Cisplatin Hypersensitivity of Testicular Germ Cell Tumors Is Determined by High Constitutive Noxa Levels Mediated by Oct-4

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Abstract

Testicular germ cell tumors (TGCT) are considered a paradigm of chemosensitive tumors. Embryonal carcinoma cells represent the pluripotent entity of TGCTs and are characterized by expression of Oct-4, a key regulator of pluripotency and a determinant of their inherent hypersensitivity to cisplatin. However, the mechanisms underlying this Oct-4-mediated sensitivity are poorly understood. We previously showed that p53 is a major player in cisplatin hypersensitivity and therefore investigated whether Oct-4 may directly affect p53 activity. Despite a significant decrease in sensitivity, depletion of Oct-4 neither did alter cisplatin-induced transactivation of p53 target genes nor its subcellular localization. These data indicate that, rather than directly modulating p53 activity, Oct-4 provides a cellular context that augments the proapoptotic activity of p53. As mitochondrial priming by the Bcl-2 family is a known determinant of chemosensitivity, we compared the constitutive levels of these proteins in Oct-4-positive and -depleted cells. We identified Noxa as the only Bcl-2 family protein to be highly correlated with Oct-4 status and cisplatin sensitivity. Compared with differentiated cells, constitutive Noxa levels were significantly higher in Oct-4-positive cell lines and cancer patient samples. Furthermore, RNA interference-mediated knockdown of Oct-4 resulted in reduced Noxa transcript, in an almost complete loss of constitutive Noxa protein and decreased cisplatin hypersensitivity to a similar extent as did Noxa depletion. In conclusion, our study indicates that Noxa is a central determinant of hypersensitivity to cisplatin. Oct-4–dependent high constitutive levels of this BH3-only protein prime embryonal carcinoma cells to undergo rapid and massive apoptosis in response to p53 activation. Cancer Res; 73(5); 1–10. ©2012 AACR.

Introduction

Testicular germ cell tumors (TGCT) are the most frequent carcinomas in young male adults with a still increasing incidence worldwide. Cisplatin-based chemotherapy cures the majority of patients even in advanced stages due to the extraordinary sensitivity of TGCTs to cisplatin. Thus, TGCTs are considered as the paradigm of a chemosensitive tumor (1). Several mechanisms have been considered to underlie this hypersensitivity, including the high apoptotic propensity of TGCT cells (2, 3).

The transcription factor Oct-4, a key regulator of pluripotency, is exclusively expressed in cells of nonmalignant pluripotent nature, that is, embryonic stem cells (ESC) and germ cells, as well as their malignant counterparts embryonal carcinoma and seminoma, where it serves as a specific malignancy marker (4, 5). Loss of Oct-4 causes a significant reduction of cisplatin hypersensitivity and was proposed to account for acquired cisplatin resistance in refractory tumors (6, 7). We and others have shown that cisplatin hypersensitivity is highly dependent on a functional p53 protein (8–10). The central role of this tumor suppressor was also shown by the finding that these cells are sensitive not only to cisplatin but also to DNA damage–independent p53 activators such as Nutlin-3 (10–12). Together, these results indicate that hypersensitivity to p53 activation requires an Oct-4–mediated cellular context. We now report that the low apoptotic threshold of Oct-4–positive TGCT cells is dictated by the constitutive presence of high Noxa protein levels.

Materials and Methods

Cell culture

Cell lines from non-seminomatous germ cell tumors were analyzed: H12.1 (embryonal carcinoma), H12.5 (embryonal carcinoma), 2102EP (embryonal carcinoma), 1777NRpmet (differentiated state), 1411HP (differentiated state), NTERA-2D1
(embryonal carcinoma), and H12.1ODM (in vitro differentiated derivative of H12.1). Origin and cultivation procedures of these cell lines were described previously (6, 10). The cell lines 833K (embryonal carcinoma), kindly provided by Prof. Andrews (University of Sheffield, Sheffield, UK), and GCT-72 (differentiated state), kindly provided by Prof. Pera (University of Southern California, Los Angeles, CA), were cultivated under same conditions. The cell line H12.1RA (in vitro differentiated derivative of H12.1) was established by treatment of H12.1 with all-trans-retinoid acid during cultivation in GCT-72–conditioned medium followed by cultivation under same condition as the other cell lines (6, 10).

H460 (lung) and A2780 (ovary) cells were obtained from the NCI-60 cell line panel and cultivated in RPMI-1640 with 10 % fetal calf serum and glutamine. Peripheral blood mononuclear cells (PBMC) and fibroblasts were isolated and maintained as described previously (13, 14).

Reagents
Cisplatin and Nutlin-3 (Sigma) were used at a concentration of 10 µmol/L, etoposide was used at 5 µmol/L, and doxorubicin at 100 ng/mL. MG132 was used at a concentration of 1 µmol/L.

Protein expression
Cells were lysed to obtain cellular protein according to standard protocols. Western blotting was conducted using a SDS-PAGE Gel Electrophoresis system.

mRNA expression
Total RNA was extracted and transcribed to cDNA according to standard protocols. Expression was analyzed using BioMark HD System (Fluidigm) according to manufacturer’s instructions. TaqMan assays were obtained from Applied Biosystems (Supplementary Table S1). The relative amount of Noxa mRNA and pre-mRNA was determined by a SYBR Green-based qPCR assay (7900HT Fast Real-Time PCR System, Applied Biosystems). Contamination of RNA samples with gDNA was excluded by DNase digestion followed by reverse transcription to cDNA. A primer pair spanning an exon-exon junction was designed to detect Noxa mRNA levels, whereas Noxa pre-mRNA was detected using primers that span an exon–intron junction (for primer sequences see Supplementary Table S2). Primers for detection of actin expression for normalization were kindly provided by Claudia Kalla (Institute of Clinical Pharmacology, Stuttgart, Germany).

Sulforhodamine B microculture colorimetric assay
IC_{50} of cisplatin and Nutlin-3 were determined as described (15).

Subcellular fractionation
Cytoplasm and nuclei were separated using Mitochondria Isolation Kit for cultured cells (Pierce) according to manufacturer’s instructions.

Apoptosis assay
Apoptosis was assessed by fluorescein isothiocyanate (FITC)-conjugated Annexin-V staining (16).

siRNA Experiments
To silence Oct-4, Noxa, Puma, and p53, we used siGenome SMARTpool siRNA (Dharmacon, for sequences see Supplementary Table S3). Bim was silenced using Bim siRNA from Santa Cruz. As a control, we used siGenome Non-Targeting siRNA #1 (Dharmacon). Cells were transfected using DharmaFECT®3 (Dharmacon). Forty-eight hours after transfection, cells were treated according to requirements. To evaluate the efficacy of siRNA silencing, protein lysates were prepared and analyzed by Western Blotting.

Tissue samples
Tissue samples from patients with TGCTs were provided by the Department of Urology, Eberhard Karls University of Tübingen (Tübingen, Germany). The local ethics committee approved the collection of patient samples (315/2012BO2 and 396/2005V), and informed consent was obtained from the patients. Specimen were frozen in liquid nitrogen immediately after surgery and stored at −80°C for further use. To obtain protein lysates for Western blotting, tissue was lysed using FastPrep-24 (MP Biomedicals) and sonication.

Statistics
Data are expressed as SD of the mean. Correlations are expressed as Spearman rank correlation coefficient.

Results
Oct-4 depletion does not compromise the p53 response to cisplatin
It has previously been shown that Oct-4 protein levels are highly correlated with cisplatin hypersensitivity in TGCT cells (6). Therefore, we first tested whether Oct-4 is also required for the more general sensitivity of TGCTs to DNA damage–independent p53 activators (10). This was addressed by assessing the impact of Nutlin-3 on NTERA-2D1 cells subjected to RNA interference (RNAi)-mediated Oct-4 knockdown. As shown in Fig. 1A, Oct-4 depletion reduced Nutlin-3–induced apoptosis to a degree comparable to that observed in cells treated with cisplatin. These results suggest that efficient induction of apoptosis by p53 requires the pluripotent, Oct-4–positive context of embryonal carcinoma cells.

Consequently, we investigated possible differences in the accumulation and activation of p53 upon cisplatin in the presence or absence of Oct-4. In response to cellular stress, p53 activation leads to induction of a wide variety of target genes. Hence, we screened a panel of 46 bona fide p53 transcriptional targets (refs. 17, 18; Supplementary Table S1) by quantitative PCR in Oct-4–positive and -deprived NTERA-2D1 cells exposed to cisplatin. A similar analysis was also conducted with the embryonal carcinoma cell line 2102EP that retains a nullipotent phenotype, allowing us to discriminate between differentiation-dependent and direct effects of Oct-4. Surprisingly, despite its pronounced impact on apoptosis, Oct-4 depletion did not compromise the p53-dependent transcriptional response to cisplatin in both cell lines (Fig. 1B). Hence, induction of a p53-mediated transcriptional program is not the primary determinant of Oct-4–mediated hypersensitivity.
Beyond its function as a transcription factor, p53 is capable of facilitating apoptosis in the cytoplasm. Thus, we monitored the accumulation of p53 upon cisplatin exposure in both nucleus and cytoplasm. Notably, upon cisplatin treatment, p53 accumulated to a similar extent in both compartments (Fig. 1C). Nevertheless, neither p53 accumulation nor its localization was significantly influenced by knockdown of Oct-4 (Fig. 1C), which further argues against a direct influence of Oct-4 on p53 activity. Together, these results indicate that rather than directly altering p53 activity, Oct-4 provides an apoptosis-prone cellular context that augments the proapoptotic efficacy of p53.

**Oct-4 determines cisplatin sensitivity by modulating the Bcl-2 family composition**

Recently, it was shown that the response to cytotoxic chemotherapy is dependent on the relative composition of Bcl-2 family proteins, which determines the apoptotic threshold (19). We therefore investigated a possible correlation of individual Bcl-2 family proteins with Oct-4 and cisplatin sensitivity in NTERA-2D1 and 2102EP cells. To that end, we silenced Oct-4 by RNAi-mediated knockdown and examined the protein levels of the proapoptotic Bcl-2 family members Bax, Bak, Noxa, Puma, Bim, and Bid, as well as the antiapoptotic members Bcl-2, Bcl-xl, Bcl-w, and Mcl-1. Notably, Noxa was the only Bcl-2 family protein that could be detected selectively in the presence of Oct-4 knockdown (Fig. 2A). The fact that this was observed in both pluripotent NTERA-2D1 and nullipotent 2102EP cells (Fig. 2A) suggests that the reduction of Noxa protein is not just a secondary effect of differentiation but a direct consequence of Oct-4 depletion. Mcl-1, the major antiapoptotic binding partner of Noxa, was also reduced upon Oct-4 knockdown (Fig. 2A). As Noxa levels became practically undetectable under such conditions (Fig. 2A), the Noxa/Mcl-1 ratio was shifted considerably in favor of Mcl-1, probably establishing a higher apoptotic threshold. Knockdown of Oct-4 also reduced the levels of the BH3-only proteins Puma and Bim, especially in 2102EP cells, although not as strongly as observed for Noxa (Fig. 2A). Notably, a role of Puma in hypersensitivity of pluripotent embryonal carcinoma cells was previously proposed (10). The protein levels of all other Bcl-2 family members analyzed did not exhibit concomitant changes in these cell lines upon Oct-4 depletion (Fig. 2A).

Next, we investigated the impact of the Oct-4-dependent Bcl-2 family proteins Noxa, Puma, and Bim on cisplatin sensitivity by modulating the Bcl-2 family proteins Noxa, Puma, and Bim.
hypersensitivity. Therefore, we transfected NTERA-2D1 and 2102EP cells with the corresponding siRNAs and obtained a similar knockdown efficiency for each siRNA (Fig. 2B, right).

Silencing of Noxa led to a marked decrease in sensitivity to cisplatin in both cell lines, comparable with that observed upon Oct-4 knockdown (Fig. 2B, left). A similar trend was also seen with Puma, although in NTERA-2D1 cells, silencing of Puma did not reduce apoptosis as efficiently as did Noxa knockdown. In contrast, Bim knockdown had only minor effects on cisplatin hypersensitivity (Fig. 2B, left). To investigate possible cross-regulation between these proapoptotic Bcl-2 family members, NTERA-2D1 cells were depleted of each protein and levels of the respective others were quantified. We found no significant cross-regulation between Noxa, Puma, and Bim (Supplementary Fig. S1).

It is of note that knockdown of either Oct-4 or Noxa reduced sensitivity of TGCTs not only to cisplatin but also to other genotoxic agents such as etoposide and doxorubicin as well as to the DNA damage–independent p53 activator Nutlin-3 (Supplementary Fig. S2). These data suggest that Oct-4 and Noxa are central mediators of the general hypersensitivity of TGCTs.

Constitutive Noxa protein levels are closely correlated with Oct-4 and cisplatin hypersensitivity in a panel of TGCT cell lines and patient samples

Noxa and Puma are induced in response to several cellular stress stimuli. However, Noxa protein was also found to be highly expressed in unstressed, undifferentiated embryonal carcinoma cells but not in cell lines derived from other tumor entities (10). We therefore investigated whether constitutive Noxa and Puma levels are maintained via transcriptional mechanisms in embryonal carcinoma cells. In contrast to Puma, Noxa transcript was expressed significantly higher in Oct-4–positive NTERA-2D1 and 2102EP cells than in Oct-4–negative lymphocytes, fibroblasts, or cancer cell lines (Fig. 3A, left). A direct effect of Oct-4 on Noxa mRNA expression was confirmed by Oct-4 knockdown experiments. Oct-4 depletion reduced Noxa transcript in NTERA-2D1 and 2102EP cells to a
level comparable with that observed in differentiated cells (Fig. 3A, left). To investigate the possibility that Oct-4 may regulate mRNA stability by posttranscriptional mechanisms, expression of Noxa pre-mRNA was examined and found to be similarly dependent on Oct-4 as was mature Noxa mRNA (Supplementary Fig. S3). This result indicates that Oct-4...
regulates Noxa gene transcription. Puma, on the other hand, was only slightly reduced upon Oct-4 depletion (Fig. 3A, left). Together, these data suggest an Oct-4-dependent transcriptional mechanism resulting in high constitutive Noxa protein levels.

The effect of Oct-4 on constitutive expression of Noxa in Oct-4–positive and -depleted embryonal carcinoma cells was even more pronounced at the protein level (Fig. 3A, right). Furthermore, the absolute levels of Noxa protein in Oct-4–positive cells were 9.2-fold (NTERA-2D1 vs. PBMCs) and 11.2-fold (2102EP vs. PBMC) higher as those of stimulated hematopoietic cells (Fig. 3A, right), which have been shown to exhibit constitutive Noxa protein levels (20), whereas the difference in transcript levels was only 2.3-fold (NTERA-2D1 vs. PBMC) and 1.3-fold (2102EP vs. PBMCs; Fig. 3A, left). It is therefore likely that Oct-4 also modulates Noxa protein translation and/or stability by a yet unknown mechanism. In contrast to Noxa, Puma levels were found to be higher only in 1 of the 2 Oct-4–positive cell lines than in differentiated cells (Fig. 3A, right).

To further investigate the correlation of Oct-4 and cisplatin sensitivity with Noxa and Puma, we analyzed constitutive protein levels of both BH3-only proteins (Fig. 3B, top left) together with cisplatin IC_{50} values (Fig. 3B, bottom left) in an extended TGCT cell line panel consisting of 5 Oct-4–negative and 5 Oct-4–positive cell lines. We confirmed a correlation of cisplatin sensitivity with Oct-4 (Spearman r = −0.842, P = 0.004; Fig. 3B, bottom right). Importantly, Noxa was significantly correlated with Oct-4 expression (Spearman r = 0.891, P = 0.001; Fig. 3B, top right) as well as with cisplatin sensitivity (Spearman r = −0.806, P = 0.007; Fig. 3B, bottom right). On the other hand, while Puma protein levels were also positively correlated with Oct-4 status, this correlation was weaker than for Noxa (Spearman r = 0.685, P = 0.035; Fig. 3B, top right), and no significant correlation was seen with cisplatin sensitivity (Spearman r = −0.527, P > 0.05; Fig. 3B, bottom right). Notably, similar results were observed with Nutlin-3 (Supplementary Fig. S4). These data further support the conjecture that Oct-4 lowers the apoptotic threshold in TGCTs primarily by maintaining high constitutive Noxa protein levels.

In addition, we sought to extend our in vitro findings to cancer patient samples. Indeed, we could confirm a close correlation between Oct-4 and Noxa protein levels in primary tissue samples derived from 5 embryonal carcinomas and 8 seminomas (Spearman r = 0.832, P < 0.0001; Fig. 3C).

**Accumulation of both Noxa and p53 is required to induce cell death in Oct-4–depleted TGCT cells**

We have previously shown that accumulation of p53 is a central determinant in TGCT hypersensitivity (10). In the present study, we established that Oct-4–positive TGCT cells are characterized by high constitutive Noxa protein levels, which seems to be a requirement for their p53-mediated hypersensitivity. Therefore, in Oct-4–depleted cells, pharmacologic induction of both Noxa and p53 protein should result in cell death. To test this hypothesis, NTERA-2D1 and 2102EP cells, respectively, were depleted of Oct-4 by RNAi-mediated gene silencing (Fig. 4, top). In contrast to cisplatin or Nutlin-3 (Fig. 1A), treatment with the proteasome inhibitor MG132 resulted in efficient induction of apoptosis in both Oct-4–depleted cell lines (Fig. 4, bottom). Importantly, MG132 treatment induced simultaneous accumulation of Noxa and p53 protein (Fig. 4, middle). Prevention of accumulation of either protein by RNAi-mediated knockdown led to increased survival upon MG132 treatment in NTERA-2D1 and 2102EP cells (Fig. 4, bottom). These data further show that both Noxa and p53 protein are required to induce apoptosis in TGCT cells.

**Discussion**

Cisplatin-based chemotherapy has been a successful cure for TGCT for decades. A variety of molecular determinants of these neoplasms were proposed to account for their exquisite sensitivity to cisplatin such as a reduced DNA repair capacity, a deregulated G1–S transition or marginal p21 expression (2, 3, 21). Much of the research done in this field has focused on p53, which is almost exclusively expressed in its wild-type conformation in TGCTs (22). We recently showed a central role for p53 in TGCT hypersensitivity to cisplatin and suggested an intrinsic sensitivity of these tumors to activation of p53, which leads to robust induction of apoptosis in this specific cellular context (10). In the present study, we found that this sensitivity is dependent on the presence of Oct-4, although p53 activation is not directly influenced by this mediator of pluripotency. Rather, by maintaining high constitutive Noxa protein levels, Oct-4 provides a cellular context that primes TGCT cells to p53-dependent apoptosis.

TGCTs derive from intrinsically apoptosis-prone tissue. Therefore, their unique responsiveness to chemotherapy might be due to inherent biological characteristics (23). An intrinsic molecular determinant of the pluripotent compartment of TGCTs is the expression of regulators of pluripotency such as Oct-4. Recent work related loss of Oct-4 to an increase in cisplatin resistance (6). In the present study, we show that Oct-4 depletion not only protected cells from cisplatin-induced cell death but also led to a reduction of the apoptotic response to other genotoxic and non-genotoxic p53 inducers (Fig. 1A and Supplementary Fig. S2). These data imply that Oct-4 mediates the general intrinsic hypersensitivity to p53 activators in pluripotent embryonal carcinoma cells. Several studies have attributed the increased susceptibility to apoptosis to overexpression of p53 (24, 25). However, we and others could show that high p53 protein levels are not a characteristic of TGCTs (10, 26). Here, we show that Oct-4 had no impact on constitutive p53 levels or on its accumulation upon cisplatin treatment (Fig. 1C). Furthermore, Oct-4 depletion did not influence the cisplatin-induced transactivation of 46 representative bona fide p53 target genes (Fig. 1B). These data indicate that the transcriptional p53 response is not substantially altered in Oct-4–positive hypersensitive TGCT cells. In addition to its capability to launch the apoptotic program through transactivation of proapoptotic target genes, p53 can directly induce apoptosis in the cytoplasm by interacting with Bcl-2 family proteins. Subcellular fractionation revealed that besides an accumulation of p53 in the nucleus, cisplatin treatment resulted in a similar accumulation in the cytoplasm (Fig. 1C), suggesting that p53 could function independently of its transcriptional activity to launch the apoptotic program in
TGCTs; this was also proposed as a proapoptotic mechanism in ESCs (27). However, Oct-4 depletion did not significantly influence subcellular distribution of p53 (Fig. 1C). Together, these data show that neither transcriptional nor cytoplasmic functions of p53 are dependent on the presence of Oct-4 in TGCT cells. As Oct-4 is central for cisplatin sensitivity, it must provide a cellular context allowing an effective proapoptotic p53 response.

Recently, Letai and colleagues established a model to explain and predict chemotherapeutic response by mitochondrial priming (19). According to this hypothesis, occupation of antiapoptotic Bcl-2 family proteins by their proapoptotic counterparts determines the proximity of a cell to the threshold of apoptosis (28). Our data show that the presence of Oct-4 in pluripotent embryonal carcinoma cells dictates a Bcl-2 family profile dominated by high constitutive Noxa protein levels, which augments the cell’s commitment to apoptosis. We found that Noxa protein levels are tightly correlated with both Oct-4 status and cisplatin sensitivity in a panel of cell lines and also in samples from primary TGCTs.

The relative levels of other Bcl-2 proteins have also previously been proposed to determine hypersensitivity in TGCTs. Several studies suggested that the enhanced sensitivity to genotoxic agents is dependent on a high Bax/Bcl-2 ratio (29) and low levels of Bcl-2 and Bcl-xL (30, 31). A chemoprotective role of Bcl-xL in these tumors has also been proposed (32). However, we found no correlation between the protein levels of Bcl-2, Bax, and Bak and Oct-4 status (Fig. 2A). Indeed, recent data showed that Bcl-2, Bcl-xL, Bax, or Bak levels were similar among TGCT cell lines with differential cisplatin sensitivity (15, 33). Together, these data indicate that Noxa is the only Bcl-2 family protein that is tightly correlated to both Oct-4 status and cisplatin sensitivity, suggesting that constitutive Noxa protein is a key determinant of the exclusive sensitivity of Oct-4-positive embryonal carcinoma cells to cisplatin. Moreover, we show that the general sensitivity of TGCTs to genotoxic and non-genotoxic agents is also dependent on Noxa (Supplementary Fig. S2). The prominent role of Noxa for TGCT hypersensitivity is further supported by the finding that Noxa protein was found to be correlated with good clinical prognosis in patients with embryonal carcinoma (34).

Noxa was shown to preferentially bind to Mcl-1 and A1 (35). The apoptotic potential of this BH3-only protein when overexpressed was therefore suggested to be quite variable due to its inability to antagonize Bcl-2 and Bcl-xL (36). However, as mentioned above, TGCTs exhibit low expression of Bcl-2 and Bcl-xL (30, 31), potentially providing an ideal premise for an increased apoptotic potential of Noxa overexpression.

Figure 4. Accumulation of both Noxa and p53 is required to induce cell death in Oct-4-depleted TGCT cells. Cells were transfected with indicated siRNAs and incubated for 48 hours before MG132 treatment. Controls reflect cells treated with a nontargeting siRNA. Efficient gene silencing was verified by Western blot analysis (top and middle). Transfected cells were treated with MG132 for 20 hours (NTERA-2D1) or 16 hours (2102EP); protein lysates were prepared and subjected to Western blot analysis to determine protein levels of p53 and Noxa (middle) and stained with Annexin-V/ FITC and propidium iodide for cell death analysis by flow cytometry (bottom). Graph reflects means ± SD of survival upon MG132 from 3 independent experiments.
Moreover, our results show a considerable shift of the Noxa/Mcl-1 ratio in favor of Mcl-1 upon Oct-4 depletion (Fig. 2A), enforcing a higher apoptotic threshold. A similar dependence on the Noxa/Mcl-1 balance was shown for HeLa and pancreatic cancer cells treated with camptothecin (37, 38). It is of note that Noxa was recently shown to be capable of binding Bcl-xL upon DNA damage and thus facilitate apoptosis induction (39), a conceivable mechanism that broadens the proapoptotic capacity of Noxa. In addition, it seems most likely that while Noxa inhibits the antiapoptotic functions of Mcl-1, a robust induction of other BH3-only proteins by p53 sequesters Bcl-xL and activates Bax and Bak. This hypothesis is supported by the fact that Puma was found to be as important as was Noxa for the hypersensitivity to cisplatin (Fig. 2B). However, constitutive Puma protein levels could not be linked to cisplatin sensitivity (Fig. 3B). These results indicate that Puma exerts its prominent proapoptotic function in TGCT cells only upon induction by p53 as previously described for other cell types (40).

Because of the established function of Oct-4 as a transcription factor, it is tempting to speculate that transcription of the Noxa gene is directly promoted by Oct-4. Interestingly, in a recent study, an Oct-4–binding site was mapped within 8 kb of the Noxa promoter (41). Moreover, Noxa mRNA was shown to be upregulated in ESCs (42) and its expression was found to be correlated with Oct-4 in this cell type (43, 44). The finding that Noxa pre-mRNA was downregulated upon Oct-4 depletion to a similar extent as the corresponding mature mRNA (Supplementary Fig. S3) suggests that Oct-4 may indeed directly regulate the transcription of the Noxa gene. However, as Oct-4 is involved in the transactivation of many genes, including other transcription factors (44), it remains possible that this regulation might as well include indirect effects.

Noxa was commonly seen as a transcriptionally activated stress-responsive gene (36) rather than a protein present at high levels in unstressed cells. However, upregulated Noxa protein levels were reported to restrain lymphocyte expansion and can be triggered by glucose deprivation (20). Together with the high constitutive protein levels reported in the present work (Fig. 3A), these data suggest an additional regulation at the protein level, for example, through the ubiquitin–proteasome system (45, 46). As Noxa protein regulation by Oct-4 was shown to be more pronounced than regulation at the mRNA level (Fig. 3A), altered Noxa protein stability cannot be excluded as a possible additional explanation for high constitutive Noxa protein levels.

We established that loss of high constitutive Noxa protein levels in Oct-4–depleted cells causes resistance to cisplatin and Nutlin-3. However, these cells were still sensitive to proteasomal inhibition by MG132 (Fig. 4). Importantly, MG132 resulted in stabilization of Noxa protein reaching levels comparable to that observed in Oct-4–positive cells. Concomitantly, p53 was stabilized upon proteasomal inhibition. RNAi-mediated knockdown of Noxa or p53, respectively, rescued Oct-4–depleted cells from cell death upon MG132 treatment (Fig. 4). These data further indicate that hypersensitivity of TGCTs is dependent on both high levels of Noxa and induction of p53.

We propose that, under basal nonstressed conditions, embryonal carcinoma cells may buffer the high apoptotic pressure driven by high constitutive Noxa levels by a parallel increase in its antiapoptotic binding partner Mcl-1. Upon cellular stress, p53-mediated induction of additional BH3-only proteins such as PUMA (10) and concomitant Mcl-1 degradation (47) may then shift the balance and lead to a rapid and robust induction of apoptosis.

In conclusion, our present study shows that Oct-4–dependent high constitutive Noxa levels determine cisplatin hypersensitivity in TGCTs. Despite the predominant role of p53 in TGCT hypersensitivity, Oct-4 does not directly influence p53 activation but rather provides an extremely low apoptotic threshold by maintaining high constitutive Noxa protein levels. Therefore, in response to cellular stress, accumulation of p53 triggers a rapid and massive induction of apoptosis.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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