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Repair shielding of platinum-DNA lesions in testicular germ cell tumors by high-mobility group box protein 4 imparts cisplatin hypersensitivity

Samuel G. Awuah*, Imogen A. Riddell*, and Stephen J. Lippard*#1

*Department of Chemistry, Massachusetts Institute of Technology, Cambridge, MA 02139; and #Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, Cambridge, MA 02139

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Cisplatin is the most commonly used anticancer drug for the treatment of testicular germ cell tumors (TGCTs). The hypersensitivity of TGCTs to cisplatin is a subject of widespread interest. Here, we show that high-mobility group box protein 4 (HMGB4), a protein preferentially expressed in testes, uniquely blocks excision repair of cisplatin-DNA adducts, 1,2-intrastrand cross-links, to potentiate the sensitivity of TGCTs to cisplatin therapy. We used CRISPR/Cas9-mediated gene editing to knockout the HMGB4 gene in a testicular human embryonic carcinoma and examined cellular responses. We find that loss of HMGB4 elicits resistance to cisplatin as evidenced by cell proliferation and apoptosis assays. We demonstrate that HMGB4 specifically inhibits repair of the major cisplatin-DNA adducts in TGCT cells by using the human TGCT excision repair system. Our findings also reveal characteristic HMGB4-dependent differences in cell cycle progression following cisplatin treatment. Collectively, these data provide convincing evidence that HMGB4 plays a major role in sensitizing TGCTs to cisplatin, consistent with shielding of platinum-DNA adducts from excision repair.

Significance

High-mobility group box protein 4 (HMGB4) is a transcription repressor preferentially expressed in the testes and binds cisplatin-damaged DNA. Investigating the DNA-damage recognition potential of HMGB4 and its relevance to cancer is of clinical importance. In this study, we found that HMGB4 regulates the sensitivity of testicular germ cell tumors (TGCTs) to cisplatin treatment. HMGB4 imparts repair shielding of platinum-DNA lesions in human TGCTs, rendering lesions inaccessible to the nucleotide excision repair (NER) machinery. Our results show that cisplatin-resistant breast cancer cells complemented with HMGB4 become sensitive to cisplatin. Furthermore, the ability of HMGB4 to modulate the cell cycle response, NER, apoptosis, and MAPK suggests a critical role for this protein in conveying cisplatin hypersensitivity in TGCTs.


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1To whom correspondence should be addressed. Email: lippard@mit.edu.

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and HMGB4 gene-edited NTera2 cells (NTera2 HMGB4+) were used. Taken together, our results point to a major role for HMGB4 in sensitizing TGCTs to cisplatin therapy.

**Results**

**HMGB4 and Cisplatin in Testicular Cancer Cells.** The structure of the ternary HMGB4–Pt-DNA complex provides a basis for understanding HMGB recognition of the platinum-induced DNA distortion (23) and led to the analysis of the protein sequence of human HMGB4 and its computed structure in complex with platinated DNA (Fig. 1). The HMGB1 phenylalanine that inserts into the hydrophobic notch created in the minor groove by the platinum 1,2-d(GpG) cross-link in the major groove is preserved in HMGB4. However, in place of two cysteine residues found at positions Cys44 and Cys22 in HMGB1, the corresponding residues in HMGB4 are Cys44 and Tyr22. This difference confers redox indifference for HMGB4 binding to cisplatin-modified DNA by eliminating the potential for disulfide bond formation that is present in HMGB1.

Because HMGB4 has not been well studied in relation to human testicular cancer, we first measured the levels of HMGB4 in a panel of TGCTs. TGCTs are classified according to their histopathology as either seminoma or nonseminoma (SI Appendix, Fig. S1A). Immunoblotting experiments revealed that different TGCTs of human origin, including the embryonic carcinomas NTera2 and Tera2 as well as the testicular teratoma GCT27, express appreciable levels HMGB4 (SI Appendix, Fig. S1B).

Cisplatin and its analogs, oxaliplatin and carboplatin, induce DNA damage by forming intrastand 1,2-d(GpG), 1,2-d(ApG), and 1,3-d(GpNpG) cross-links, with fewer interstrand and DNA–protein cross-links. There is substantial evidence that HMGB4 binds 1,2-d(GpG) cross-links in vitro (27). To investigate the effect of cisplatin on HMGB4 in testes, NTera2 and GCT27 cells were treated with 2 μM of the drug for several time points and the protein level was monitored by Western blotting. Cisplatin treatment reduced HMGB4 expression over time by comparison with controls (Fig. 2), a phenomenon possibly associated with posttranslational modification of the target protein (28).

To investigate the alternative possibility that the diminution in HMGB4 recognition by the antibody after 72 h was a consequence of HMGB4 interaction with cisplatin-DNA adducts, we treated NTera2 and GCT27 cells with cisplatin and a Re(V) complex (I) that induces necroptosis but not DNA cross-link formation (Fig. 2) (29). Necroptosis is a specialized caspase-independent pathway of programmed necrosis that relies on the signaling activity of receptor interacting serine/threonine protein kinase 1 and 3 (RIP1, RIP3) to phosphorylate each other to form necrosomes (30, 31). Subsequent phosphorylation of mixed lineage kinase domain (MLKL) by necrosomes facilitates necroptosis. Characteristic features of necroptosis are swollen organelles and disintegrated plasma membrane. As seen in Fig. 2, there was no significant difference in HMGB4 recognition in NTera2 cells treated with I.

**CRISPR/Cas9 KO of HMGB4 in Human Testicular Embryonic Carcinoma.** The Cas9 endonuclease of *Streptococcus pyogenes* (SpCas9) associated with CRISPR can be reprogrammed to target genomic loci of mammalian cells in a specific fashion using single guide RNA (sgRNA) (32–34). We used this gene-editing strategy to target HMGB4 in the human embryonic testicular cancer cell line, NTera2. Using RT-PCR, we observed a >80% change in HMGB4 mRNA expression levels in the KO relative to the parental NTera2 cells (Fig. 3A). We also confirmed a corresponding decrease in HMGB4 protein levels using Western blot analysis of the single clone HMGB4 KO NTera2 cells (Fig. 3C). The KO cell lines afford us a biological tool to explore our hypothesis that HMGB4 conveys DNA-repair shielding, which leads to sensitization of TGCTs to cisplatin treatment.

**Loss of HMGB4 Desensitizes TGCTs to Cisplatin.** To assess the involvement of HMGB4 in mediating cellular sensitivity to cisplatin, we investigated the effect of HMGB4 on the viability of human TGCT cells treated with cisplatin using RNAi knockdown or CRISPR/Cas9 KO of HMGB4 in NTera2 cells. First, we used RNAi-mediated knockdown to obtain NTera2 cells with varying HMGB4 expression levels. As confirmed by quantitative PCR (qPCR) and Western blot, we generated NTera2 cell lines that enabled the correlation of HMGB4 expression with cisplatin sensitivity in an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay in HMGB4−/−, NTera2 and GCT27 cells with cisplatin and a Re(V) complex (I) that induces necroptosis but not DNA cross-link formation (Fig. 2) (29). Necroptosis is a specialized caspase-independent pathway of programmed necrosis that relies on the signaling activity of receptor interacting serine/threonine protein kinase 1 and 3 (RIP1, RIP3) to phosphorylate each other to form necrosomes (30, 31). Subsequent phosphorylation of mixed lineage kinase domain (MLKL) by necrosomes facilitates necroptosis. Characteristic features of necroptosis are swollen organelles and disintegrated plasma membrane. As seen in Fig. 2, there was no significant difference in HMGB4 recognition in NTera2 cells treated with I.

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assay (SI Appendix, Fig. S2). Notably, reduced levels of HMGB4 in silenced Ntera2 cells conferred relative cisplatin resistance compared with WT cells, and a correlation was observed between cellular resistance and HMGB4 expression level. Second, we investigated cisplatin sensitivity in HMGB4 KO Ntera2 cells from different clones compared with WT Ntera2 cells. We observed a 4.5-fold difference in cisplatin sensitivity of the KO compared with WT Ntera2 cells (Fig. 4 A and B). This result underscores the ability of HMGB4 to increase cellular sensitivity to cisplatin.

Next, we investigated the ability of HMGB4 to promote cisplatin-induced apoptosis in Ntera2 and CRISPR/Cas9-mediated HMGB4 KO Ntera2 cells. Taking advantage of the translocation of phosphatidylserine to the exterior of apoptotic cells for annexin V recognition, we used a dual-staining annexin V/SYTOX green apoptosis dead cell assay to study the apoptotic behavior of Ntera2 and Ntera2 HMGB4−/− after 72 h of exposure to cisplatin (Fig. 4 C and D). The apoptotic and necrotic populations in Ntera2 HMGB4−/− cells were reduced by approximately twofold compared with WT cells, indicative of the role of HMGB4 in effecting cell death by cisplatin-induced apoptosis.

To further confirm our findings, we carried out two complementation studies to ensure that the observed desensitization of Ntera2 to cisplatin is a consequence of the loss of HMGB4. We transiently transfected Ntera2 HMGB4−/− cells with a plasmid encoding HMGB4 and assessed the cellular response of the transfected cells to cisplatin. The HMGB4 transfected cells restored sensitivity to cisplatin to the level of WT Ntera2 cells (Fig. 5). In addition, the triple-negative breast cancer cell line MDA-MB-231, known to be highly resistant to cisplatin treatment, was transfected with the HMGB4 cDNA. HMGB4 expression was confirmed by Western blot and cisplatin sensitivity was assessed by an MTT assay (SI Appendix, Fig. S3). The IC50 (50% inhibitory concentration) values generated from dose–response curves showed twofold enhanced sensitivity for MDA-MB-231 transfected cells over WT cells. Collectively, our results establish that HMGB4 plays a role in sensitizing TGCTs to cisplatin.

Because HMGB4 specifically recognizes and binds 1,2-(GpG) Pt-DNA cross-links, we studied the effect of a monofunctional platinum agent on Ntera2 and Ntera2 HMGB4−/− cells. Monofunctional platinum adducts, unlike their bifunctional counterparts, do not significantly distort the 3D structure of duplex DNA (35). Therefore, if sensitization of TGCTs to cisplatin arises from HMGB4 recognition and repair shielding of the 1,2(GpG) cisplatin-DNA cross-link, monofunctional adducts would not elicit the same response. Indeed, phenanthriplatin, a highly potent antitumor agent developed within our laboratory (36), did not discriminate between the two different isogenic lines, at least with respect to IC50 values (SI Appendix, Fig. S4). This result supports our hypothesis that sensitivity of TGCTs to monofunctional platinum complexes is independent of HMGB4.

**HMGB4 Dictates Cell-Cycle–Specific DNA-Repair Events.** Cellular DNA is under constant threat by endogenous and exogenous agents that inflict damage. In response, cells deploy sophisticated repair machinery to remove cytotoxic lesions and maintain genomic integrity. DNA repair is regulated throughout the cell cycle. We therefore examined the role of HMGB4 in cell cycle progression after exposure of both Ntera2 and Ntera2 HMGB4−/− cells to cisplatin. It is conceivable that lack of repair in Ntera2 cells resulting from the repair-shielding role of HMGB4 will block S-phase progression because the lesions impede DNA polymerases (37). To investigate this possibility, we treated Ntera2 and Ntera2 HMGB4−/− cells with cisplatin (2 μM) and used flow cytometry to assay cell cycle progression using ModFit. Ntera2 cells displayed a clear S-phase delay 24 and 48 h following cisplatin treatment, whereas Ntera2 HMGB4−/− cells did not (SI Appendix, Fig. S5). These data support the hypothesis that HMGB4 shields repair, inducing S-phase blockage and imparting cisplatin sensitivity to testicular cancer cells. We found that the G0/G1 and S-phase checkpoints of Ntera2 HMGB4−/− cells did not change significantly at various time points, suggesting that, in the absence of HMGB4, DNA damage repair is unimpeded and S-phase blockage is not observed, properties that confer cellular resistance to cisplatin.

**Shielding of DNA-Excision Repair by Constitutive HMGB4 Is Important for Sensitivity of TGCTs to Cisplatin.** Excision repair has long been known as the major mechanism for removing the major DNA damage caused by cisplatin (38). It is well conserved across mammalian species, from rodent to human (39). Given the risk of cisplatin-resistant relapses, we investigated the role of HMGB4 on the sensitivity of TGCTs to cisplatin. The IC50 values from the dose–response curves shown in A. WT Ntera2 cells were used as control. Experiments were performed in triplicate. Data are represented as mean ± SD. (C) Flow cytometry experiment measurement of the extent of apoptosis in Ntera2 (Upper) and Ntera2 HMGB4−/− (Lower), untreated or treated with cisplatin (1 μM, 72 h) using APC-annexin V and SYTOX green as the markers. (D) Quantification of apoptosis and necrosis in Ntera2 and Ntera2 HMGB4−/− cells as determined in C.
adducts formed by cisplatin (19, 38). To study the role of NER in TGCTs, we examined the biological consequences of cisplatin treatment on the expression of genes encoding proteins involved in the repair complex. The repair mechanisms comprise damage recognition by proteins, unwinding or opening of the DNA helical structure, excision of the damaged DNA strand by endonucleases, and the subsequent action of DNA polymerases and ligases to complete the repair using the undamaged strand as template (39). With the use of qPCR to interrogate selected genes within the NER pathway, we found that endonucleases XPF-ERCC1 (xeroderma pigmentosum group F) repair complex and ERCC1-XPF (xeroderma pigmentosum group G) mRNA transcripts were consistently up-regulated by approximately twofold in NTera2 HMGB4−/− relative to NTera2 cells, following treatment with cisplatin (SI Appendix, Fig. S6 A–D). Consistent with the report that mouse HMGB4 acts as a transcription repressor (26), this difference might result from HMGB4 transcriptionally regulating NER-associated proteins required to repair cisplatin-DNA adducts and that its absence in Ntera2 HMGB4−/− cells derepresses excision repair. In addition, the mRNA levels of XPC (xeroderma pigmentosum group C), which is considered to function in DNA-damage recognition within the global genome repair arm of NER (GG-NER), showed a sustained increase over a 48-h period in NTera2 HMGB4−/− compared with WT NTera2 cells.

Given that XPB (xeroderma pigmentosum group B) is a helicase in transcription factor II human (TFIIH) that facilitates DNA duplex unwinding for transcription and repair within the NER machinery, we investigated the role of XPB in NTera2 and NTera2 HMGB4−/− cell lines following cisplatin treatment. We used a small molecule inhibitor, spironolactone (SI Appendix, Fig. S7A), to inactivate XPB and evaluated cisplatin-induced cytotoxicity by MTT (SI Appendix, Fig. S7B). In addition, using shRNA to target the XBP gene, we knocked down XBP (SI Appendix, Fig. S7C) and examined the response of the mutated NTera2 and NTera2 HMGB4−/− cells to cisplatin, as shown in SI Appendix, Fig. S7D. A threefold increase in sensitivity was observed in NTera2 HMGB4−/− having reduced XBP compared with the XBP-containing parental HMGB4−/− cell line (SI Appendix, Fig. S7D). Reduced XBP activity in NTera2 showed little effect on the cellular sensitivity to cisplatin (SI Appendix, Fig. S7E).

To further validate these results, we studied the impact of HMGB4 on DNA repair using a human excision nuclease assay performed with cell-free extracts (CFEs) derived from NTera2 and NTera2 HMGB4−/− cells. The assay of cisplatin-damaged DNA repair was carried out by first preparing the substrates shown in Fig. 6A, formed by annealing and ligation a short oligomer containing cisplatin 1,2-d(GpG) cross-links with four other overlapping oligomers, as previously described (19). The oligomer containing the Pt damage was labeled with [γ−32P] ATP such that the 146-bp duplexes contained a radiolabel at the fifth phosphodiester bond 5′ to the cisplatin d(GpG) adduct. When these substrates were incubated with NTera2 and NTera2 HMGB4−/− CFE, the adducts were released in oligonucleotides that were mainly 25- to 30-nt long (Fig. 6B), as observed by polyacrylamide gel electrophoresis. The removal of cisplatin-modified fragments requires active nucleotide excision repair in the TGCTs with depleted HMGB4. In support of the repair-shielding hypothesis, we observed that, in NTera2 cells with normal HMGB4 levels, there was reduced 25- to 30-nt fragment removal compared with results for NTera2 HMGB4−/− cells (Fig. 6C). Site-specifically platinated control oligonucleotides prepared in an identical manner failed to produce the characteristic 25- to 30-nt fragments when incubated without CFE.

**Discussion**

Understanding the high cure rates of TGCTs by cisplatin is of longstanding interest and could be of clinical value for treatment of refractory solid tumors. Given that cisplatin forms both intra- and interstrand cross-links on DNA that ultimately result in apoptosis if not removed, the observation that DNA-recognition proteins, including HMG-family proteins, can block the repair pathway is of potential clinical relevance. We previously demonstrated that HMG proteins potentiate sensitivity of mammalian cells to cisplatin by binding to 1,2-intrastrand d(GpG) cross-links using excision nuclease assays (40), which implies that NER proteins may be implicated in regulating cisplatin sensitivity in malignancies. Here, we expanded our investigation of HMG proteins to include HMGB4, a testes-specific protein involved in spermatogenesis.

In this study, we report an unexplored role of HMGB4 in sensitizing TGCTs to cisplatin at the molecular level. Higher IC50 and
lower apoptosis induction associated with CRISPR/Cas9-mediated HMGB4 KO Ntera2 cells reveals increased cisplatin resistance in the absence of HMGB4. Genome-wide screens in Saccharomyces cerevisiae identified genes that, when disrupted, confer cisplatin resistance (41). Several of the identified genes had not been previously linked to cisplatin resistance and functioned in RNA Pol II-dependent gene regulation, DNA repair, and genome stability. Complementation of individual inactivated genes eradicated cisplatin resistance. In a similar manner, we here identify the specific involvement of HMGB4 in conferring sensitivity in the cisplatin-resistant human breast cancer cell line (MDA-MB-231) by complementation with a HMGB4-encoding plasmid (SI Appendix, Fig. S3). The sensitivity doubled upon expression of the protein. Our observation that the cisplatin-resistant phenotype of the deleted HMGB4 cell line is due to the specific disruption of HMGB4 suggests that changes to HMGB4 in testicular germ cell tumors, such as posttranslational modifications, may also confer cisplatin resistance. Given that HMGB1 undergoes hyperacetylation and phosphorylation upon interaction with cisplatin-DNA adducts (28), the same or similar behavior seems highly plausible for HMGB4, considering that the two proteins share significant homology. The clinical consequence is that patients with TGCTs with cisplatin resistance are likely to have HMGB4 mutations or modifications. To explore this possibility, we are currently studying human biopsy samples from patients with TGCTs experiencing cisplatin-resistant phenotypes to determine whether they can be correlated with HMGB4 levels and associated mutations or modifications. Furthermore, the parallel of HMGB4 expression levels, as quantified by qRT-PCR, in transient knockdown cells with their cytotoxicity profiles verifies the specific involvement of HMGB4 in determining cisplatin sensitivity. For TGCTs, this report demonstrates that the platinum DNA-damage recognition protein, HMGB4, correlates with cisplatin sensitivity. Accumulation of cells at the G2/M cell cycle transition reflects unrepaired Pt-DNA lesions in Ntera2 HMGB4-proficient cells and follows delayed S phase after cisplatin treatment. The Pt lesions block DNA polymerases required for replication (42) and the transcription of the mitotic spindle apparatus needed for cell division. In cells containing HMGB4, failure to repair the damaged DNA during G1 results in replication stalling and ultimately leads to cell death. Conversely, in HMGB4-deficient cells, we propose that DNA damage is sufficiently well repaired during the G1 phase by unimpeached NER proteins to account for the unchained S phase observed. Notably, no significant accumulation of cells at G2/M over the analysis period following cisplatin treatment was observed in Ntera2 HMGB4<sup>−/−</sup>. In further support of this argument, a persistent G1 phase was observed, as indicative of growth and NER activity.

The involvement of HMGB4 in sensitizing TGCTs to cisplatin prompted our investigation of DNA repair mediated by NER in human TGCT cells. Inefficient repair of cisplatin-induced DNA damage in TGCTs has been associated with reduced XPA protein levels (43). It is possible that HMGB4 interacts with XPA to shield repair activity, although careful experiments are needed to support this presumption. Considering that cisplatin is an effective anticancer drug used to cure metastatic testicular cancer, our understanding of whether or not the predominant DNA lesion, the 1,2-d(GpG) intrastrand cross-link, is an important substrate for human extrusion nucleases has clinical therapeutic implications not only for testicular neoplasms but other cancer types. In this study, we used the excision nuclease assay to demonstrate that cisplatin-induced 1,2-d(GpG) intrastrand cross-links are substrates for a human excision repair system derived from the embryonic carcinoma cells, Ntera2, and related genetically modified Ntera2 HMGB4<sup>−/−</sup>. The detection of radiolabeled 25- to 30-nt-long products generated by excinuclease activity supports our repair-shielding hypothesis. The result demonstrates specific inhibition of repair of the 1,2-d(GpG) intrastrand cisplatin cross-link by HMGB4. This result is consistent with our previous report showing that yeast mutants lacking the HMG-domain protein Ixr1 were significantly less sensitive to cisplatin compared with WT cells (18).

Whereas numerous studies have investigated the effect of genotoxic drugs on the NER machinery (7), little work has been done in elucidating the effect of DNA-recognition proteins. We therefore, evaluated the ability of HMGB4 to impart an effect on DNA-repair processes, particularly NER. The structure-specific endonucleases, XPG and ERCC1–XPF, which cleave damaged DNA strands on the 3′ and 5′ sides of the Pt-DNA lesion, respectively, are key excision proteins within the NER pathway. ERCC1 has also been proposed as a predictive marker to assess the therapeutic benefit of cisplatin-based chemotherapy in a personalized medicine setting (44). In TGCTs, up-regulation of ERCC1 and XPF has been associated with disease progression (45). In our quest to understand how HMGB4 affects protein regulation in the NER pathway, we confirmed that the absence of HMGB4 in testicular cancer cells facilitates the action of XPG, ERCC1–XPF endonucleases, as well as XPC, consistent with their up-regulation in gene profiling studies. It is possible that cells lacking HMGB4 readily detect DNA damage and up-regulate other transcription factors for their excision repair.

As already mentioned, recruitment of NER proteins to Pt-DNA damage sites involves TFIH, which has XBP as a principal component. Our examination of Ntera2 and Ntera2 HMGB4<sup>−/−</sup> cells with reduced levels of XBP shows differential sensitivity to cisplatin treatment. This result suggests that the loss of HMGB4 and NER genes, in particular those involved in the initiation steps including XBP, are required for cisplatin sensitivity. However, exclusive loss of HMGB4 in Ntera2 cells is sufficient to induce significant resistance to cisplatin. It would be of interest to analyze patient genomic data from TGCTs treated with cisplatin to determine possible correlation with HMGB4 levels. Experiments of this kind are currently in progress.

In Ntera2 cells, which are cisplatin sensitive relative to Ntera2 HMGB4<sup>−/−</sup>, p-ERK1/2 (phosphorylated extracellular signal-regulated kinase) and p-c-Jun (phosphorylated c-Jun) expression decreased over a 72-h period following cisplatin treatment. Interestingly, basal levels of p-ERK1/2 and p-c-Jun were relatively diminished in Ntera2 HMGB4<sup>−/−</sup> compared with normal Ntera2 cells (SI Appendix, Fig. S9). Additionally, the proapoptotic protein expression for Ntera2 HMGB4<sup>−/−</sup> cells treated with cisplatin was significantly lower than that of the WT cells, whereas the antiapoptotic Bcl-xL protein expression was higher in Ntera2 HMGB4<sup>−/−</sup> than in Ntera2 cells (SI Appendix, Fig. S10). This imbalance supports the differential apoptosis response observed for the TGCT cells under study.

In conclusion, our results are consistent with a repair-shielding model in which HMGB4 recognizes and binds cisplatin-DNA adducts, such as d(GpG) and d(GpGpG), stalling the NER machinery recruited to the damage site, which otherwise would have excised and ultimately repaired the damage. Although we do not rule out transcriptional activity imposed by HMGB4 to regulate genes in the NER pathway, we conclude, based on the present findings, that a significant component of the hypersensitivity of TGCTs to cisplatin is a result of repair shielding by HMGB4. We speculate that HMGB4 may also impart transcriptional activity by regulating NER genes based on the evaluation of mRNA transcripts in Ntera2 or Ntera2 HMGB4<sup>−/−</sup> treated with cisplatin. Consistent with this suggestion is the G2/M accumulation in HMGB4-proficient Ntera2 cells and the relatively lower levels of XPC, XPG, ERCC1, and XPF mRNA transcripts in Ntera2 cells compared with HMGB4<sup>−/−</sup> cells, which leads to significant sensitivity of the former to cisplatin.

Materials and Methods

All cell lines were grown at 37°C under a humidified atmosphere of 5% (vol/vol) CO<sub>2</sub>. The TGCT, Ntera2, and the breast cancer cell line MDA-MB-231 were purchased from American Type Culture Collection. An Ntera2 HMGB4<sup>−/−</sup> cell line was generated via CRISPR/Cas9-mediated KO. Detailed experimental procedures, including gene editing, transfection, cell cycle, apoptosis, and human excision nuclease assays, are provided in SI Appendix.
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