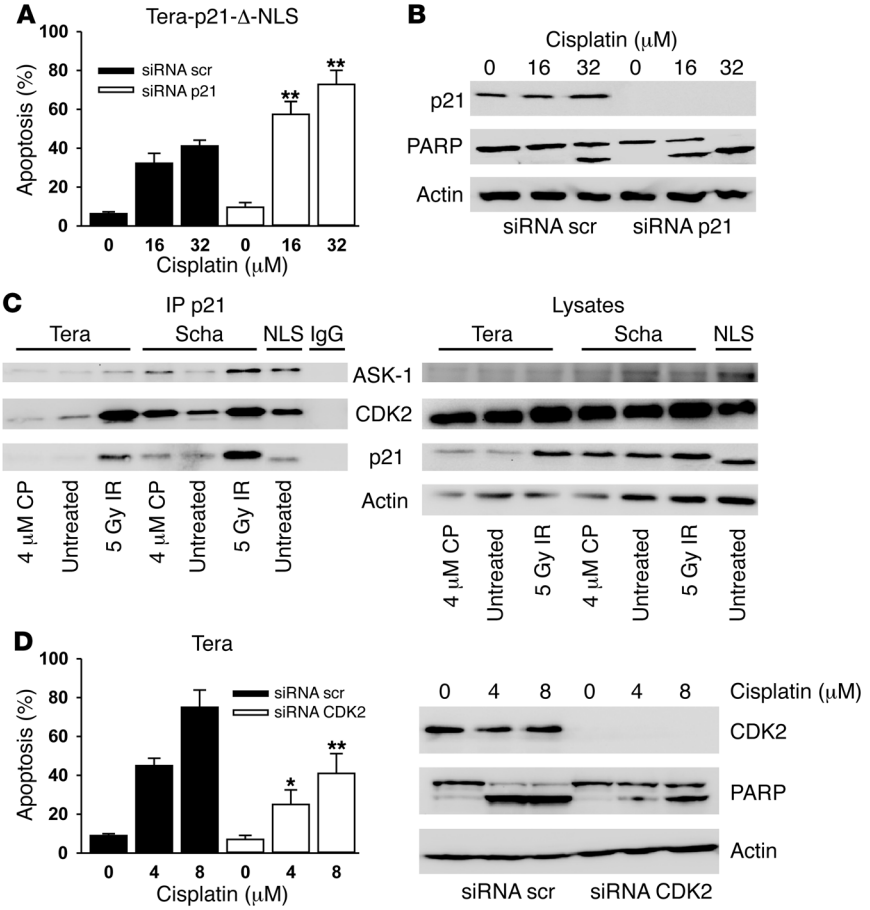

Figure 3
Overexpression of cytoplasmic p21 protects against cisplatin-induced apoptosis via complex formation of p21 with CDK2 and ASK1. (**A**) p21 siRNA–mediated downregulation of p21-ΔNLS in Tera-p21-Δ-NLS sensitizes for cisplatin-induced apoptosis. (**B**) WB analysis showing downregulation of p21-ΔNLS and enhanced cisplatin-induced cleavage of PARP in Tera-p21-ΔNLS after treatment with p21 siRNA. (**C**) EC cells were harvested 24 hours after γ irradiation or cisplatin treatment. Cell lysates were subjected to p21 IP. Immunoblotting was performed using anti-p21, anti-CDK2, and anti-ASK1 antibodies. In Scha and Tera-p21-ΔNLS, higher amounts of p21 are precipitated and more CDK2 and ASK1 are coprecipitated compared with Tera, whereas in irradiated Tera and Scha, almost similar levels of p21, CDK2, and ASK1 are coprecipitated. The data presented are representative of 3 independent experiments. (**D**) CDK2 acts proapoptotic after 24 hours cisplatin treatment in Tera. Decreased apoptotic response and increased PARP cleavage after successful downregulation of CDK2. $*P < 0.05$; $**P < 0.01$ compared with matching siRNA scrambled control. Data are represented as mean ± SD.

ization of p21 (Supplemental Figure 5D). In addition, Akt1–3 suppression sensitized 2102EP and Scha for cisplatin-induced apoptosis (Figure 4D).

Enhanced cytoplasmic p21 positivity and low levels of apoptosis were found in Tera and Scha 24 hours after γ irradiation (Figure 1E and Supplemental Figure 4D). The combined treatment of γ irradiation with either LY294002 or triciribine for 24 hours resulted in less p-p21, predominantly nuclear localization of p21, and augmented apoptosis in both Tera and Scha (Supplemental Figure 4, B and D, and Supplemental Figure 5E). In Tera-p21-ΔNLS, lacking the phosphorylation site Thr145, treatment with the PI3K/Akt inhibitors, however, had no effect on the cytoplasmic localization of p21-ΔNLS (Supplemental Figure 4C) and treatment with the inhibitors did not lead to sensitization to cisplatin (data not shown). These results show that the sensitizing effect of PI3K/Akt inhibitors on cisplatin-induced apoptosis is mediated via dynamic changes in p21 localization.

Cytoplasmic expression of p21 and p-p21 in the EC component of chemorefractory TC patients. Next, we investigated the p21 and p-p21 (Thr145) expression patterns in EC containing TC tissue from 23 patients with metastatic disease who were cured and considered sensitive to cisplatin-containing chemotherapy and from 7 patients with metastatic disease not cured and considered refractory to cisplatin-containing chemotherapy. Histological analysis after H&E staining was used to distinguish the various TC components. In addition, Oct4 (also known as POU5F1 and Oct3/4) immunohistochemistry (IHC) was used as marker for embryonal pluripotency (16). Mature teratoma showed an intense p21 and p-p21 staining, whereas no staining of Oct4 was noticed (Supplemental Figure 6A). EC components from TC patients, sensitive to cisplatin-containing chemotherapy, were negative for p21 in 23 (100%) and negative for p-p21 in 21 (91%) of the patients (Figure 5A and Supplemental Figure 6A). In contrast, the EC component of refractory TC patients stained positive for p21/p-p21 in 6 out of 7 (86%) patients, including 2 patients with EC as the only component (Figure 5B). Positive staining for p21/p-p21 in the EC component was significantly more often observed in refractory TC patients compared with TC patients sensitive to cisplatin-containing chemotherapy ($P < 0.0003$, Fisher's exact test). Moreover, IF showed that the localization of p21/p-p21 in the EC component of refractory patients was mainly cytoplasmic, whereas the localization of p21/p-p21 in teratomas is more nuclear (Figure 5C). In addition, presence of Ki-67 staining indicated proliferation instead of cell-cycle arrest in abundantly p21/p-p21–positive EC (Supplemental Figure 6B). Taken together, our results show that cytoplasmic p21 and p-p21 positivity of EC components in TC patients is associated with the response (poor outcome) to cisplatin-containing chemotherapy.

Reduced levels of Oct4 and miR-106b family members cause high cytoplasmic p21 expression in cisplatin-resistant EC cells. Involvement of miRNAs belonging to the miR-106b seed family in regulating p21/*CDKN1A* and cell-cycle control has previously been demonstrated (30, 31). Therefore, we examined the expression levels of the miR-106b seed family in the EC cell lines. The intrinsically resistant Scha and 2102EP cell lines, with high p21 levels, showed lower levels of the

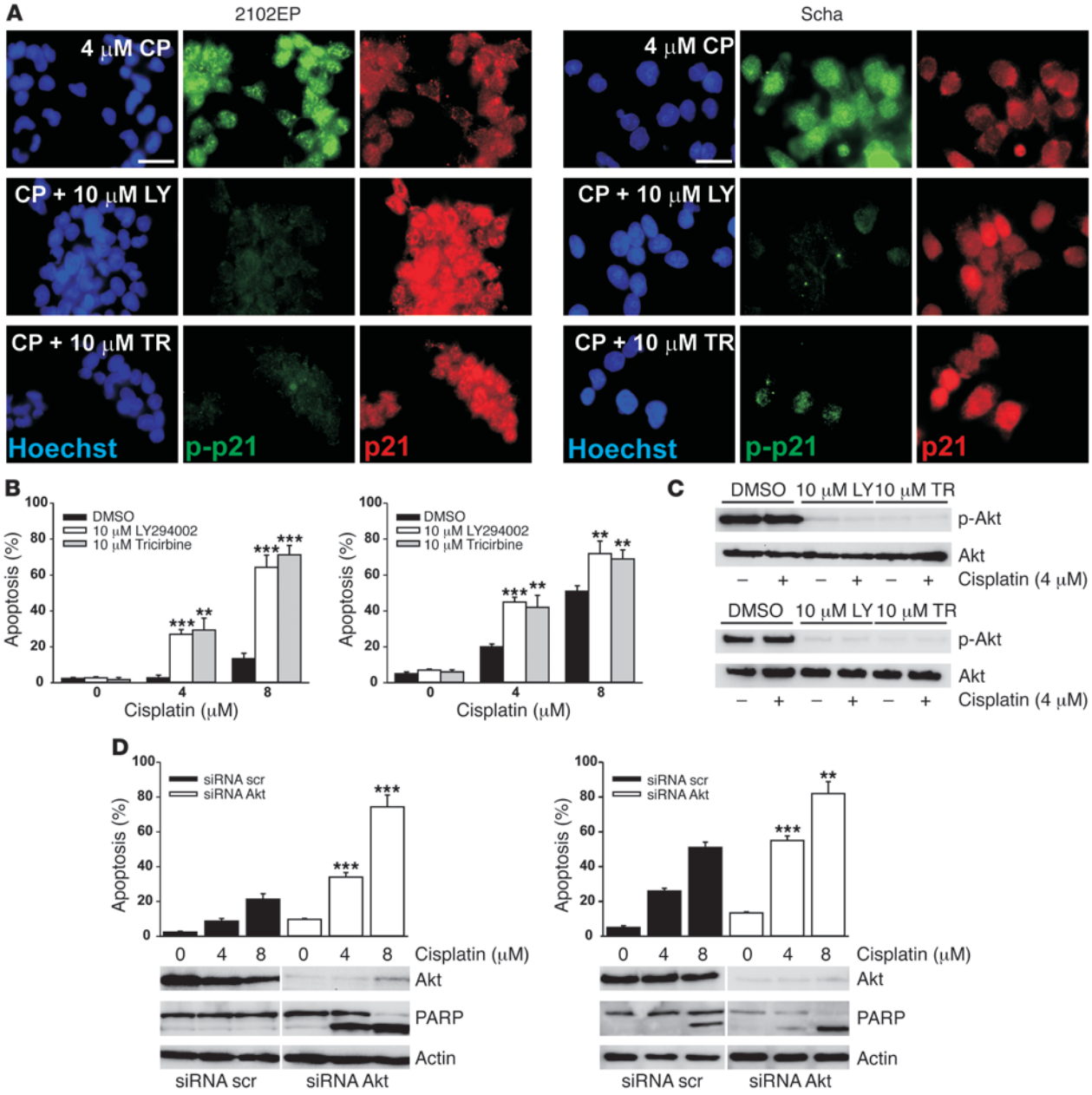

Figure 4

Dephosphorylation of p-p21, nuclear localization of p21, and sensitization for cisplatin after combined treatment with LY294002 or triciribine. (**A**–**C**) 24 hours after cisplatin treatment in combination with either 10 μM LY294002 (LY) or 10 μM triciribine (TR) decreased phosphorylated levels of p21 (Thr145), whereas localization of p21 is more pronounced in the nucleus of 2102EP and Scha (**A**) and dephosphorylation of p-Akt occurred after treatment with LY294002 or triciribine in Scha and 2102EP (**B**). This treatment led to increased apoptosis induction in 2102EP and Scha (**C**). $^{**}P < 0.01$; $^{***}P < 0.001$ compared with matching DMSO control. (**D**) siRNA against Akt1–3 sensitizes intrinsically resistant EC cell lines for cisplatin. Akt1–3 downregulation increased apoptosis induction and enhanced PARP cleavage after cisplatin treatment in 2102EP and Scha. $^{**}P < 0.01$; $^{***}P < 0.001$ compared with matching siRNA scrambled control. Data are represented as mean ± SD. Scale bars: 30 μm.

miR-106b seed family compared with the cisplatin-sensitive Tera and 833KE cell lines (Figure 6A and Supplemental Figure 6C). Subsequently, we used synthetic anti-miRNA to specifically inhibit the miR-106b seed family and to demonstrate its relation with localization and expression level of p21 in EC cell lines. In Tera cells, transfection with synthetic anti-miRNA against miR-17-5p, miR-20a, miR-93, miR-106a, or miR-106b resulted in a massive increase in cytoplasmic p21 levels compared with control (Figure 6, B and D). Although treatment with synthetic anti–miR-17-5p resulted in enhanced p21 levels in Tera, Tera-CP, Scha, and 2102EP, the strongest enhancement of p21 was found in Tera and Tera-CP (Figure 6C), which may at least in part be explained by the higher miR-17-5p levels in these cell lines (Figure 6A and ref. 32). The strongly enhanced cytoplasmic p21 levels in Tera following synthetic anti–miR-17-5p

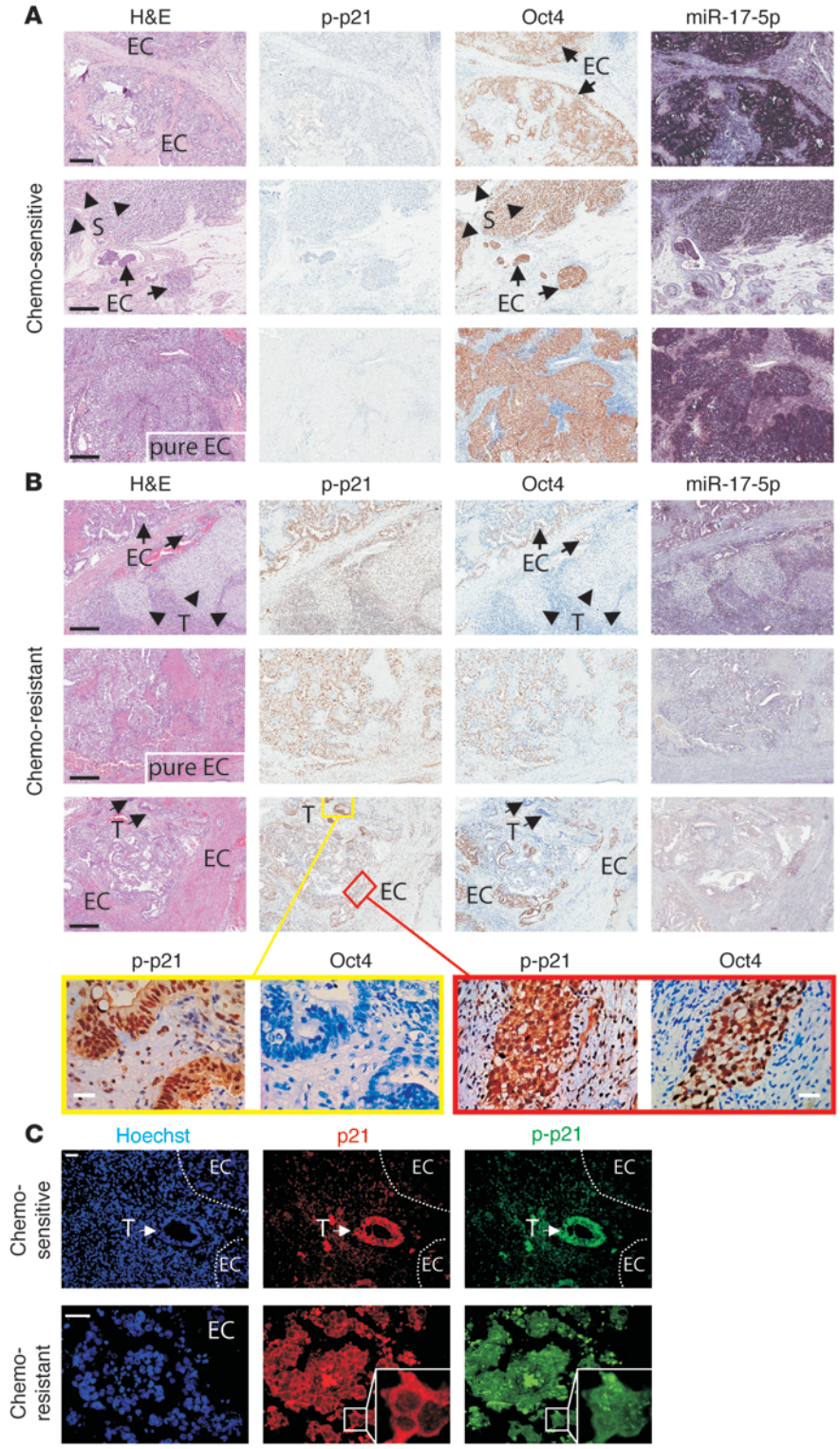

Figure 5
Cytoplasmic p21 expression in ECs of nonresponding TC patients. (**A**) EC or seminoma (S) components of chemosensitive patients stain negative for p21 (IHC) and intensively positive for Oct4 (IHC) and miR-17-5p (ISH). Representative examples of staining are shown. Scale bars: 300 μm. (**B**) The EC component of patients refractory to cisplatin-containing chemotherapy, in contrast with responders, stains in the majority of cases positive for p21 and less positive for Oct4 and miR-17-5p. All teratoma component (T) stains positive for p21/p-p21 and negative for Oct4 and miR-17-5p. Representative examples of staining are shown. Scale bars: 300 μm. Pictures of the panels with higher magnification were made with Leica DM3000 (Leica Microsystems). Scale bar: 30 μm. (**C**) Immunofluorescent staining showing that p21 is nuclear localized in mature teratoma and negative in chemosensitive EC patients, whereas p21 is cytoplasmic localized in the EC component of refractory patients. Representative example of stainings is shown. Scale bar: 30 μm. Insets represent a selected area of the original image as indicated, digitally magnified, ×4.

treatment completely blocked cisplatin-induced apoptosis (Figure 6, D and E). Suppression of cytoplasmic p21 with siRNA or shuttling of p21 to the nucleus with LY294002 resensitized synthetic anti–miR-17-5p–treated Tera cells to cisplatin (Figure 6F and Supplemental Figure 6D). Overexpression of pre–miR-17-5p resulted in lower p21 levels and an increase in cisplatin-induced apoptosis in Scha and 2102EP cells (Figure 6G), demonstrating the important causal relation among the presence of miR-17-5p, reduced p21 expression, and high cisplatin sensitivity.

We performed in situ hybridization (ISH) for miR-106a and miR-17-5p on paraffin-embedded formalin-fixed tumor tissue from patients with multicomponent (primary) TC to define in

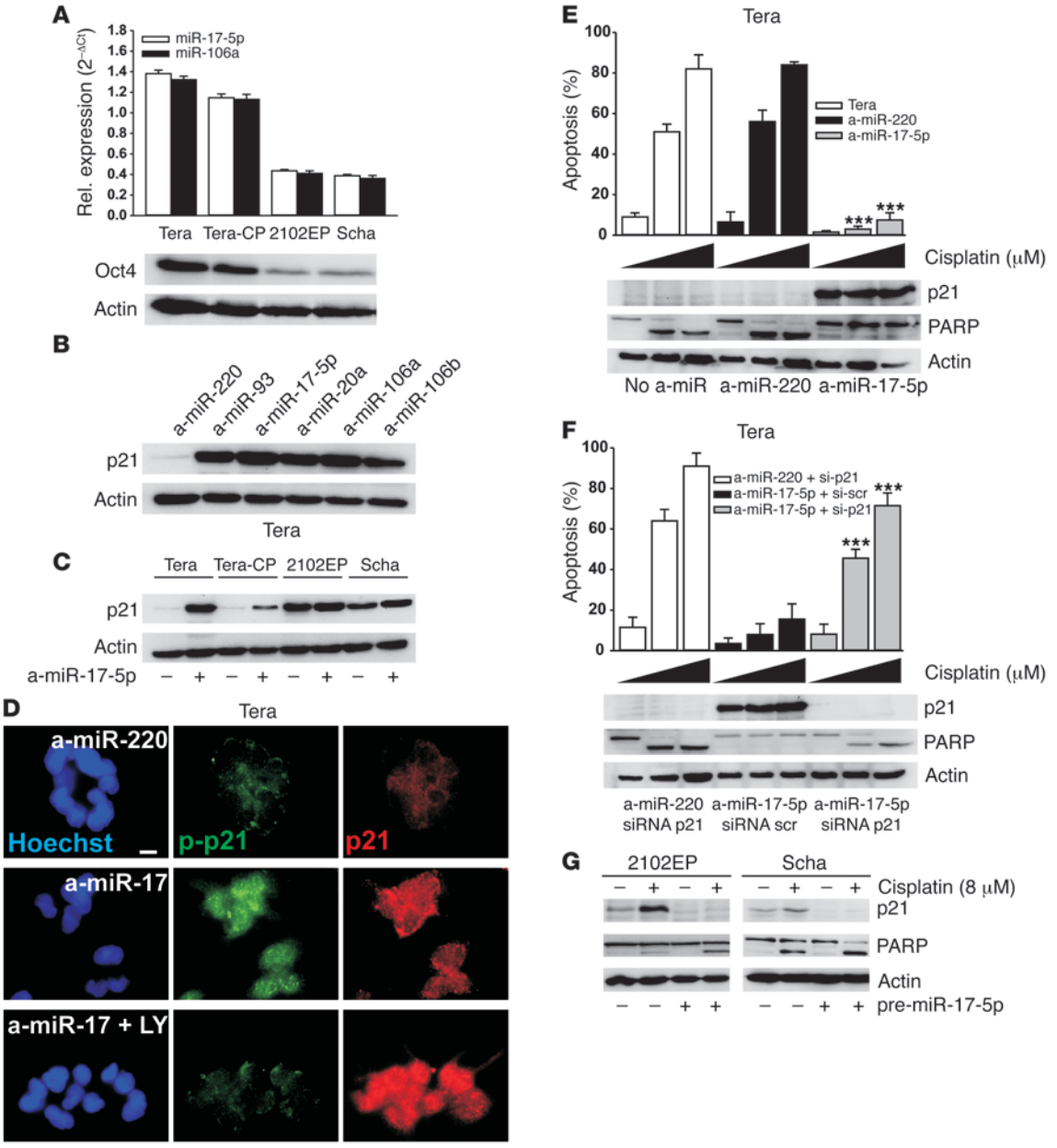

Figure 6

Positivity for p21 and p-p21 protein is inversely associated with the expression of the miR-106b seed family in ECs. (**A**) Quantitative RT-PCR on miR-106b seed family expression in EC cell lines. Note that the intrinsic cisplatin-resistant EC cell lines (2102EP and Scha) have lower levels of the miR-106b seed family and lower Oct4 expression compared with cisplatin-sensitive EC cell lines (Tera and 833KE). All reactions were run in triplicate. miRNA expression was normalized to RNU48 expression, resulting in a ΔCt from which the $2^{-\Delta Ct}$ value was derived and depicted. (**B**) Enhanced p21 levels in Tera 24 hours after treatment with synthetic anti–miR-106b seed family members. Synthetic anti–miR-220 was used as negative control, since miR-220 expression was not detectable in TC (32). (**C**) Differences in p21 enhancement in EC cells 24 hours after synthetic anti miR-17-5p treatment. (**D**) Levels of cytoplasmic localized p21 were enhanced after treatment with synthetic anti–miR-17-5p (a-miR-17), compared with control (a-miR-220). Treatment with 10 μM LY294002 (LY) decreased phosphorylated levels of p21 (Thr145) and p21 became more localized in the nucleus of synthetic anti-miR-17-5p transfected Tera cells. Scale bar: 30 μm. (**E**) Synthetic anti–miR-17-5p strongly upregulates p21 and reduces cisplatin-induced apoptosis and PARP cleavage in Tera. Cells were treated with 0, 4, and 8 μM cisplatin. ***$P < 0.001$ compared with matching anti-miR-220 control. (**F**) Reduced apoptosis induction and reduced PARP cleavage depend on upregulation of p21 by synthetic anti–miR-17-5p, as shown by cotransfection with siRNA against p21. Cells were treated with 0, 4, and 8 μM cisplatin. ***$P < 0.001$ compared with matching anti–miR-17-5p plus scrambled siRNA. (**G**) Lower expression levels of p21 and enhanced PARP cleavage 24 hours after cisplatin treatment in pre–miR-17-5p transfected Scha and 2102EP cells. Data are represented as mean ± SD.

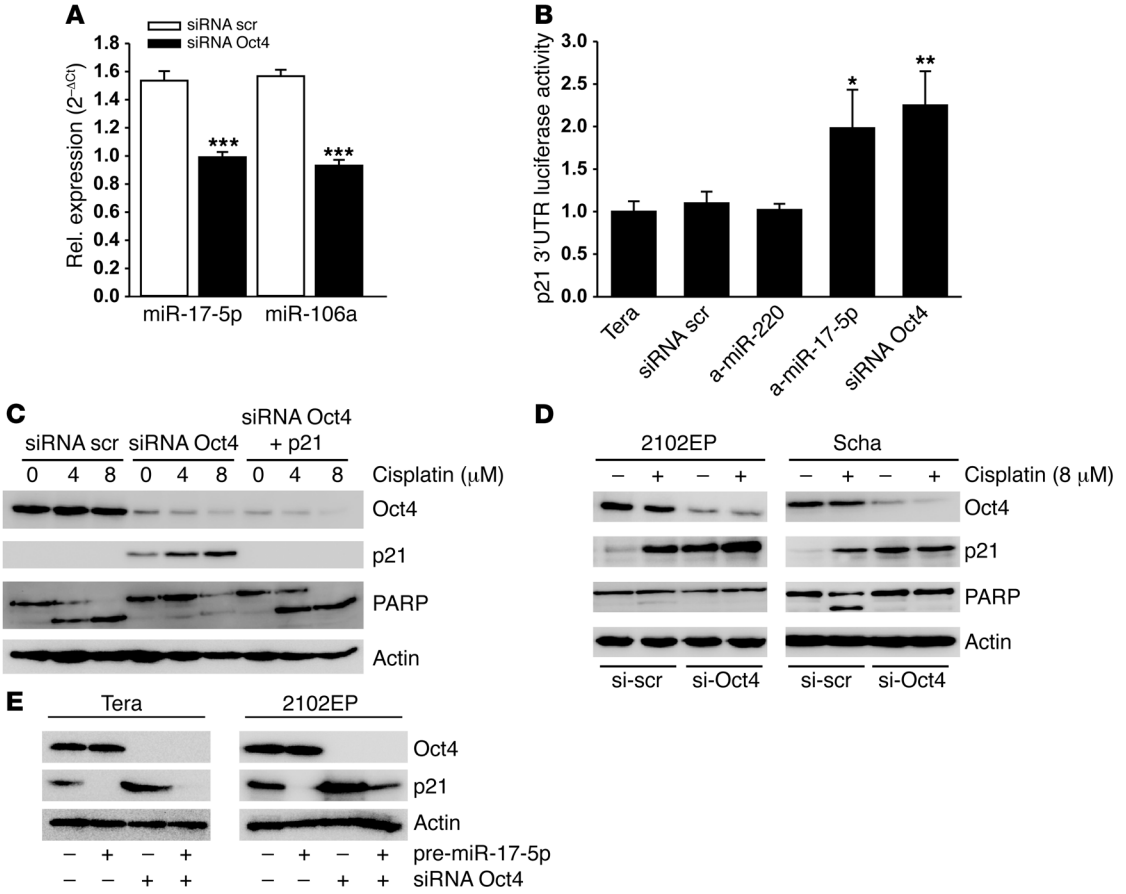

Figure 7

Oct4 regulates the expression level of the miR-106b seed family in ECs. (**A**) Quantitative RT-PCR on miR-106b seed family expression in EC cell lines, showing less expression of the miR-106b seed family in Oct4 suppressed cells after 48 hours of treatment with siRNA against Oct4 compared with control. ***$P < 0.001$ compared with control. (**B**) Reporter assay showing increased p21-3′ UTR–dependent luciferase translation in cells 48 hours after cotransfection of psiCHECK2-p21-3′ UTR with either anti–miR-17-5p or Oct4 siRNA. Bars indicate Firefly luciferase activity normalized to Renilla luciferase activity. *$P < 0.05$; **$P < 0.01$. (**C** and **D**) Oct4 suppression leads to upregulation of p21 and concomitantly resistance to cisplatin in Tera (**C**), 2102EP, and Scha cells (**D**), while downregulation of p21 with siRNA-resensitized Oct4-suppressed Tera cells for cisplatin (**C**). (**E**) Oct4 suppression leads to upregulation of p21 in Tera and 2102EP cells. Pre–miR-17-5p transfection abolished p21 expression in Tera and 2102EP cells and diminished p21 upregulation after Oct4 suppression to less than basal p21 expression levels. Data are represented as mean ± SD.

vivo the relation between p21/p-p21 expression and the presence of miR-106b seed family members in the EC component. A strong positive staining for miR-17-5p, miR-106a, and Oct4 but not for p21/p-p21 was found in the EC components of chemosensitive patients. In contrast, mature teratomas showed no staining of miR-17-5p, miR-106a, and Oct4 while staining intensively positive for p21/p-p21 (Figure 5A and Supplemental Figure 6A). On the contrary, in chemorefractory TC patients, almost no miR-17-5p staining was observed in EC components, while p21/p-p21 was clearly detectable. Moreover, the staining intensity of Oct4 in the EC component was less pronounced in refractory patients compared with chemotherapy-sensitive patients (Figure 5B). Additionally, the expression levels of both Oct4 and miR-106b seed family members were lower in the intrinsically resistant cell lines Scha and 2102EP compared with the cisplatin-sensitive cell line Tera (Figure 6A). These results suggest a relation between the reduced expression of Oct4 and the miR-106b seed family member in refractory ECs. In murine embryonic stem cells, Oct4 has been implicated in controlling expression of several miRNA families, including miR-17~92 and miR-106a~363 cluster of the miR-106b seed family (33). We downregulated Oct4 in Tera cells with siRNA in order to establish the direct relation among Oct4, miR-106b, and p21 expression in ECs. Suppression of Oct4 lowered the expression level of miR-106a, miR-17-5p, and miR-20a compared with control Tera cells (Figure 7A and Supplemental Figure 6E) and resulted in enhanced p21-3′ UTR dependent luciferase translation (Figure 7B) and robust induction of p21 (Figure 7C and Supplemental Figure 6F). A similar enhancement of p21-3′ UTR–dependent luciferase translation was observed following transfection of Tera cells with synthetic anti–miR-17-5p (Figure 7B). Furthermore, Oct4 suppression in Tera, Scha, and 2102EP resulted in an upregulation of p21 and a concomitant resistance to cisplatin, which could be reverted by p21 downregulation (Figure 7, C and D). Sustained miR-17-5p expression using pre–miR-17-5p precluded the induction of p21 protein even in the context of Oct4 knockdown in Tera and 2102EP cells (Figure 7E). Some p21 expression, however, was

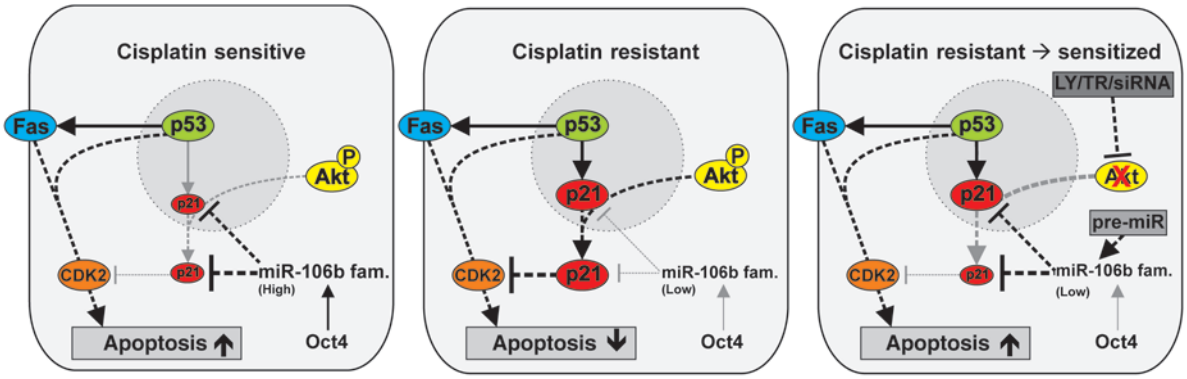

Figure 8

Proposed simplified model showing the mechanisms through which cytoplasmic p21 can inhibit cisplatin-induced apoptosis in EC cells. Cisplatin-induced DNA damage activates p53, which in turn transcribes *CDKN1A* (p21) and activates the Fas apoptosis pathway and the mitochondrial apoptosis pathway. Activated Akt is important for dynamic shuttling of p21 from the nucleus toward the cytoplasm, where p21 can block apoptosis. miR-106b seed family members (miR-106b fam.) are involved in regulating p21 expression levels. Oct4 regulates the expression levels of the miR-106b seed family members. Cisplatin-sensitive cells are characterized with high levels of Oct4 and miR-106b family members and as a consequence low amounts of cytoplasmic p21, resulting in cisplatin-induced apoptosis. Cisplatin-resistant cells have lower levels of Oct4 and miR-106b family members and high amounts of cytoplasmic p21, resulting in CDK2 inhibition and concomitantly moderate levels of cisplatin-induced apoptosis. Interestingly, deactivation of Akt with LY294002 (LY), triciribine (TR), or siRNA against Akt sensitized cisplatin-resistant cells. Deactivation of Akt leads to nuclear localization of p21, which in turn is no longer capable of blocking cisplatin-induced apoptosis. Overexpression of pre–miR-17-5p in cisplatin-resistant cells resulted in lower p21 levels and an increase in cisplatin-induced apoptosis. Dotted lines indicate interaction, whereas solid lines indicate (p53-induced) transcription.

still observed in 2102EP cells, albeit at a lower level compared with control 2102EP cells, indicating that not all effects of Oct4 on p21 expression are mediated by the miR-106b seed family. Overall, these results reveal an important role of Oct4 in regulating p21 expression via the miR-106b seed family in ECs.

Discussion

The functionality of many proteins is thought to be related to their intracellular localization. Nuclear localized p21 is known to control the cell cycle and DNA replication, whereas cytoplasmic p21 has been implicated in the inhibition of apoptosis. Here, we have demonstrated that high levels of cytoplasmic localized p21 protect EC cells against cisplatin-induced apoptosis. Furthermore, we dissected the pathway involved in the regulation of p21 expression levels, i.e., OCT4 and the miR-106b family, and identified the key protein causing the cytoplasmic localization of p21. Our results indicate that p-Akt–mediated p21 phosphorylation is essential for p21 localization in the cytoplasm. Inhibition of p-Akt retained p21 in the nucleus, resulting in less p21 complex formation with CDK2 and sensitized EC cells to cisplatin-induced apoptosis. In cisplatin-sensitive EC cell lines and EC components of chemosensitive TC patients, p21 is not commonly expressed, which is related to the expression levels of miR-106b seed family members and Oct4. On the contrary, in cisplatin-resistant EC cell lines and EC components of refractory TC patients, indicating clinical cisplatin resistance, cytoplasmic p21 expression was clearly detectable and related to reduced Oct4 and miR-106b expression. Therefore, we conclude that high levels of cytoplasmic localized p21 are important determinants of resistance to cisplatin-based chemotherapy in ECs (Figure 8).

We recently demonstrated that cisplatin induces apoptosis mainly in a Fas-dependent manner in EC cells (20), while enhanced p21 expression protects EC cells against Fas-mediated apoptosis (7). This protection might be mediated via the inhibition of caspase 3 (34), CDK2, and ASK1 by cytoplasmic p21. We have no indication that p21 binds to caspase 3 and inhibits activation in irradiated EC cells (7) or cisplatin-treated EC cells (data not shown). In the present paper, we show that endogenous cytoplasmic p21 forms a complex with CDK2 and ASK1 in EC cells. In EC cells expressing low p21 levels, CDK2 downregulation strongly reduced the cisplatin-induced apoptotic response. Moreover, relocalization of p21 to the nucleus using LY294002, which completely abolished p21-CDK2 complexes, as well as suppression of p21 made initially cisplatin-resistant cells sensitive to cisplatin in a CDK2-dependent manner, indicating the importance of cytoplasmic CDK2 as a proapoptotic factor in cisplatin-treated EC cells. The proapoptotic signaling of CDK2 is not clear yet. It has been reported that cytoplasmic CDK2 activity is induced in a caspase-dependent way following activation of the Fas pathway (35) and leads to depolarization of the mitochondrial membrane potential (36) by cytoplasmic CDK2-mediated translocation of Bax to the mitochondria (36, 37). Alternatively, functional interaction of CDK2 with FOXO1 after DNA damage can be an important mechanism to enhance, among others, Fas ligand expression (38). Taken together, these results indicate that the high cytoplasmic p21 levels, in complex with cytoplasmic CDK2, protect against cisplatin-induced apoptosis in EC cells.

In the present study, in vivo relevance of p21 expression was demonstrated in the EC component of TC patients with refractory disease, being positive for p21, whereas no p21 was detected in the EC component of patients with chemosensitive tumors. Lack of p21 staining in the EC component but strongly positive p21 staining in mature teratoma components has been frequently observed (9, 14, 15, 18). Teratomas, however, predominately show a nuclear localization of p21. Moreover, teratomas in contrast with ECs express Rb (17, 39), suggesting a putative role for p21 in cell-cycle control in these nongrowing or rarely growing tumors, also known as growing teratoma syndrome (17). Furthermore, terato-

mas are, possibly via p21-mediated cell-cycle arrest, not responsive to treatment with cisplatin (8, 15–17). Interestingly, p21 staining in the EC component of refractory patients was mainly localized in the cytoplasm of EC cells. In addition, Ki-67 staining revealed no G1 arrest, but proliferation in ECs with abundant expression of p21, indicating a similar role in vivo for high levels of cytoplasmic p21 in the protection against cisplatin-induced apoptosis, as we have demonstrated for cisplatin-resistant EC cell lines. Cytoplasmic p21 expression has been related to drug resistance in several other types of cancer (11). Previous studies in non-TC cell types have shown that phosphorylation of Thr145 in the p21-NLS by p-Akt is important for dynamic shuttling of p-p21 between the nucleus and the cytoplasm (11, 27). PTEN can prevent phosphorylation of Akt in EC cells (29). Virtually all ECs (cell lines and TC patients), however, lack PTEN expression (29), which is shown to be important for the development of invasive TCs (29, 40). Here, we show that relocalization of p21 to the nucleus by dephosphorylation of p21, using the PI3K inhibitor LY294002, the Akt inhibitor triciribine, or siRNA against Akt, sensitized cells for cisplatin-induced apoptosis. Phosphorylation of Akt might be inhibited by targeting tyrosine kinase membrane receptors such as KIT and ERBB2, that have been implicated in TC (8, 41–46). An even more direct approach to targeting Akt phosphorylation, besides triciribine, is the use of PI3K/Akt inhibitors that are currently in clinical development (47). However, the efficacy of the various subclasses of PI3K or Akt inhibitors to relocalize p21 and subsequently enhance cisplatin sensitivity in refractory TC patients needs to be further elucidated.

The role of miRNAs in tumorigenesis is attracting increasing attention, and miRNAs have actually been implicated in TC/EC carcinogenesis (48, 49). The miR-106b seed family is potentially oncogenic (50) and has been implicated in embryonal stem cells in their maintenance and control of differentiation (51). ECs share many similarities with embryonic stem cells (16, 32) including their miRNA expression profile (32). Interestingly, our results indicate differences in expression levels of p21-3′ UTR regulating miR-106b seed family members between intrinsically cisplatin-resistant EC and cisplatin-sensitive EC cell lines. Additionally, we report that high levels of the miR-106b seed family members are causing low levels of (cytoplasmic) p21 in EC cells and consequently sensitivity to cisplatin. In contrast, low levels of the miR-106b seed family members were associated with high levels of (cytoplasmic) p21 in EC cells and ECs of refractory TC patients. In murine embryonic stem cells, miR-17~92 and miR-106a~363 clusters of the miR-106b seed family are under control of embryonic stem cell factor Oct4 (33). ECs, like embryonic stem cells, also express a cassette of pluripotency genes (16, 32, 52), where Oct4 is supposed to be the key pluripotency regulator (52). Here, we show that expression of members of the miR-106b seed family colocalizes with Oct4 expression in TC. Our results indicate that Oct4 regulates expression of miR-17-5p, miR-106a, and miR-20a, belonging to the miR-106b seed family, via the p21-3′ UTR. Furthermore, in EC cells expressing high basal levels of Oct4, overexpression of miR-17-5p completely precluded p21 induction when Oct4 was suppressed. Similar results were obtained with EC cells expressing lower basal levels of Oct4; however, some p21 expression was still detectable in these cells after combined transfection of Oct4 siRNA and pre–miR-17-5p. This suggests that besides the miR-106b seed family, the regulation of p21 expression through Oct4 may be more complex, involving other factors as well. In summary, we provide evidence that Oct4 regulates expression of p21-3′ UTR targeting miR-106b seed family members and thus indirectly p21 levels and cisplatin sensitivity in EC cells.

In conclusion, we demonstrate an important mechanism in ECs that causes elevated cytoplasmic p21 levels and consequently results in cisplatin resistance. Moreover, we provide a target to overcome resistance, which may form the basis for the development of novel therapeutic approaches to alleviate cisplatin resistance in TCs or other cisplatin-resistant types of solid tumors.

Methods

Cell lines and reagents. A well-defined panel of cisplatin-sensitive and cisplatin-resistant human EC cell lines, all expressing wild-type p53, were used (7, 20, 22, 23, 53). The 2102EP cell line was obtained from L. Looijenga (Department of Pathology, Erasmus Medical Center, Rotterdam, the Netherlands). Tera, Tera-CP, 2102EP, Scha and 833KE, and the human breast carcinoma cell line MCF-7 (used as a control) were cultured in RPMI 1640 medium (Gibco; Invitrogen) supplemented with 10% FCS (SanBio) at 37°C in a humidified atmosphere with 5% CO_2. Cisplatin was purchased from Bristol-Myers Co., LY294002 from Cell Signaling, and Triciribine from Biomol.

Drug sensitivity assay and apoptosis. Drug sensitivity testing was performed as described previously (20, 53). Acridine orange fluorescent staining of nuclei was used to distinguish apoptotic from vital cells as described previously (7, 20, 54).

WB and preparation of fractionated proteins. Lysates were examined by WB as described previously (7, 20). Nuclear and cytosolic protein fractions were prepared as described previously (7). Antibodies used are listed in Supplemental Methods.

Immunofluorescence. Cells were fixed with 4% paraformaldehyde and blocked with 0.1% Triton X-100, 1% BSA, and 1% normal goat serum (NGS) in PBS. This was followed by immunostaining with the corresponding antibodies and counterstaining with Alexa Fluor goat secondary antibodies (Molecular Probes; Invitrogen). Finally, cells were stained with Hoechst 33258 (Molecular Probes; Invitrogen) and mounted with Vectashield (Vector Laboratories). The Quantimet 600S system (Leica Microsystems) was used for analysis. The data were exported as bmp files and processed using Corel Photo-Paint X4 (Corel Corp.).

Caspase 3 activity. Caspase 3 was assayed according to the manufacturer's instructions using DEVD-MCA (Zebra Bioscience BV). Fluorescence was monitored in an FL600 Fluorimeter Bio-Tek plate reader (Beun de Ronde).

Tera-p21-Δ-NLS stably transduced cell line. pMSCV-p21-ΔNLS, pMSCV-IGFP, and pCL-Ampho were provided by J.J. Schuringa and H. Schepers (Hematology Research, University Medical Center Groningen). For viral production, 293T cells were transfected with pCL-Ampho and MSCV-IGFP either with or without p21-ΔNLS. GFP-positive Tera cells were sorted on a fluorescent-activated cell sorter (MoFlow; Cytomation).

RNA interference, miRNA antisense, and transfections. Specific siRNA and negative control (scrambled) were purchased from Eurogentec. Synthetic anti-miRNA was purchased from IDT and pre–miR-17-5p from Ambion. All sequences and methods are listed in Supplemental Methods. For the luciferase reporter assay, Tera cells were transfected with psiCHECK2-p21-3′ UTR using Fugene, lysates were made, and assay was performed as described previously (55).

IP. Cells (10^7) were harvested, washed with ice-cold PBS, and lysed in 500 μl lysis buffer (20 mM Tris HCl, pH 7.6, 150 mM NaCl, 0.2% NP-40, protease inhibitor COMPLETE, 1 mM PMSF, 1 mM NaF, and 1 mM DTT). Lysates were clarified, and protein concentration was equalized with Bradford and incubated for 16 hours with a mixture of agarose-conjugated anti-p21 (F5 and C19; Santa Cruz Biotechnology Inc.). Immuno-

complexes were washed 5 times and eluted with 0.5 M glycine/HCl pH 2.4, mixed 1:1 with standard 2× WB sample buffer, and examined by WB analysis as described above.

Patients, IHC, and miRNA ISH. General patient characteristics and histopathology are summarized in Supplemental Table 1. The TC patients with chemosensitive disease have been reported before (56). From the patients with TC diagnosed and treated at our institution between 1985 and 2007 with refractory disease (defined as patients not achieving a response on initial treatment or renewed elevation of tumor marker levels within 4 weeks after completion of chemotherapy), patients with sufficient EC component in their tumor samples ($n = 7$) were selected. TC specimens were used to represent all histological subtypes of the primary tumor (YS, yolk sac tumor; ChC, choriocarcinoma; T, mature teratoma/immature teratoma; S, seminoma) and each of the 3 different prognosis groups according to the IGCCCG classification (57). The studies were approved by the medical ethical committee of University Medical Center Groningen, and all patients gave informed consent prior to study entry. For each patient, representative paraffin-embedded tumor material was collected and serial 3-μm sections were cut.

For IHC, sections were deparaffinized in xylene and rehydrated in alcohol; antigen retrieval was performed followed by blocking of endogenous peroxidase (30 min, 3% H_2O_2). Subsequently, slides were incubated for 1 hour with the primary antibodies Oct4 (C20; Santa Cruz Biotechnology Inc.), p21 (EA10, Oncogene), p-p21 (Thr145; Santa Cruz Biotechnology Inc.), and Ki-67 (MIB-1; Dako) and counterstained with HRP-conjugated secondary antibodies (Dako). DAB was used as chromagen to visualize peroxidase activity. Counterstaining was performed with hematoxylin. Immunoglobulin class-matched control sera were used as negative controls. Normal colon and normal skin served as a positive control for p21.

miRNA ISH was performed as reported previously (58). For both ISH and IHC, slides were scanned using ScanScope CS System (Aperio) and pictures were taken with the ImageScope software package (Aperio).

RNA isolation and TaqMan miRNA quantitative PCR. Total RNA was isolated and miRNA-106b family expression was measured as previously described (55). The miRNA expression was normalized to RNU48 expression, resulting in a ΔCt from which the $2^{-\Delta Ct}$ value was derived and depicted.

Statistics. Results of at least 3 experiments are expressed as mean ± SD. Student's unpaired t test was used to compare values of test and control samples. Differences were considered significant at $P < 0.05$. Fisher's exact χ^2 test was used for categorical patient variables. Differences were considered significant with 2-sided test at $P < 0.05$.

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