Oct4, miR-106b family and p-Akt regulated cytoplasmic p21$^{cip1/waf1}$ expression levels determine cisplatin-resistance in human testicular cancer

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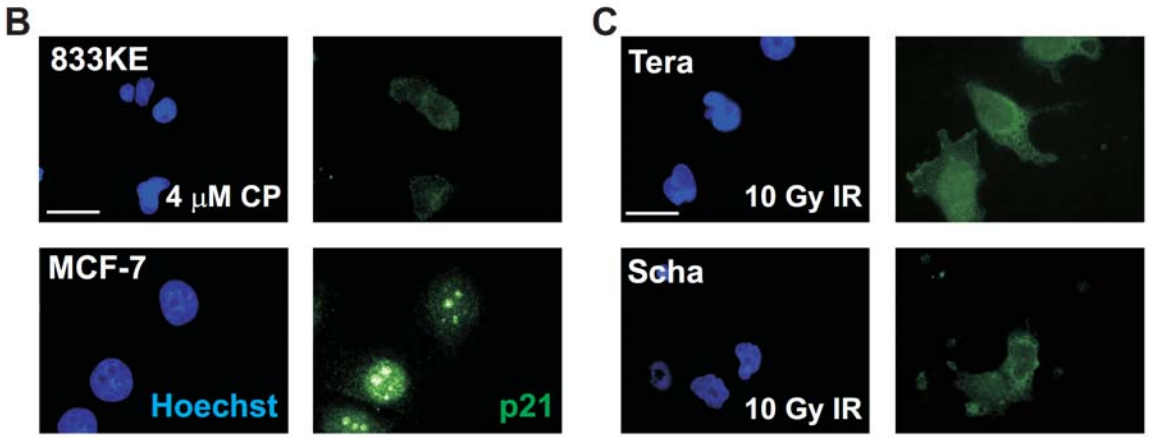
Supplemental Figure 1

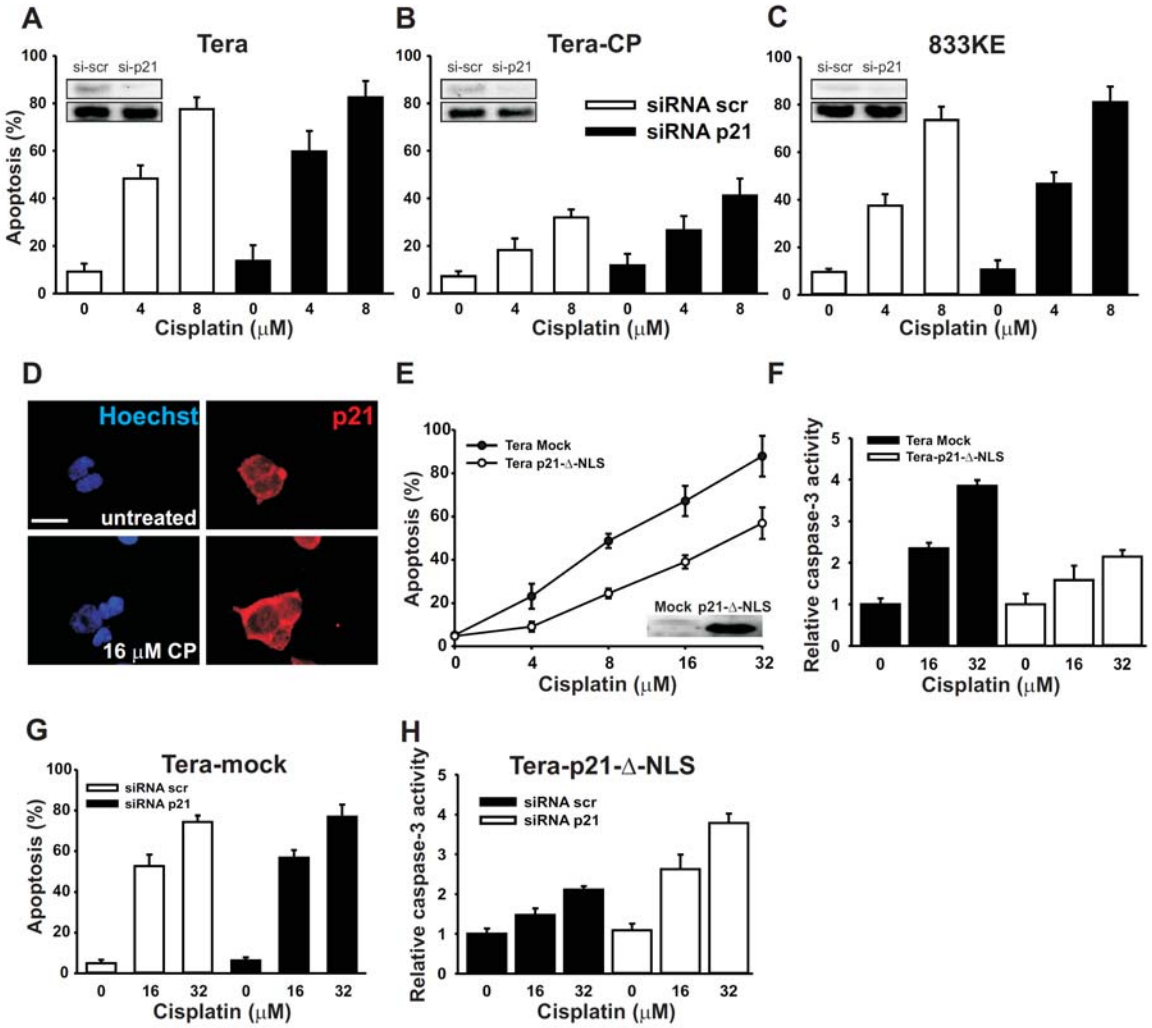
A

	IC_{50} cisplatin (μM)	Model
Tera	0.69 ± 0.11	Sensitive
833KE	1.04 ± 0.09	Sensitive
Tera-CP	2.10 ± 0.16	Acquired resistant
Scha	2.90 ± 0.84	Intrinsic resistant
2102EP	3.80 ± 0.49	Intrinsic resistant



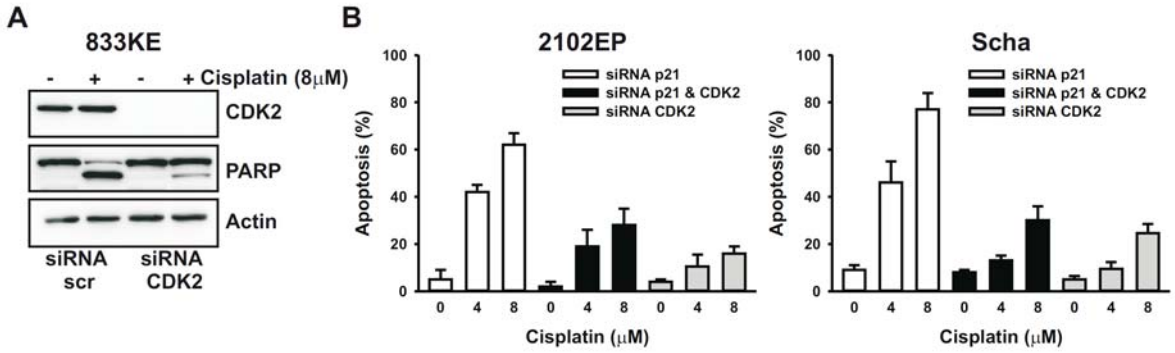
(**A**) IC_{50} values for cisplatin were calculated from the graph in Figure 1A. (**B-C**) Localization of p21 was determined with immuno-fluorescence; 24h after cisplatin treatment p21 is,cytoplasmic localized in 833KE, using the breast carcinoma cell line MCF-7 as a control for nuclear staining after chemotherapy treatment (Menendez et al. 2005);(Panno et al. 2006) Scale bar, 30 μm. (**B**). 24h after gamma-irradiation (IR) p21 is localized in the cytoplasm of Tera and Scha (**C**) Scale bar, 30 μm.

Supplemental Figure 2



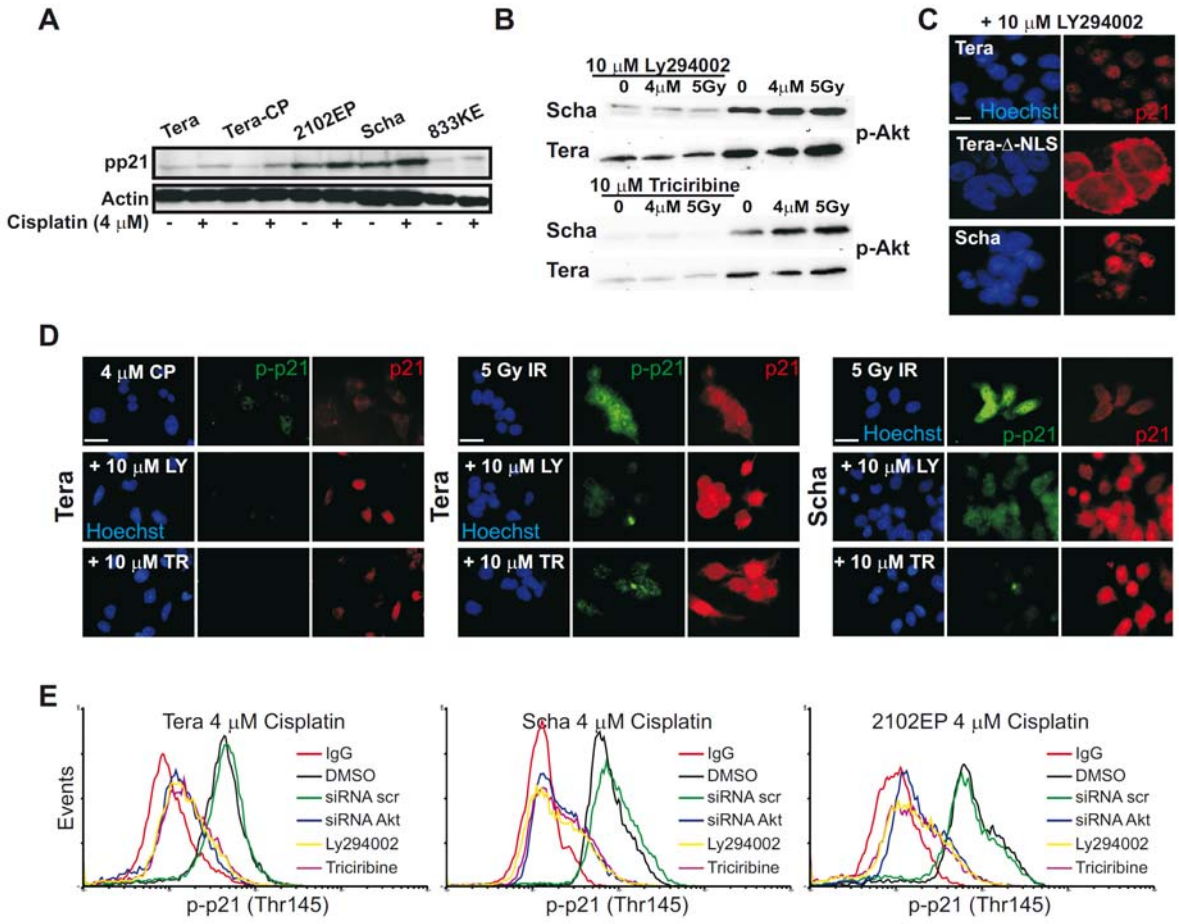
(A-C) No significant effect of p21 siRNA on cisplatin induced apoptosis in Tera, Tera-CP and 833KE compared to scr siRNA. Values are the mean ± SD of three independent experiments. Inset shows successful p21 suppression. (**D**) p21-ΔNLS is localized in the cytoplasm of Tera-p21-ΔNLS cells when untreated or treated with cisplatin (CP). Scale bar, 30 μm. (**E,F**) Tera-p21-Δ-NLS is less sensitive for cisplatin compared to Tera Mock as shown by a lower percentage of apoptotic cells (**E**) and reduced caspase 3 activation (**F**) following cisplatin treatment for 24 h. Values are the mean ± SD of three experiments. (**G**) No significant effect of p21 siRNA on cisplatin induced apoptosis in Tera Mock to scr siRNA. (**H**) Enhanced caspase 3 activity in Tera-p21-ΔNLS after treatment with p21 siRNA. Values are the mean ± SD of three experiments.

Supplemental Figure 3



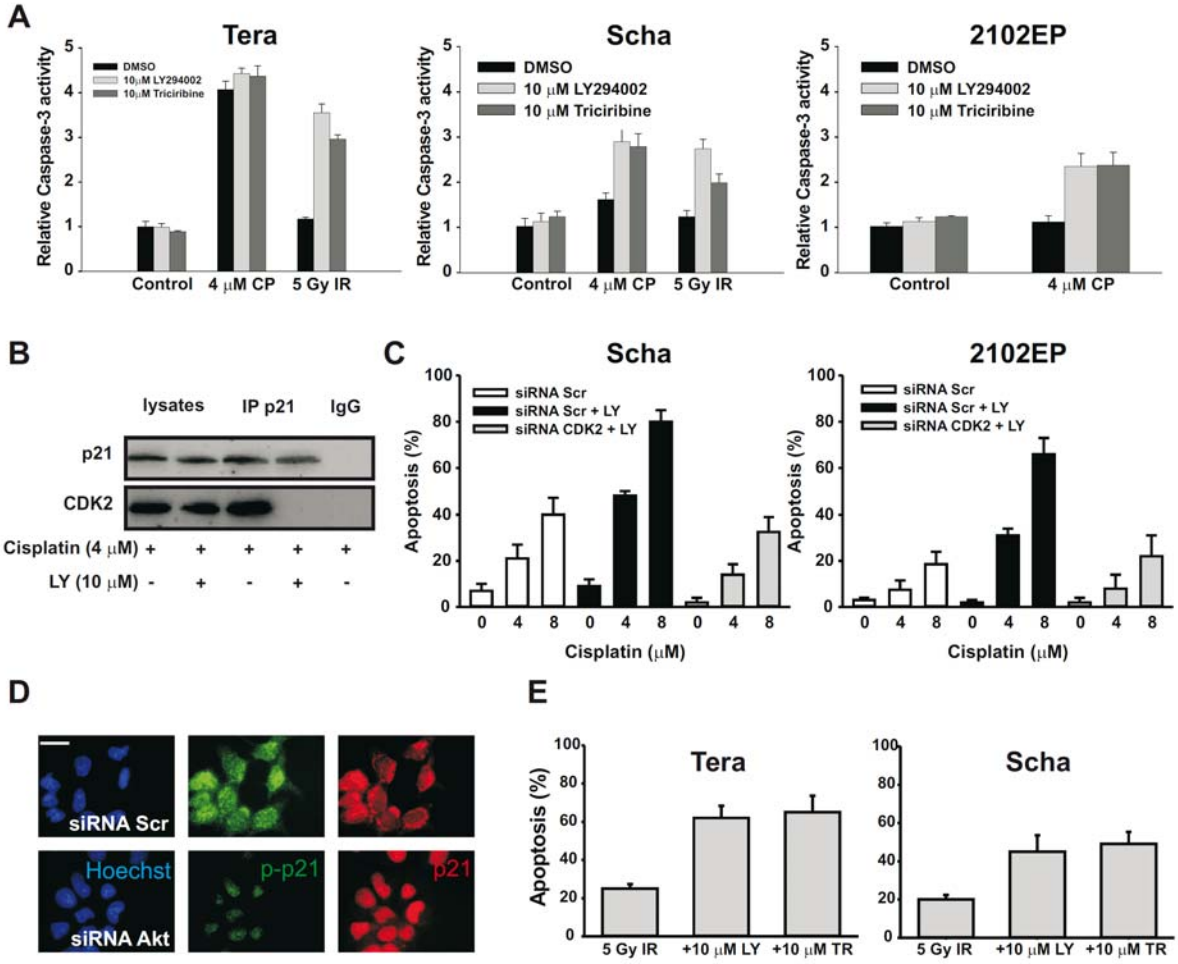
(**A**) Decreased PARP cleavage after successful downregulation of CDK2 24h after cisplatin treatment of 833KE. **(B**) Dramatically reduced apoptosis response after co-transfection with CDK2 and p21 siRNA in 2102EP and Scha cells.

Supplemental Figure 4



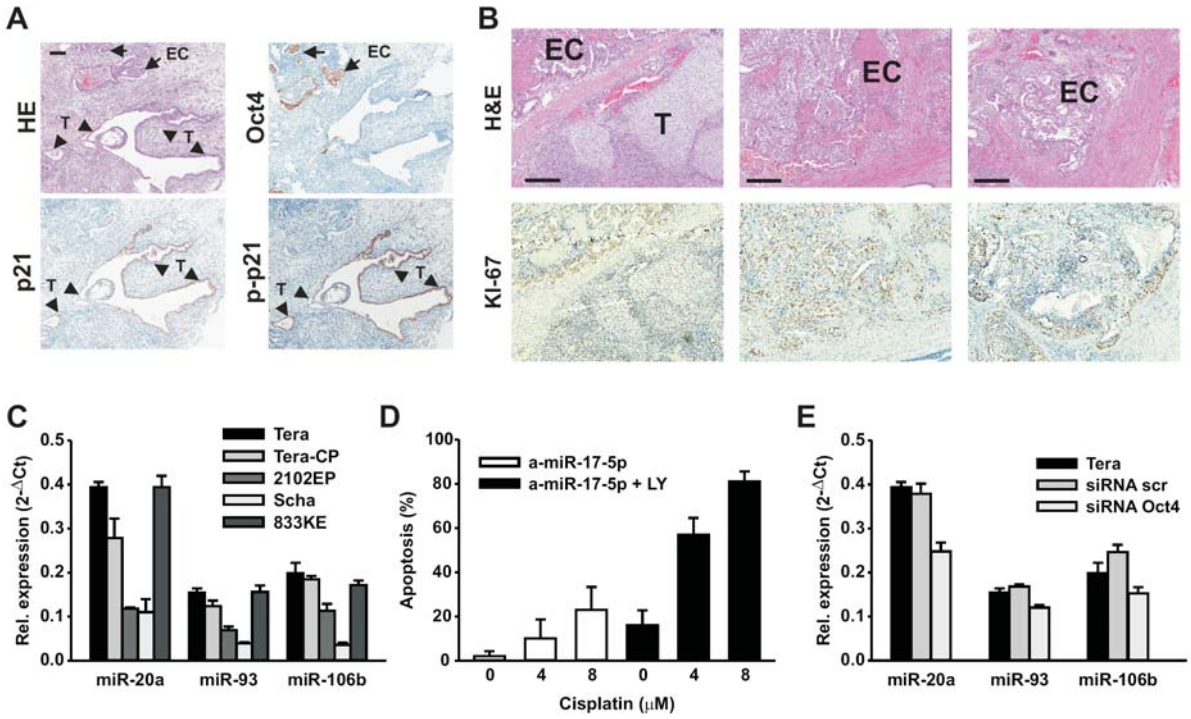
(**A**) Difference in p-p21 levels of the EC cell lines treated and untreated with cisplatin. Note that intrinsic cisplatin-resistant EC cell lines (2102EP and Scha) have higher basal and cisplatin-induced p21/p-p21 levels compared to cisplatin-sensitive EC cell lines. (**B**) Following treatment with cisplatin or gamma-irradiation of EC cells p-Akt is induced, whereas dephosporylation of p-Akt occurred after treatment with Ly294002 or Triciribine in Scha and Tera. (**C**) After treatment with 10 μM LY294002 for 24h, p21 is localized in the nucleus of Tera and Scha, whereas this treatment in Tera-p21-Δ-NLS had no effect on p21-Δ-NLS localization. Scale bar, 30 μm. (**D**) 24h after treatment with gamma-irradiation in combination with either 10 μM LY294002 (LY) or 10 μM Triciribine (TR), phosphorylated levels of p21(Thr145) decreased and p21 is more pronounced localized in the nucleus of Tera and Scha. Scale bar, 30 μm. (**E**) Dephosporylation of p-Akt and downregulation of Akt reduces the levels of p-p21.

Supplemental Figure 5



(**A**) Enhanced caspase 3 activity after the combined treatment of Scha and 2102EP with Ly294002 or Triciribine and cisplatin, while there is no increase in activity in Tera in combination with cisplatin. The combined treatment for 24h of LY294002/Triciribine and gamma-irradiation enhanced caspase 3 activity in both Tera and Scha. (**B**) Treatment with LY294002 (LY) resulted in the loss of complex formation between p21 and CDK2 in Scha cells. (**C**) Suppression of CDK2 considerably reduced the increase in apoptosis induction of 2102EP and Scha cells upon combined treatment with cisplatin and LY294002 (LY). (**D**) Downregulation of Akt reduced the levels of p-p21 and resulted in a more pronounced nuclear localization of p21 in 2102EP. Scale bar, 30 μm. (**E**) The combined treatment of 24h LY294002/Triciribine and irradiation sensitized both Tera and Scha for gamma-irradiation induced apoptosis. Values are the mean ± SD of three independent experiments.

Supplemental Figure 6



(A) Mature teratoma component (T) stains positive for p21/p-p21 but negative Oct4, whereas the embryonal carcinoma component (EC) stains negative for p21/p-p21 and intensively positive for Oct4 using IHC. A representative sample is shown. Scale bar, 300 μm. (**B**) Presence of Ki-67 staining indicating proliferation instead of cell cycle arrest in abundant p21 positive EC. (**C**) 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 family compared to cisplatin-sensitive EC cell lines (Tera and 833KE). All reactions were run in triplicate. The miRNA expression was normalized to RNU48 expression resulting in a ΔCt from which the $2^{-\Delta Ct}$ value was derived and depicted. (**D)** Treatment with LY294002 (LY) resensitized synthetic anti-miR-17-5p transfected Tera cells to cisplatin. (**E**) Quantitative RT-PCR on miR-106b seed family expression in EC cell lines, showing less expression of the miR-106b family in Oct4 suppressed cells, after 48h of treatment with siRNA against Oct4, compared to control.

Supplemental Table 1 Patient characteristics

	Chemo-sensitive disease	Refractory disease
No. of patients	31	7
Median age at start of chemo-therapy (range)	29 (17-53)	31 (19-44)
Histology *		
EC (pure component)	23 (5)	7 (2)
YS (pure component)	6 (1)	1 (0)
ChC (pure component)	6 (0)	2 (0)
T (pure component)	17 (0)	4 (0)
S (pure component)	7 (3)	0 (0)
Prognosis group **		
Good	22	1
Intermediate	5	2
Poor	4	4
Disease outcome		
Death of disease	0	7
Alive with disease	0	0
No evidence of disease	31	0

*single or multiple components; each component listed

** International Germ Cell Cancer Classification (IGCCCG 1997)

Supplemental Experimental Procedures

The following antibodies were used: mouse anti p21 (F5, Santa Cruz), mouse anti p53 (DO-1, Santa Cruz), mouse anti β-Actin (MP Biomedicals), mouse anti pRB (IF8, Santa Cruz), rabbit anti-Parp (Roche Diagnostics), mouse anti ASK1 (F9, Santa Cruz), goat anti CDK2 (M2, Santa Cruz), rabbit anti p-p21 (Thr145, Santa Cruz), rabbit anti p-Akt/Akt (Cell Signalling), goat anti Oct4 (C20, Santa Cruz) and rabbit anti caspase 3 (cell Signalling). The antibody binding was eventually determined using horseradish peroxidase (HRP)-conjugated secondary antibodies (DAKO) and visualised with the POD chemoluminescence kit (Roche Diagnostics). WB membranes were imaged with Molecular Imager Gel Doc XR System (Biorad). Equal protein loading was checked for with the Bradford total protein assay, Ponceau S staining of the blots and ß-actin immunobloting.

RNA interference & miRNA antisense

EC cells were transfected in 6 well plates with 5 µl of 20µM siRNA duplex or miRNA antisense using Oligofectamine reagent according to the manufacturer's instructions (Invitrogen). After 24h, cells were treated with cisplatin. 24h after the treatment cells were harvested for protein isolation. Alternatively, in order to perform an apoptosis assay, at 24h after transfection, cell were harvested and plated in 96-well plate. The day after, cells were treated with cisplatin.

p21-I (Spierings et al., Oncogene, 2004)	5'-CUUCGACUUUGUCACCGAGdTdT (sense)
	5'-CUUACGCUGAGUACUUCGAdTdT (anti-sense)
P21-II (Spierings et al., Oncogene, 2004)	5'-GACCAUGUGGACCUGUCACTdT (sense)
	5'-GUGACAGGUCCACAUGGUCdTdT (antisense)
CDK2 (Mitra et al., Cell Cycle, 2006)	5'-GCCAGAAACAAGUUGACGGGAdTdT (sense)
	5'-UCCCGUCAACUUGUUUCUGGCdTdT (anti-sense)
CDK2 II (Cai D, et al. Cancer Res 2006)	5'-GGUGGUGGCGCUUAAGAAAdTdT (sense)
	5'-UUUCUUAAGCGCCACCACCdTdT (anti-sense)
Akt1 (Kim et al., Cancer Res, 2005)	5'-GGAGGGUUGGCUGCACAAAdTdT (sense)
	5'-UUUGUGCAGCCAACCCUCCdTdT (anti-sense)
Akt2 (Kim et al., Cancer Res, 2005)	5'-CUUCUCCGUAGCAGAAUGCdTdT (sense)
	5'-GCAUUCUGCUACGGAGAAGdTdT (anti-sense)
Akt3 (Kim et al., Cancer Res, 2005)	5'-CUGGAGGCCAAGAUACUUCdTdT (sense)
	5'-GAAGUAUCUUGGCCUCCAGdTdT (anti-sense)
Oct4 I (Matin et al., Stem Cells,2004)	5'-CAUGUGUAAGCUGCGGCCCdTdT (sense)
	5'-GGGCCGCAGCUUACACAUGdTdT (anti-sense)

Oct4 II (Matin et al., Stem Cells,2004)	5'-AGCAGCUUGGGCUCGAGAAdTdT (sense)
	5'-UUCUCGAGCCCAAGCUGCUdTdT (anti-sense)

Quantitative real-time PCR for p21 (CDKN1A)

Total RNA was isolated using the RNeasy Midi Kit according to the manufacturer's instructions (Qiagen). cDNA was synthesized from total RNA as described by the manufacturer's protocol (Life Technologies) using oligo dT primers and M-MLV transcriptase. Quantitative real-time PCR was performed using SYBR Green qPCR SuperMix (Invitrogen). P21 mRNA levels were normalized to the level of GAPDH in the same sample. Results of at least 3 experiments in duplicate are expressed as mean ± SD. Used primers:

GAPDH For: CACCACCARGGAGAACGCTGG

GAPDH Rev: CCAAAGTTGTCATGGATGACC

P21 For: CCTGTCACTGTCTTGTACCCT

P21 Rev: GCGTTTGGAGTGGTAGAAATCT

Facs analysis p-p21 (Thr145)

For FACS analysis of the levels of phosphorylated p21 EC cells were seeded in 6 wells plates and 24h after cisplatin or gamma-irradiation treatment the cells were collected, washed with PBS and fixed with Methanol/Aceton for 20 min at room temperature. Fixed cells were washed 2 times with 1% BSA in PBS followed by immunostaining with p-p21 antibody in 1% BSA in PBS and counterstained with Alexa-Fluor secondary antibody. Fluorescence intensity was detected with the FACS-Calibur (Becton Dickinson).

Supplemental references

IGCCCG. 1997. International Germ Cell Consensus Classification: a prognostic factor-based staging system for metastatic germ cell cancers. International Germ Cell Cancer Collaborative Group. *J. Clin. Oncol.* **15**: 594-603.

Menendez JA, Mehmi I, Lupu R. 2005. Heregulin-triggered Her-2/neu signaling enhances nuclear accumulation of p21WAF1/CIP1 and protects breast cancer cells from cisplatin-induced genotoxic damage. *Int. J. Oncol.* **26**: 649-659.

Panno ML, Giordano F, Mastroianni F, Morelli C, Brunelli E, Palma MG, Pellegrino M, Aquila S, Miglietta A, Mauro L, Bonofiglio D, Ando S. 2006. Evidence that low doses of Taxol enhance the functional transactivatory properties of p53 on p21 waf promoter in MCF-7 breast cancer cells. *FEBS Lett.* **580**: 2371-2380.