MOLECULAR AND CELLULAR BIOLOGY, May 2006, p. 3935–3941 Vol. 26, No. 10
0270-7306/06/$08.00+0 doi:10.1128/MCB.26.10.3935–3941.2006
Copyright © 2006, American Society for Microbiology. All Rights Reserved.

Early Embryonic Lethality Due to Targeted Inactivation of DNA Ligase III†

Nahum Puebla-Osorio,[1] Devin B. Lacey,[1] Frederick W. Alt,[2] and Chengming Zhu[1]*

Department of Immunology, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, Texas 77030,[1] and The CBR Institute for Biomedical Research, Howard Hughes Medical Institute, Children's Hospital, and Harvard Medical School, Boston, Massachusetts 02115[2]

Received 6 December 2005/Returned for modification 21 December 2005/Accepted 2 March 2006

DNA ligases catalyze the joining of strand breaks in the phosphodiester backbone of duplex DNA and play essential roles in DNA replication, recombination, repair, and maintenance of genomic integrity. Three mammalian DNA ligase genes have been identified, and their corresponding ligases play distinct roles in DNA metabolism. DNA ligase III is proposed to be involved in the repairing of DNA single-strand breaks, but its precise role has not yet been demonstrated directly. To determine its role in DNA repair, cellular growth, and embryonic development, we introduced targeted interruption of the DNA ligase III (LIG3) gene into the mouse. Mice homozygous for LIG3 disruption showed early embryonic lethality. We found that the mutant embryonic developmental process stops at 8.5 days postcoitum (dpc), and excessive cell death occurs at 9.5 dpc. LIG3 mutant cells have relatively normal XRCC1 levels but elevated sister chromatid exchange. These findings indicate that DNA ligase III is involved in essential DNA repair activities required for early embryonic development and therefore cannot be replaced by other DNA ligases.

All eukaryotic organisms have multiple DNA ligases. Three genes, ligase I (LIG1), LIG3, and LIG4, have been identified in vertebrate cells. Although these DNA ligases are related in sequence and structure, each ligase functions distinctly in DNA metabolism (for reviews, see references 11, 32, 36, 44, 47, and 48). DNA ligase I is the key ligase for DNA synthesis (25, 44), whereas DNA ligase IV is required for nonhomologous end-joining and V(D)J recombination during T and B lymphocyte development (1, 12, 14). Both ligases are conserved in all eukaryotes. DNA ligase III, a ligase unique to vertebrates, is thought to function in base excision repair (BER) and other DNA single-stranded break (SSB) repairs (11, 46).

DNA ligase III is found in two isoforms, ligase IIIα and ligase IIIβ, produced by alternative pre-mRNA splicing (7, 18, 52). The two spliced variants of ligase III differ at their C termini (31). DNA ligase IIIα contains a unique BRCT motif, whereas DNA ligase IIIβ is a shorter polypeptide that lacks a BRCT motif. DNA ligase IIIα is ubiquitously expressed, suggesting a role in broader DNA repair processes, while ligase IIIβ is expressed uniquely in pachytene spermatocytes and haploid spermatids of the testes, implying a specific role in meiotic recombination (31). Both forms of DNA ligase III have a putative zinc finger near the N terminus. Similar structures have been found in other proteins such as poly(ADP-ribose) polymerase (PARP), which is known as a sensor for DNA nicks (39). Several studies have demonstrated the interaction between DNA ligase III and PARP1 and PARP2 (3, 24, 35), thus suggesting a role for DNA ligase III in SSB repair (19, 30, 38, 39). DNA ligase III also contains mitochondrion-targeting sequences. By initiating translation at two different AUG codons, DNA ligase III can be targeted to either the nucleus or the mitochondria (22, 23, 34). Therefore, DNA ligase III may also function in the replication and repair of mitochondrial DNA.

A recent study showed DNA ligase III directly interacts with tyrosyl phosphodiesterase 1 in the SSB repair complex, which is important for maintaining the genomic integrity of developing neurons (9). Furthermore, DNA ligase III may act as a backup ligase in the repair of DNA double-stranded breaks (50), especially when the main ligase for the nonhomologous end-joining pathways, ligase IV, is deficient. Also, the LIG3 gene likely encodes DNA ligase II, a proteolytic product derived from DNA ligase III, found to be active in the liver (18, 42, 51).

All DNA ligases function together with its "partner" protein: ligase I associates with replication factor proliferating cell nuclear antigen (PCNA) (25, 45), and ligase IV associates with XRCC4 (13–15, 37). Ligase IIIα associates with DNA repair protein XRCC1 (4, 5, 33) via BRCT motifs (20, 56). A study showed that a mutation in the BRCT motif of XRCC1 abolishes the SSB DNA repair activity of the protein, specifically at the G_1 phase of the cell cycle (38). XRCC1-deficient cells have a decreased level of DNA ligase III compared to normal cells and are hypersensitive to many DNA-damaging agents, particularly alkylating agents. XRCC1 mutant cells also show an increased level of sister chromatid exchanges (SCE), which demonstrate a general status of DNA instability, and are defective in joining SSBs (5, 6, 27, 43). All these results strongly suggest the DNA ligase III and XRCC1 complex plays an important role in the repair of SSBs.

Besides its association with DNA ligase III, XRCC1 is able to interact with many additional proteins, such as DNA polymerase β (pol β), PARP1, PARP2, polynucleotide kinase (3, 16, 21, 53), PCNA (10), and major apyrimidinic endonuclease (APE) (49). This indicates XRCC1 is the centerpiece for hold-

* Corresponding author. Mailing address: Department of Immunology, Unit 902, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030. Phone: (713) 563-3255. Fax: (713) 563-3357. E-mail: czhu@mdanderson.org.

† Supplemental material for this article may be found at http://mcb.asm.org/.

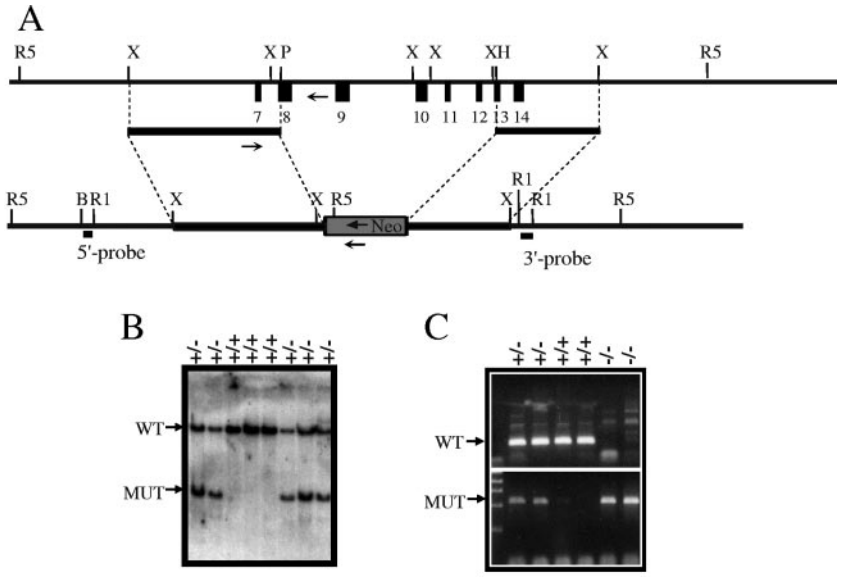

FIG. 1. Targeted disruption of murine DNA ligase III. (A) Partial genomic structure of the mouse LIG3 gene. There is a total of 20 exons in this gene; exons 7 to 14 are shown. A 5.3-kb fragment containing exons 8 to 13 is replaced by a neo-resistant gene in a reversed orientation. Probes (both 3′ and 5′) used for genotyping by Southern blotting are shown, and the primers for PCR genotyping are marked with arrows. Restriction sites on the map: X, XbaI; P, PstI; H, HindIII; R1, EcoRI; R5, EcoRV. (B) Results of genotyping of ES cells by Southern blotting. WT, germ line band of 19.3 kb. MUT, targeted band of 7.4 kb identified by the 3′ probe. (C) Results of genotyping of embryos by PCR. Primers specific for germ line sequences and targeted mutant sequences are shown.

ing together protein complexes in SSB repair (2) or for assembling multiple complexes and therefore functioning independently of DNA ligase III.

Currently, LIG3-deficient mice or cells have not yet been described, but it has been shown that deficiency of XRCC1 leads to early embryonic lethality at 6.5 days postcoitum (dpc) through 7.5 dpc (40, 41). One can predict that LIG3-deficient mice have a similar phenotype. Here, we report the first mutation of the LIG3 gene by gene targeting. We found mutant mice homozygous for LIG3 inactivation are indeed early embryonic lethal but survive slightly longer than XRCC1-deficient embryos.

MATERIALS AND METHODS

Construction of an LIG3-targeting vector. A targeting vector was designed on the basis of known cDNA sequences, genomic library screening, and known gene structure. A 5.3-kb fragment containing exons 8 to 13, which includes the active site for ligase III, was replaced with the neomycin phosphotransferase (neo)-resistant gene in the opposite transcriptional orientation (Fig. 1A).

Generation of LIG3-deficient embryonic stem cells and mice. The targeting construct was transfected into embryonic stem (ES) cells via electroporation, and homologous recombinant ES cells were screened by Southern blotting using probes that are 5′ and 3′ to the targeted arms (Fig. 1A) and the probe within the neo-resistant gene. Positive ES colonies were then subcloned and karyotyped for chromosomal counting. Targeted ES cells with normal chromosomes were injected into blastocysts to generate chimeric mice. Male chimeric mice were bred with 129sv females to generate heterozygous mice. The experiments involving the animal subjects were performed according to M. D. Anderson Cancer Center animal use protocol guidelines and were approved by the institutional animal care and use committee.

Southern blotting, Western blotting, and PCR genotyping. DNA samples from ES cells or mouse tails were digested with EcoRV, and Southern blot analyses were performed with 5′, 3′, and neo probes (Fig. 1B). Western blot analysis was performed in extracts obtained from 8.5-dpc mutant, heterozygous, and wild-type embryos with primary antibodies against ligase III (Transduction Labs, San Jose, CA) and XRCC1 (Bethyl Laboratories, Montgomery, TX). For PCR genotyping, primers 5′ and 3′ to the replaced regions together with primers from the neo-resistant gene region were used to detect targeted mutations. PCR primers within the replaced region were used to detect the wild-type allele (Fig. 1C). Primer sequences were as follows: neo-resistant primer CZ43, CGCAGCGCATCGCCT TCTATCGC; 5′ primer CZ58, CGGCTGATCAAACATGATCTGAAGATG; 5′ replaced primer CZ94, CAGGCGTCATCAGTGAGG CCTGGAC.

RNA interference. A pair of complementary 56-mer oligonucleotides were synthesized, annealed, and ligated into pSuppressor-Neo vector (Imgenex, San Diego, CA). This small interfering RNA system is able to produce a small hairpin RNA with an 8-nucleotide loop and a 19-nucleotide double-stranded region matching the human DNA ligase III gene near the 5′ end. Small interfering RNA vector DNA with or without inserts was introduced into HeLa cells by electroporation. The cells were selected in G418, and the knockdown cells were screened by Western blotting. The sense strand of oligonucleotide (CZ125) sequence is 5′-TCGAGTCCTAGCTACAACACGAAGGAGTACTGCTTCGT GTTGTAGCTAGGATTTTT.

Histological analysis. After dissection, mutant and wild-type embryos were fixed in paraformaldehyde and embedded in paraffin. Samples were then sectioned, mounted onto positively charged glass slides, and stained with hematoxylin and eosin.

TUNEL assays. Terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL; Promega, Madison, WI) was performed on embryonic tissue, including a DNase-treated positive control. Briefly, tissue sections were pretreated with proteinase K and DNA strand breaks were labeled with fluorescein-12-dUTP. The green fluorescence of apoptotic cells in a red background (propidium iodide) was detected by fluorescence microscopy.

SCE assay. Embryos (8.5 dpc) from heterozygous breeding were cultured in the presence of bromodeoxyuridine (BrdU) for 48 h. After 2 h of Colcemid (Gibco BRL) incubation, the embryos were placed in hypotonic solution, fixed, and spread onto slides. The slides were stained with Hoechst 33258 and exposed to UV light. The slides were stained with Giemsa, mounted with antifading agent and 4′,6′-diamidino-2-phenylindole (DAPI; Vector), and analyzed using fluorescence microscopy. The average SCE per chromosome was determined as a ratio of the number of SCEs to the total number of chromosomes.

RESULTS

Targeted disruption of the LIG3 gene. To disrupt the LIG3 gene we designed a targeting vector, as illustrated in Fig. 1A. The 5.3-kb PstI-HindIII fragment containing six exons of LIG3 encoding amino acid residues 451 to 553, including the con-

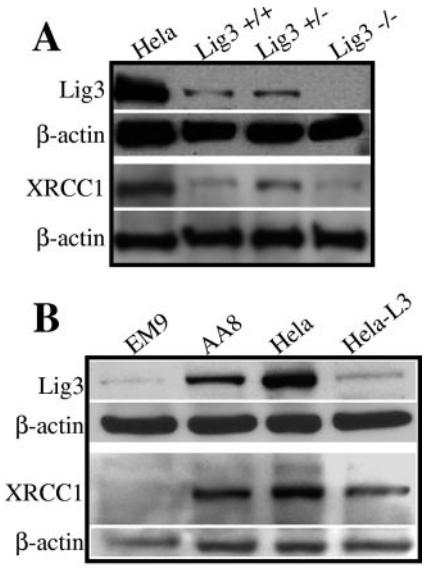

FIG. 2. Western blotting analysis. (A) Extracts from 8.5-dpc embryos with different genotypes were detected for their DNA ligase III and XRCC1 expression, with HeLa cell extract as a control. (B) Detection of DNA ligase III and XRCC1 in cell extracts from XRCC1-deficient EM9 cells and its wild-type control, AA8, as well as LIG3 knockdown cells and its control, HeLa cells.

served active site (KYDGER, residues 512 to 517), was replaced with the neo gene under the control of the phosphoglycerate kinase promoter. Mouse ES cells were transfected with the targeting vector, and neo-resistant clones were selected. ES cell colonies were screened by Southern blotting using an external 3′ probe (Fig. 1B); positive colonies were confirmed with 5′ and internal neo probes. Six positive ES colonies were selected and confirmed, five of which showed normal chromosomal counts by karyotyping (data not shown). After injecting positive targeted cell lines into host blastocysts, chimeric male mice were generated from all three targeted ES cell lines. The chimeric mice from each ES cell line were then bred to wild-type female mice, and all transmitted the targeted mutation to their F_1 progenies, confirmed by PCR genotyping (Fig. 1C). Heterozygous offspring from these breedings were phenotypically indistinguishable from each other.

Western blotting analysis was performed to determine that the targeted disruption of LIG3 generates a null mutation. As demonstrated in Fig. 2A, anti-DNA ligase III antibody clearly detected DNA ligase III from HeLa cells as well as wild-type and LIG3$^{+/-}$ embryos, but DNA ligase III was undetectable in LIG3$^{-/-}$ embryos, indicating that indeed the targeted inactivation of LIG3 generates a null mutation.

Normal XRCC1 expression in LIG3-deficient cells. XRCC1 is a known DNA ligase III cofactor and is required for the stabilization of DNA ligase III. We next asked whether the XRCC1 level is altered in DNA ligase III-deficient cells. Western blotting analysis of 8.5-dpc embryos revealed similar XRCC1 protein levels in DNA ligase III null cells (Fig. 2A). To confirm this result, we made DNA ligase III knockdown cells using RNA interference in HeLa cells to significantly reduce the DNA ligase III expression (Fig. 2B). Consistent with the mouse knockout results, the XRCC1 level is compatible with normal controls in LIG3 knockdown cells (Fig. 2B). As a control, XRCC1-deficient EM9 cells do not exhibit XRCC1 expression and show a dramatic reduction of DNA ligase III (Fig. 2B). Our results indicate that although XRCC1 is required to stabilize DNA ligase III, DNA ligase III deficiency does not affect XRCC1 stabilization and expression.

Inactivation of the DNA ligase III gene and resultant early embryonic lethality. Mice heterozygous for the LIG3 mutation were fertile and did not display phenotypic abnormalities. Additionally, no evidence of early susceptibility to cancer or any other disease was observed in these mice within the first 20 months of life. Our attempts to breed heterozygous mice to generate homozygous LIG3 mutant mice yielded no viable LIG3$^{-/-}$ pups (Table 1) in nearly 800 young mice genotyped by Southern blotting or PCR. These results clearly demonstrate DNA ligase III is essential for mouse embryonic development and survival.

To further determine the effects of LIG3 gene inactivation on embryonic development, embryos or yolk sacs were collected from heterozygous mice crossbred at different days of gestation and genotyped by PCR. We first dissected 48 embryos at 7.5 dpc from six different pregnant female mice (Table 1). Eight of the 48 embryos (16.7%) were homozygous for the LIG3 mutation, 6 (12.5%) were wild type, and 34 (70.8%) were heterozygous for the LIG3 mutation. Morphologically, 7.5-dpc mutant embryos were very similar to the wild-type and heterozygous LIG3 embryos (see Fig. S1 in the supplemental material), with the exception that the mutant embryos were generally smaller (approximately one-third the normal size). At 8.5 dpc, we dissected 14 homozygous mutant embryos from a total of 128 embryos (10.9%), or less than 25% of what was expected, according to Mendelian segregation. In contrast, the numbers of both wild-type (32%) and heterozygous (57%) embryos were higher than expected. Despite their lower numbers and smaller sizes, surviving mutant embryos at 8.5 dpc were morphologically similar to the wild-type embryos (see Fig. S1 in the supplemental material). At 9.5 dpc, we were able to dissect 10 homozygous mutant embryos from a total of 79 embryos (12.6%), and these mutant embryos were very different morphologically from normal 9.5-dpc embryos; the mutant embryos remained in the dorsal curvature, resembling 8.5-dpc rather than normal 9.5-dpc embryos. In contrast, the 9.5-dpc wild-type and heterozygous LIG3 embryos all had ventral curvatures (Fig. 3A). This indicated that mutant embryonic development stopped at 8.5 dpc. At 10.5 dpc, resorption and empty deciduae were observed at a relatively higher frequency,

TABLE 1. Early embryonic lethality of DNA ligase III-deficient mice[a]

Developmental status (no.) of mice[c]	No. expected/no. observed for genotype:		
	+/+	+/−	−/−
Live born (782)	195.5/248	391/534	195.5/0
E10.5 (57)	14.25/20	28.5/28	14.25/6 + 3[b]
E9.5 (79)	19.75/23	39.5/46	19.75/10
E8.5 (128)	32/41	64/73	32/14
E7.5 (48)	12/6	24/34	12/8

[a] Expected and observed genotypes of live-born pups and embryos from heterozygous mating.
[b] Three had empty deciduae.
[c] E10.5, embryonic day 10.

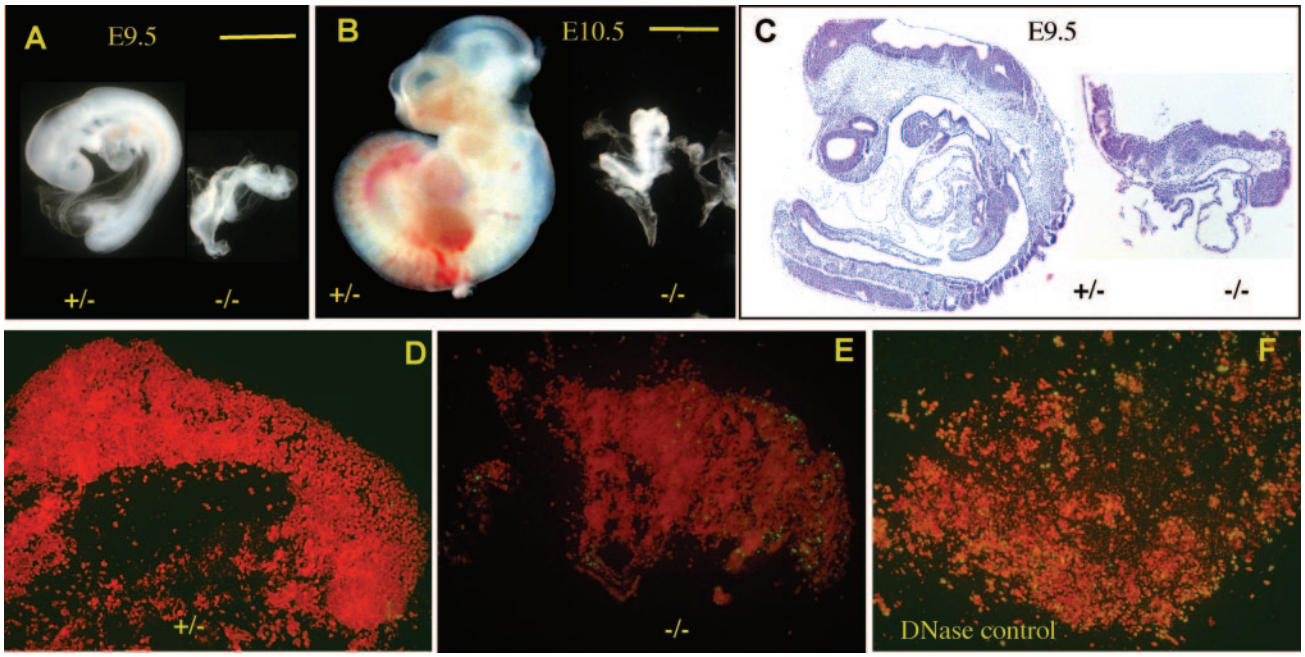

FIG. 3. LIG3 deficiency and the resultant disruption of embryonic development at 8.5 dpc and excessive apoptosis at 9.5 dpc. A morphological comparison of wild-type (left) and mutant (right) embryos at 9.5 dpc (A) and 10.5 dpc (B). Yellow bars, 1 mm. (C) Section of wild-type (left) and mutant (right) embryos at 9.5 dpc stained with hematoxylin and eosin. TUNEL assays were performed on sections of wild-type (D) and mutant (E) embryos. Apoptotic cells are visible as green fluorescence, and nuclei are labeled with phosphatidylinositol staining. As a positive control, a section of wild-type embryos was treated with DNase I prior to the TUNEL assay (F).

and mutant embryos were dramatically different from normal embryos. Remarkably, these embryos remained in the dorsal curvature and had the size of 8.5-dpc embryos (Fig. 3B). Therefore, our findings demonstrate the LIG3 gene, like its associated factor XRCC1, is essential for early embryonic development. XRCC1 is critical for gastrulation, and its deficiency results in embryonic mortality at 7.5 dpc (40). However, LIG3-deficient embryos die after gastrulation on 9.5 through 10.5 dpc, indicating disruption of embryonic development at 8.5 dpc. Together, our results imply XRCC1 plays an additional role in early embryonic development independent of DNA ligase III, especially during gastrulation.

Elevated level of SCE in DNA ligase III-deficient cells. The hallmark of XRCC1-deficient cells is an increased level of SCE. We asked the extent to which the SCE level is also elevated in DNA ligase III-deficient cells. After being cultured in BrdU for 48 h, 8.5-dpc embryos were harvested to make chromosomal spreads. Several spreads were scored from wild-type embryos, but only a few were visible from mutant embryos (see Fig. S2 in the supplemental material). This is likely the result of poor growth of mutant embryos and limited uptake of BrdU. We were not able to score the actual number of SCE due to the poor quality of the image, although we did observe abnormal SCE from mutant embryos, as demonstrated in Fig. S2 in the supplemental material. To further confirm this observation, additional SCE assays were performed in DNA ligase III knockdown HeLa cells. Compared to vector control HeLa cells, an increased level of SCE was clearly observed in LIG3 knockdown cells (Fig. 4). We randomly scored 25 chromosomal spreads from vector control HeLa cells and LIG3 knockdown cells, and the average SCE per chromosome was 0.24 and 0.78, respectively. Therefore, we conclude that DNA ligase III deficiency also leads to abnormal sister chromatid exchange.

LIG3 deficiency results in excessive apoptosis at 9.5 dpc. To further characterize the LIG3 mutant cells, we attempted to culture 9.5-dpc embryos to obtain embryonic fibroblasts. Although these cells were easily grown from wild-type and heterozygous embryos, homozygous mutant embryos did not produce fibroblasts in three different attempts using six mutant embryos. We reasoned that at 9.5 dpc, embryonic development stops and many cells start to die, most likely due to the accumulation of unrepaired DNA damage. To further determine cell survival at 9.5 dpc in mutant embryos, we examined the level of apoptotic cells in the mutant embryos by using a TUNEL assay. As shown in Fig. 3E, the number of apoptotic cells in the mutant embryos was significantly higher than that of the wild-type or heterozygous embryos, indicating that at 9.5 dpc homozygous mutant embryos have undergone extensive apoptosis.

DISCUSSION

In this report, we described the first targeted knockout of the DNA ligase III gene; our results reveal DNA ligase III is essential for early embryonic development in the mouse. Interruption of embryonic development in LIG3 mutants occurs at 8.5 dpc, followed by massive apoptosis at 9.5 dpc and ultimately death, which is likely a result of unrepaired DNA damage. Consistent with this notion, we found the LIG3-deficient cells showed an elevated SCE level. Our results also demonstrate the LIG3 deficiency does not affect the stability of

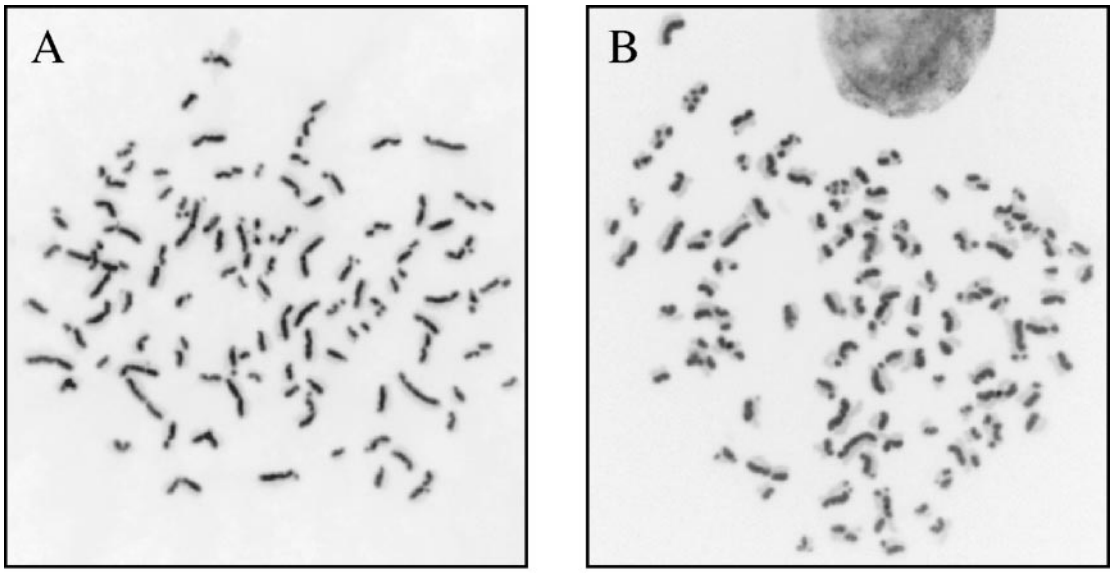

FIG. 4. SCE assays. Depicted are HeLa cells (A) and their counterpart LIG3 knockdown cells (B) showing elevated levels of SCE in LIG3 knockdown cells.

XRCC1, and LIG3-deficient embryos are able to develop into later stages than those of XRCC1-deficient embryos. This indicates that in addition to being a member of the DNA ligase III complex, XRCC1 also works independently of the LIG3 gene, and this function is essential for gastrulation. Furthermore, LIG3-deficient embryos die earlier than all other DNA ligase-deficient embryos, establishing the critical role DNA ligase III plays during the early embryonic development, demonstrating that its unique function cannot be performed by other DNA ligases.

Some DNA repair activities, such as BER, are essential for the early development of the embryo and its subsequent survival. Base excision repair is the major pathway for the repair of DNA base damage caused by genotoxins, such as alkylating and oxidizing agents. This pathway is initiated by the removal of damaged bases by DNA glycosylase, followed by the generation of SSB by APE1, and finally repaired by the SSB repair machinery, which includes XRCC1, PARP-1, pol β, and DNA ligase III (2). In addition, oxidative agents may also damage deoxyribose, which will lead to SSBs. Targeted inactivation of key components of BER, such as XRCC1, APE1, or pol β, results in early embryonic mortality from 5.5 to 7.5 dpc (17, 28, 40, 55), suggesting that embryonic cells suffering from DNA damage during normal development can only be repaired by BER. Other DNA repair genes associated with early embryonic mortality due to deficiency include XPD and the homologous recombination factors Rad51, Brca1, and Brca2 (8, 26, 29). Together, these findings imply that base modification and strand breaks occur frequently during early embryonic development. The most probable source of this DNA damage is oxidizing agents, which are produced by normal metabolic processes and are known to be able to modify bases as well as attack deoxyribose, both of which will lead to SSBs. Deficiency in DNA repair leads to the accumulation of DNA damage, activating cellular responses resulting in DNA repair and cellular survival.

Remarkably, other DNA repair pathways are not as critical for embryonic development in the early stages. Such pathways include nucleotide excision repair, DNA mismatch repair, and nonhomologous end joining, all of which are important for late embryonic development and survival. This indicates either certain types of DNA damage occur more frequently during early stages of development or redundant repair pathways exist for certain types of damage.

Our findings suggest that a deficiency in BER activity allows the accumulation of damaged cells to reach a critical level, thus halting embryonic development and leading to the death of the embryo. As we observed, LIG3-deficient embryos die approximately at 8.5 dpc, which is a later stage than XRCC1-deficient embryos, most likely due to DNA ligase functioning in the last step of the repair process. Therefore, the initial responses to DNA damage, such as the activation of the protein XRCC1 and other early factors, may still be intact and able to initiate repair of damaged DNA. This may also delay cell death, while normal embryonic development continues to a limited extent. It is further of note that all embryonic development stops at 8.5 dpc in all $LIG3^{-/-}$ embryos, indicating this critical developmental stage is absolutely sensitive to the accumulation of DNA damage in cells.

In addition to the aforementioned role in the maintenance of nuclear genomic integrity by repairing damaged DNA, the ligases possess the ability to maintain the mitochondrial genome. The mitochondrial form of DNA ligase is generated by alternative initiation of translation to the product that carries mitochondrial targeting sequences. Examples include the yeast CDC9 gene, which is a DNA ligase I homolog (54), and the LIG3 gene in mice and humans (22). Antisense-RNA experiments have shown ligase III as an important factor in repairing DNA breaks and in maintaining DNA content in mitochondria (22, 23). These studies suggest ligase III-deficient cells lack the ability for repairing DNA and thus have unstable mitochondria. It may also explain the early embryonic mortality of

LIG3$^{-/-}$ embryos and the inability to establish a ligase III-deficient cell line (LIG3$^{-/-}$ cells). However, our results show it is unlikely DNA ligase III is required for cell viability, despite failure to generate null ES cells screened by high G418 selection or second-allele targeting (results not shown) and/or by growing mutant mouse blastocysts from heterozygous crossbreeding (data not shown). We did not observe 8.5-dpc LIG3$^{-/-}$ embryos displaying elevated levels of apoptosis (data not shown) but found embryos showing increased levels of apoptosis at 9.5 through 10.5 dpc, thus suggesting embryonic development continues until 8.5 dpc. We argue that LIG3$^{-/-}$ embryos failing to develop beyond 8.5 dpc might be due to accumulation of unrepaired DNA damage rather than due to unstable mitochondrial DNA. Abnormal SCE observed in LIG3-deficient cells supports this notion. Taken together, our results demonstrate for the first time that the targeted inactivation of DNA ligase III leads to early embryonic mortality.

ACKNOWLEDGMENTS

We thank Laurie Davidson and Dan Foy for their assistance with the mice, Hui Pang for her technical assistance, Melanie Dujka for editing, and Miles Wilkinson, Randy Legerski, Philip Carpenter, and Allen McDonald for their critical reading of the manuscript.

This work was partially supported by a Cancer Research Institution fellowship, an institutional grant from The University of Texas M. D. Anderson Cancer Center, and a grant from the Leukemia Research Foundation (to C.Z.). F.W.A. is a Howard Hughes Medical Institute Investigator. This work is partially supported by NIH grant AI35714 (to F.W.A.).

REFERENCES

1. **Barnes, D. E., G. Stamp, I. Rosewell, A. Denzel, and T. Lindahl.** 1998. Targeted disruption of the gene encoding DNA ligase IV leads to lethality in embryonic mice. Curr. Biol. **8:**1395–1398.
2. **Caldecott, K. W.** 2001. Mammalian DNA single-strand break repair: an X-ra(y)ted affair. Bioessays **23:**447–455.
3. **Caldecott, K. W., S. Aoufouchi, P. Johnson, and S. Shall.** 1996. XRCC1 polypeptide interacts with DNA polymerase beta and possibly poly (ADP-ribose) polymerase, and DNA ligase III is a novel molecular 'nick-sensor' in vitro. Nucleic Acids Res. **24:**4387–4394.
4. **Caldecott, K. W., C. K. McKeown, J. D. Tucker, S. Ljungquist, and L. H. Thompson.** 1994. An interaction between the mammalian DNA repair protein XRCC1 and DNA ligase III. Mol. Cell. Biol. **14:**68–76.
5. **Caldecott, K. W., J. D. Tucker, L. H. Stanker, and L. H. Thompson.** 1995. Characterization of the XRCC1-DNA ligase III complex in vitro and its absence from mutant hamster cells. Nucleic Acids Res. **23:**4836–4843.
6. **Cappelli, E., R. Taylor, M. Cevasco, A. Abbondandolo, K. Caldecott, and G. Frosina.** 1997. Involvement of XRCC1 and DNA ligase III gene products in DNA base excision repair. J. Biol. Chem. **272:**23970–23975.
7. **Chen, J., A. E. Tomkinson, W. Ramos, Z. B. Mackey, S. Danehower, C. A. Walter, R. A. Schultz, J. M. Besterman, and I. Husain.** 1995. Mammalian DNA ligase III: molecular cloning, chromosomal localization, and expression in spermatocytes undergoing meiotic recombination. Mol. Cell. Biol. **15:**5412–5422.
8. **de Boer, J., I. Donker, J. de Wit, J. H. Hoeijmakers, and G. Weeda.** 1998. Disruption of the mouse xeroderma pigmentosum group D DNA repair/basal transcription gene results in preimplantation lethality. Cancer Res. **58:**89–94.
9. **El-Khamisy, S. F., G. M. Saifi, M. Weinfeld, F. Johansson, T. Helleday, J. R. Lupski, and K. W. Caldecott.** 2005. Defective DNA single-strand break repair in spinocerebellar ataxia with axonal neuropathy-1. Nature **434:**108–113.
10. **Fan, J., M. Otterlei, H. K. Wong, A. E. Tomkinson, and D. M. Wilson, III.** 2004. XRCC1 co-localizes and physically interacts with PCNA. Nucleic Acids Res. **32:**2193–2201.
11. **Fan, J., and D. M. Wilson, III.** 2005. Protein-protein interactions and post-translational modifications in mammalian base excision repair. Free Radic. Biol. Med. **38:**1121–1138.
12. **Frank, K. M., J. M. Sekiguchi, K. J. Seidl, W. Swat, G. A. Rathbun, H. L. Cheng, L. Davidson, L. Kangaloo, and F. W. Alt.** 1998. Late embryonic lethality and impaired V(D)J recombination in mice lacking DNA ligase IV. Nature **396:**173–177.
13. **Grawunder, U., M. Wilm, X. Wu, P. Kulesza, T. E. Wilson, M. Mann, and M. R. Lieber.** 1997. Activity of DNA ligase IV stimulated by complex formation with XRCC4 protein in mammalian cells. Nature **388:**492–495.
14. **Grawunder, U., D. Zimmer, S. Fugmann, K. Schwarz, and M. R. Lieber.** 1998. DNA ligase IV is essential for V(D)J recombination and DNA double-strand break repair in human precursor lymphocytes. Mol. Cell **2:**477–484.
15. **Grawunder, U., D. Zimmer, P. Kulesza, and M. R. Lieber.** 1998. Requirement for an interaction of XRCC4 with DNA ligase IV for wild-type V(D)J recombination and DNA double-strand break repair in vivo. J. Biol. Chem. **273:**24708–24714.
16. **Gryk, M. R., A. Marintchev, M. W. Maciejewski, A. Robertson, S. H. Wilson, and G. P. Mullen.** 2002. Mapping of the interaction interface of DNA polymerase beta with XRCC1. Structure (Cambridge) **10:**1709–1720.
17. **Gu, H., J. D. Marth, P. C. Orban, H. Mossmann, and K. Rajewsky.** 1994. Deletion of a DNA polymerase beta gene segment in T cells using cell type-specific gene targeting. Science **265:**103–106.
18. **Husain, I., A. E. Tomkinson, W. A. Burkhart, M. B. Moyer, W. Ramos, Z. B. Mackey, J. M. Besterman, and J. Chen.** 1995. Purification and characterization of DNA ligase III from bovine testes. Homology with DNA ligase II and vaccinia DNA ligase. J. Biol. Chem. **270:**9683–9690.
19. **Ikejima, M., S. Noguchi, R. Yamashita, T. Ogura, T. Sugimura, D. M. Gill, and M. Miwa.** 1990. The zinc fingers of human poly(ADP-ribose) polymerase are differentially required for the recognition of DNA breaks and nicks and the consequent enzyme activation. Other structures recognize intact DNA. J. Biol. Chem. **265:**21907–21913.
20. **Krishnan, V. V., K. H. Thornton, M. P. Thelen, and M. Cosman.** 2001. Solution structure and backbone dynamics of the human DNA ligase IIIalpha BRCT domain. Biochemistry **40:**13158–13166.
21. **Kubota, Y., R. A. Nash, A. Klungland, P. Schar, D. E. Barnes, and T. Lindahl.** 1996. Reconstitution of DNA base excision-repair with purified human proteins: interaction between DNA polymerase beta and the XRCC1 protein. EMBO J. **15:**6662–6670.
22. **Lakshmipathy, U., and C. Campbell.** 1999. The human DNA ligase III gene encodes nuclear and mitochondrial proteins. Mol. Cell. Biol. **19:**3869–3876.
23. **Lakshmipathy, U., and C. Campbell.** 2000. Mitochondrial DNA ligase III function is independent of Xrcc1. Nucleic Acids Res. **28:**3880–3886.
24. **Leppard, J. B., Z. Dong, Z. B. Mackey, and A. E. Tomkinson.** 2003. Physical and functional interaction between DNA ligase IIIα and poly(ADP-ribose) polymerase 1 in DNA single-strand break repair. Mol. Cell. Biol. **23:**5919–5927.
25. **Levin, D. S., A. E. McKenna, T. A. Motycka, Y. Matsumoto, and A. E. Tomkinson.** 2000. Interaction between PCNA and DNA ligase I is critical for joining of Okazaki fragments and long-patch base-excision repair. Curr. Biol. **10:**919–922.
26. **Lim, D. S., and P. Hasty.** 1996. A mutation in mouse rad51 results in an early embryonic lethal that is suppressed by a mutation in p53. Mol. Cell. Biol. **16:**7133–7143.
27. **Ljungquist, S., K. Kenne, L. Olsson, and M. Sandstrom.** 1994. Altered DNA ligase III activity in the CHO EM9 mutant. Mutat. Res. **314:**177–186.
28. **Ludwig, D. L., M. A. MacInnes, Y. Takiguchi, P. E. Purtymun, M. Henrie, M. Flannery, J. Meneses, R. A. Pedersen, and D. J. Chen.** 1998. A murine AP-endonuclease gene-targeted deficiency with post-implantation embryonic progression and ionizing radiation sensitivity. Mutat. Res. **409:**17–29.
29. **Ludwig, T., D. L. Chapman, V. E. Papaioannou, and A. Efstratiadis.** 1997. Targeted mutations of breast cancer susceptibility gene homologs in mice: lethal phenotypes of Brca1, Brca2, Brca1/Brca2, Brca1/p53, and Brca2/p53 nullizygous embryos. Genes Dev. **11:**1226–1241.
30. **Mackey, Z. B., C. Niedergang, J. M. Murcia, J. Leppard, K. Au, J. Chen, G. de Murcia, and A. E. Tomkinson.** 1999. DNA ligase III is recruited to DNA strand breaks by a zinc finger motif homologous to that of poly(ADP-ribose) polymerase. Identification of two functionally distinct DNA binding regions within DNA ligase III. J. Biol. Chem. **274:**21679–21687.
31. **Mackey, Z. B., W. Ramos, D. S. Levin, C. A. Walter, J. R. McCarrey, and A. E. Tomkinson.** 1997. An alternative splicing event which occurs in mouse pachytene spermatocytes generates a form of DNA ligase III with distinct biochemical properties that may function in meiotic recombination. Mol. Cell. Biol. **17:**989–998.
32. **Martin, I. V., and S. A. MacNeill.** 2002. ATP-dependent DNA ligases. Genome Biol. **3:**3005.1–3005.7.
33. **Nash, R. A., K. W. Caldecott, D. E. Barnes, and T. Lindahl.** 1997. XRCC1 protein interacts with one of two distinct forms of DNA ligase III. Biochemistry **36:**5207–5211.
34. **Perez-Jannotti, R. M., S. M. Klein, and D. F. Bogenhagen.** 2001. Two forms of mitochondrial DNA ligase III are produced in Xenopus laevis oocytes. J. Biol. Chem. **276:**48978–48987.
35. **Schreiber, V., J. C. Ame, P. Dolle, I. Schultz, B. Rinaldi, V. Fraulob, J. Menissier-de Murcia, and G. de Murcia.** 2002. Poly(ADP-ribose) polymerase-2 (PARP-2) is required for efficient base excision DNA repair in association with PARP-1 and XRCC1. J. Biol. Chem. **277:**23028–23036.
36. **Shuman, S., and B. Schwer.** 1995. RNA capping enzyme and DNA ligase: a superfamily of covalent nucleotidyl transferases. Mol. Microbiol. **17:**405–410.
37. **Sibanda, B. L., S. E. Critchlow, J. Begun, X. Y. Pei, S. P. Jackson, T. L.**

Blundell, and L. Pellegrini. 2001. Crystal structure of an Xrcc4-DNA ligase IV complex. Nat. Struct. Biol. **8:**1015–1019.

38. **Taylor, R. M., D. J. Moore, J. Whitehouse, P. Johnson, and K. W. Caldecott.** 2000. A cell cycle-specific requirement for the XRCC1 BRCT II domain during mammalian DNA strand break repair. Mol. Cell. Biol. **20:**735–740.
39. **Taylor, R. M., J. Whitehouse, E. Cappelli, G. Frosina, and K. W. Caldecott.** 1998. Role of the DNA ligase III zinc finger in polynucleotide binding and ligation. Nucleic Acids Res. **26:**4804–4810.
40. **Tebbs, R. S., M. L. Flannery, J. J. Meneses, A. Hartmann, J. D. Tucker, L. H. Thompson, J. E. Cleaver, and R. A. Pedersen.** 1999. Requirement for the Xrcc1 DNA base excision repair gene during early mouse development. Dev. Biol. **208:**513–529.
41. **Tebbs, R. S., L. H. Thompson, and J. E. Cleaver.** 2003. Rescue of Xrcc1 knockout mouse embryo lethality by transgene-complementation. DNA Repair (Amsterdam) **2:**1405–1417.
42. **Teraoka, H., T. Sumikawa, and K. Tsukada.** 1986. Purification of DNA ligase II from calf thymus and preparation of rabbit antibody against calf thymus DNA ligase II. J. Biol. Chem. **261:**6888–6892.
43. **Thompson, L. H., K. W. Brookman, N. J. Jones, S. A. Allen, and A. V. Carrano.** 1990. Molecular cloning of the human XRCC1 gene, which corrects defective DNA strand break repair and sister chromatid exchange. Mol. Cell. Biol. **10:**6160–6171.
44. **Timson, D. J., M. R. Singleton, and D. B. Wigley.** 2000. DNA ligases in the repair and replication of DNA. Mutat. Res. **460:**301–318.
45. **Tom, S., L. A. Henricksen, M. S. Park, and R. A. Bambara.** 2001. DNA ligase I and proliferating cell nuclear antigen form a functional complex. J. Biol. Chem. **276:**24817–24825.
46. **Tomkinson, A. E., L. Chen, Z. Dong, J. B. Leppard, D. S. Levin, Z. B. Mackey, and T. A. Motycka.** 2001. Completion of base excision repair by mammalian DNA ligases. Prog. Nucleic Acid Res. Mol. Biol. **68:**151–164.
47. **Tomkinson, A. E., and D. S. Levin.** 1997. Mammalian DNA ligases. Bioessays **19:**893–901.
48. **Tomkinson, A. E., and Z. B. Mackey.** 1998. Structure and function of mammalian DNA ligases. Mutat. Res. **407:**1–9.
49. **Vidal, A. E., S. Boiteux, I. D. Hickson, and J. P. Radicella.** 2001. XRCC1 coordinates the initial and late stages of DNA abasic site repair through protein-protein interactions. EMBO J. **20:**6530–6539.
50. **Wang, H., B. Rosidi, R. Perrault, M. Wang, L. Zhang, F. Windhofer, and G. Iliakis.** 2005. DNA ligase III as a candidate component of backup pathways of nonhomologous end joining. Cancer Res. **65:**4020–4030.
51. **Wang, Y. C., W. A. Burkhart, Z. B. Mackey, M. B. Moyer, W. Ramos, I. Husain, J. Chen, J. M. Besterman, and A. E. Tomkinson.** 1994. Mammalian DNA ligase II is highly homologous with vaccinia DNA ligase. Identification of the DNA ligase II active site for enzyme-adenylate formation. J. Biol. Chem. **269:**31923–31928.
52. **Wei, Y. F., P. Robins, K. Carter, K. Caldecott, D. J. Pappin, G. L. Yu, R. P. Wang, B. K. Shell, R. A. Nash, P. Schar, D. E. Barnes, W. A. Haseltine, and T. Lindahl.** 1995. Molecular cloning and expression of human cDNAs encoding a novel DNA ligase IV and DNA ligase III, an enzyme active in DNA repair and recombination. Mol. Cell. Biol. **15:**3206–3216.
53. **Whitehouse, C. J., R. M. Taylor, A. Thistlethwaite, H. Zhang, F. Karimi-Busheri, D. D. Lasko, M. Weinfeld, and K. W. Caldecott.** 2001. XRCC1 stimulates human polynucleotide kinase activity at damaged DNA termini and accelerates DNA single-strand break repair. Cell **104:**107–117.
54. **Willer, M., M. Rainey, T. Pullen, and C. J. Stirling.** 1999. The yeast CDC9 gene encodes both a nuclear and a mitochondrial form of DNA ligase I. Curr. Biol. **9:**1085–1094.
55. **Xanthoudakis, S., R. J. Smeyne, J. D. Wallace, and T. Curran.** 1996. The redox/DNA repair protein, Ref-1, is essential for early embryonic development in mice. Proc. Natl. Acad. Sci. USA **93:**8919–8923.
56. **Zhang, X., S. Morera, P. A. Bates, P. C. Whitehead, A. I. Coffer, K. Hainbucher, R. A. Nash, M. J. Sternberg, T. Lindahl, and P. S. Freemont.** 1998. Structure of an XRCC1 BRCT domain: a new protein-protein interaction module. EMBO J. **17:**6404–6411.