Meiotic Knockdown and Complementation Reveals Essential Role of RAD51 in Mouse Spermatogenesis

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SUMMARY

Meiotic homologous recombination (HR) is important for proper chromosomal segregation during gametogenesis and facilitates evolutionary adaptation via genomic reshuffling. In most eukaryotes, HR is mediated by two recombinases, the ubiquitous RAD51 and the meiosis-specific DMC1. The role of RAD51 in mammalian meiosis is unclear and study of its function is limited due to embryonic lethality of RAD51 knockouts. Here, we developed an in-vivo meiotic knockdown and protein complementation system to study RAD51 during mouse spermatogenesis. We show that RAD51 is crucial during meiotic prophase and its loss leads to depletion of late prophase I spermatocytes through a p53-dependent apoptotic pathway. This phenotype is distinct from that observed in the DMC1 knockdown. Our meiotic knockdown and complementation system establishes an experimental platform for mechanistic studies of meiotic proteins with unknown functions or essential genes for which a testis-specific knockout is not possible.

Graphical Abstract

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SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures and 7 figures.

AUTHOR CONTRIBUTIONS
J.D., O.V., and R.D.C.-O. conceived and designed the experiments. J.D. performed most of the experimental work and data analysis. O.V. performed experiments on protein purification and characterization. S.P. and J.D. performed the animal surgery and microinjection. J.D. and O.V. wrote the manuscript with input from R.D.C.-O.

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INTRODUCTION

Meiotic HR is indispensable for generating new combinations of DNA sequences to enable populations to adapt during the course of evolution. It is also essential for maintaining the karyotype of an organism and for preventing aneuploidy by ensuring proper segregation of homologous chromosomes. Meiotic HR is initiated by the formation of DNA double-strand breaks (DSBs), catalyzed by the topoisomerase-like enzyme SPO11 (Baudat et al., 2000; Keeney et al., 1997; Romanienko and Camerini-Otero, 2000). After the resection of DNA, homologous recombinases RAD51 and DMC1 polymerize on the single stranded DNA produced at the ends of DSBs. The resulting nucleoprotein filaments invade homologous dsDNA to form D-loop structures that are essential intermediates in the initiation of DNA strand exchange between homologous DNA molecules (Neale and Keeney, 2006).

RAD51 and DMC1 share around 50% identity at the amino acid level. While bound to DNA, they form very similar filamentous structures (Masson and West, 2001; Sheridan et al., 2008) and both these proteins are localized to meiotic chromosomes (Tarsounas et al., 1999). DMC1 is a meiosis-specific recombinase, while RAD51 is also required for mitotic HR (Shinohara et al., 1992). The role of DMC1 and RAD51 in meiotic HR and the extent of their involvement in this process remain unclear. Previously, we proposed that the intrinsic resistance of DMC1 strand invasion intermediates to dissociation might be essential for the proper segregation of homologous chromosomes in meiosis (Bugreev et al., 2013).

In the SK1 strain of budding yeast, dmc1 mutants arrest at meiotic prophase I, accumulate persistent DSBs, show delayed homolog pairing and synapsis, and exhibit severely reduced crossover formation (Bishop et al., 1992; Shinohara et al., 1997). Biochemical studies suggest that interhomolog recombination is disrupted in these dmc1 mutants, while intersister recombination still can be initiated (Schwacha and Kleckner, 1997). On the other hand, rad51 mutants show delayed meiotic progression, as well as a reduction in spore formation and viability. The rad51 mutants show defects in meiotic HR, including the

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accumulation of DSBs, a delay in homologous pairing and synapsis, and a reduction in interhomolog recombination and crossover formation (Schwacha and Kleckner, 1997; Shinohara et al., 1997; Shinohara et al., 1992). However, in the BR background both rad51 and dmc1 mutants exhibit meiotic defects different from those in the SK1 strain (Rockmill and Roeder, 1994; Rockmill et al., 1995; Tsubouchi and Roeder, 2003). Thus, the strain-dependent meiotic phenotypes of budding yeast rad51 and dmc1 mutants pose an additional difficulty in understanding the relative contributions of RAD51 and DMC1.

In fission yeast, dmc1 mutants do not show prophase arrest and rad51 mutants exhibit a more severe phenotype (decrease in spore viability and meiotic recombination) than that observed in the dmc1 mutants (Grishchuk et al., 2004; Shinohara and Shinohara, 2004). In the flowering plant Arabidopsis thaliana, dmc1 mutants show reduced fertility, while rad51 null mutants are completely sterile and show severe DNA fragmentation (Couteau et al., 1999; Li et al., 2004). In mammals, both male and female DMC1 knockout mice are sterile (Pittman et al., 1998; Yoshida et al., 1998). In DMC1 knockout females, oogenesis is aborted in embryos; adult ovaries are devoid of oocytes although a few are present in prenatal and newborn ovaries (Pittman et al., 1998; Yoshida et al., 1998; Di Giacomo et al., 2005). In DMC1 knockout males, spermatocytes arrest at the zygotene stage without synapsis. The function of RAD51 in mammalian meiotic HR remains unclear due to the embryonic lethality of RAD51 knockout mice (Lim and Hasty, 1996; Tsuzuki et al., 1996) and the lack of a conditional knockout.

A possible alternative approach for studying the function of RAD51 in mammalian meiosis is through testis-specific knockdowns. Previous attempts to silence meiotic genes with short hairpin RNAs (shRNAs) using seminiferous tubule microinjection combined with in vivo electroporation have seen only partial success due to inefficient targeting to spermatocytes (Blanchard and Boekelheide, 1997; Gonzalez-Gonzalez et al., 2008; Ikawa et al., 2002; Ogawa et al., 1997; Shibuya et al., 2014; Shoji et al., 2005). In this study, we developed a system for in vivo knockdown of genes in mouse testis, using microinjection of small interfering RNAs (siRNAs) into the seminiferous tubules. We used Accell siRNAs (Dharmacon) that have been proved to be delivered into different types of cells and in-vivo tissues without additional reagents, viral vectors, or instruments (see company’s website for references, http://dharmacon.gelifesciences.com/accell-sirna-references/). Our approach is therefore less invasive and easier to implement. It also overcomes the major limitation of previous methods that resulted in predominant targeting to Sertoli cells. Furthermore, we have established a protein microinjection system to rescue the knockdown phenotype and to exclude possible off-target effects of knockdown experiments. Using siRNA knockdowns in seminiferous tubules of mouse testis, we were able to successfully reduce the amount of endogenous RAD51. We found that RAD51 knockdown depletes late prophase I spermatocytes, probably through a p53-dependent apoptotic pathway. In surviving knockdown pachytene cells, sex-chromosome asynapsis is increased and crossover formation is reduced. Our results indicate that RAD51 plays an essential role in the progression of mammalian meiosis, and it provides a framework for further molecular studies on RAD51 in mammalian meiotic HR.
RESULTS

Depletion of DMC1 in Mouse Testis with an in-vivo Knockdown Approach

To determine the role of RAD51 in mammalian meiotic HR, we developed an in-vivo knockdown system based on the microinjection of siRNAs into seminiferous tubules of mouse testis. We carried out siRNA injections in pups of 8 days post-partum (dpp) or 20 dpp. In C57BL/6 mice, male germ cells enter meiosis around 8 dpp and a second wave of spermatogenesis starts around 21 dpp; the length of prophase I is about 10 days (Goetz et al., 1984). Hematoxylin and eosin (H&E) staining of tissue sections was used for the morphological analysis, and the stage of spermatocytes was determined based on the morphology of the nuclei (Ahmed and de Rooij, 2009). Briefly, in leptotene spermatocytes chromosome threads start to appear; in zygotene the chromatin threads become thicker and heavily stained; in pachytene spermatocytes, nuclei are more round and the chromatin becomes large cords (Figure 1B). We also used Immunostaining of SYCP3 and γH2AX to help further confirm the staging of each population. By monitoring fluorescent signals from DY-547-labeled control non-targeting siRNA, we observed that the Accell siRNA signal persisted in the spermatocytes for over 2 weeks (data not shown). Preliminary tests showed that the injection of non-targeting siRNAs at concentrations as high as 100 μM and filling up to 90% of the surface seminiferous tubule volume did not cause major morphological defects in the injected testes. 7.3 ± 6.6 % (mean ± SD) of tubules in testes of pups injected at 8 dpp and 4.4 ± 4.3 % of tubules in testes of pups injected at 20 dpp show tissue damage manifested as empty tubules with loss of both somatic and germ cells (Figure S1). The proportion of tubules showing tissue damage is quantified in each sample (Figure S1).

To evaluate our in-vivo knockdown system, we first knocked down DMC1 with siRNA and compared the phenotype with that of DMC1-knockout mice (Pittman et al., 1998; Yoshida et al., 1998). In the pups injected with 100 μM of DMC1 siRNAs at 8 dpp, we assessed the effect of siRNA knockdown 10 days after injection, when most spermatocytes have progressed to late-pachytene/diplotene in the wild type. In tissue sections, about 50% of tubules injected with DMC1 siRNA showed a reduction in DMC1 protein staining, and most spermatocytes were observed arresting at late-zygotene/early-pachytene in these DMC1 downregulated tubules (Figure 1A). Morphological analysis further validated that 54.3 ± 13.9 % (including 2.6 ± 3.0 % tissue damage) (Figure 2 and Figure S1) of DMC1-siRNA injected tubules showed a significant increase of zygotene spermatocytes and a decrease of pachytene/diplotene spermatocytes (Figure 1B and Figure S2A), suggesting a phenotype of zygotene arrest in DMC1 knockdown spermatocytes that is similar to that seen in DMC1 knockout male mice (Pittman et al., 1998; Yoshida et al., 1998). In addition, as found in DMC1 knockout samples, chromosome asynapsis was observed in a large proportion of cells in spermatocyte chromosome spreads of DMC1 knockdown samples (data not shown). Increased apoptosis was detected by the TUNEL assay in some tubules with arrested spermatocytes (Figure 1C and Figure S3). These results indicate that successful silencing of meiotic genes in vivo can be achieved by siRNA microinjection in mouse testes.
In vivo Knockdown of RAD51 in Mouse Testis Results in a Depletion of Late Prophase I Spermatocytes

We microinjected RAD51 siRNAs in the testes of 8 dpp or 20 dpp mice, and these days coincide with each of the first two waves of spermatogenesis. To validate the effectiveness of the siRNA-driven RAD51 knockdown, we used mRNA fluorescent in situ hybridization (FISH) to examine the RAD51 transcript level in the siRNA-injected testes. While over 95% of the control sections showed a strong RAD51 mRNA FISH signal in both somatic and germ cells, FISH staining was observed only in 10–20% of sections prepared from RAD51 siRNA-injected testes (Figure S4A). Immunofluorescence microscopy of tissue sections using RAD51 specific antibody showed reduced RAD51 fluorescence intensities in about 60% of tubules injected with RAD51 siRNA; the other 40% of tubules presented normal level of RAD51 protein staining, and they showed wild-type like phenotype (Figure 3A). Moreover, in RAD51 siRNA injected samples we observed less RAD51 foci in chromosome spreads prepared from zygotene spermatocytes. (Figure 3B). This confirms siRNA efficiently lowers RAD51 transcript levels, although in some tubules RAD51 protein presented before injection may persist.

Morphological analysis of RAD51 siRNA injected testes showed about 60% tubules (59.6 ± 11.7 % including 4.0 ± 4.0 % tissue damage in samples injected at 8 dpp, and 62.7 ± 12.7 % including 4.9 ± 4.2 % tissue damage in samples injected at 20 dpp) (Figure 2 and Figure S1) had a typical phenotype of primary spermatocyte depletion as showing acellular space between the basal membrane and the lumen (Figure 3C). This phenotype was seen as early as 7 days and as late as 14 days after injection. Analysis of cell populations further confirmed a significant reduction of prophase I spermatocytes in RAD51 knockdown tubules with this spermatocyte depletion phenotype (Figure S2B). The efficacy of RAD51 siRNA was examined by evaluating the dose-dependent response using 10 to 100 μM of siRNAs. The results showed that injections of 25 μM to 100 μM of RAD51 siRNA generated indistinguishable knockdown phenotypes (data not shown). Furthermore, significantly increased apoptosis was observed in RAD51 knockdown affected tubules (Figure 3D and Figure S3). No visible changes in the staining of γH2AX or DMC1 was observed in RAD51 knockdown affected tubules, and the number of DMC1 foci was normal in zygotene spermatocytes from RAD51 knockdown samples (Figure S4B).

We further examined the effect of siRNA knockdown on the cell populations in the tubules. For these analysis we only used tubules showing a spermatocyte depletion phenotype. Previous attempts to deliver shRNA/DNA in testes resulted in preferential targeting of Sertoli cells, which are required for normal spermatogenesis progression all through the meiosis (Blanchard and Boekelheide, 1997; Gonzalez-Gonzalez et al., 2008; Ikawa et al., 2002). However, we observed no difference between Sertoli cell populations in the RAD51-knockdown and control tubules (Figure S2B). In samples collected from testes of pups injected at 20 dpp, the existing spermatids are likely derived from spermatocytes that progressed beyond prophase before injection, and thus are not affected by RAD51 siRNA knockdown. Therefore, as expected, no reduction was observed in the amount of round spermatids in the testis tubules from pups injected at 20 dpp (Figure S2B). This result further indicates that the Sertoli cells remain functional and support spermatogenesis in RAD51-
knockdown tubules. The phenotype of spermatocyte depletion was observed in RAD51 knockdown samples collected from 7 days after injection. In the RAD51-knockdown tubules, prophase I spermatocytes in zygotene and later stages were significantly reduced (Figure S2A). This depletion was not observed in RAD51 knockdown samples collected after 4 days of injection. The knockdown tubules were wild-type looking, and zygotene spermatocytes were still present at that stage. These data suggest that RAD51 knockdown induces apoptosis during early stages of prophase I. A similar phenotype was not seen in the DMC1-knockdown tubules. DMC1-knockdown tubules showed a significant increase of zygotene spermatocytes compared to the control, however post-pachytene spermatocytes were eliminated (Figure S2A). An increase of spermatogonia and pre-leptotene to leptotene spermatocytes was observed in RAD51-knockdown tubules (Figure S2). In DMC1-knockdown tubules, an increase of pre-leptotene to leptotene spermatocytes was also observed (Figure S2A). One of the important mitotic functions of RAD51 is the restoration of stalled replication forks (Henry-Mowatt et al., 2003). To evaluate replication in RAD51-knockdown tubules, we used thymidine analogue, 5-ethynyl-2-deoxyuridine (EdU) to label the actively replicating DNA in pre-leptotene spermatocytes (Boateng et al., 2013). The result showed that no significant decrease in replication was observed in the RAD51-knockdown tubules (Figure S4C).

In affected RAD51-knockdown tubules, a few post-zygotene cells were observed. These cells showed normal autosomal chromosomal synapsis (Figure 4A and Figure S5A), however, increased sex-chromosome asynapsis was observed (17%, N=307, compared to the wild type 9%, N=303); although a normal sex body formed (determined by γH2AX staining) (Figure S5A). The true proportion of sex-chromosome asynapsis in knockdown samples is likely higher because unaffected cells will be disproportionally counted in spermatocyte chromosome spreads. In addition to sex-chromosome asynapsis, RAD51-knockdown pachytene cells positive for H1t (a marker for mid-late pachytene) had fewer MLH1 foci (a marker of crossovers) (Figure 4B and Figure S5B). No persistent γH2AX, DMC1 or RPA was observed. These results indicate that normal meiotic HR is not completed in the surviving late-stage RAD51-knockdown spermatocytes. Furthermore, in samples collected 14 days after injection, spermatids were observed in most tubules injected with control siRNA, but not in those injected with RAD51 siRNA. In fact, RAD51 knockdown spermatocytes did not progress beyond metaphase I (Figure 3C). This observation further indicates that there is a meiotic defect in those RAD51 knockdown cells escaping early-prophase I apoptosis. However, it is not clear if these cells reflect the phenotypic heterogeneity of RAD51 knockdown, or are the result of incomplete knockdown of RAD51.

Depletion of p53 Prevents Spermatocyte Depletion in RAD51-Knockdown Testes by Suppressing Apoptosis

Disruption of Rad51 in mice results in embryonic lethality early in development; however, RAD51-knockout embryos survive for longer periods if the p53 protein is also absent (Lim and Hasty, 1996). Physical interactions between RAD51 and p53 in mitotic cells have been also documented (Buchhop et al., 1997; Linke et al., 2003; Sturzbecher et al., 1996), so to examine the involvement of p53 in the RAD51 knockdown-induced apoptotic pathway, we
generated p53 and RAD51 double-knockdown testes by co-injecting seminiferous tubules with two siRNAs (50 μM for each). In the double-knockdown testes collected 10 days after injection, over 90% of total tubules lacked p53 with far fewer apoptotic cells (Figure 5, and Figure S3). Furthermore, late prophase I spermatocyte depletion was observed only in about 15 % of injected tubules (15.4 ± 9.4% including 3.1 ± 3.2 % tissue damage in samples injected at 8 dpp, and 15.5 ± 9.2 % including 4.0 ± 5.2 % tissue damage in samples injected at 20 dpp), compared to 60 % in tubules injected with RAD51 siRNA alone (Figure 2, Figure 5, and Figure S1). Increased H1t positive pachytene spermatocytes were observed in p53 and RAD51 double-knockdown samples compared to RAD51 single-knockdown samples (data not shown). Similar results were obtained when both RAD51 and p53 were inactivated by injecting RAD51 siRNA into the testes of p53-knockout mice, and no difference was seen between the p53+/− and p53−/− mice (Figure S3 and Figure S6A).

Together, these data suggest that even partial depletion of p53 may allow cells escape from the early-prophase I apoptosis caused by RAD51 knockdown, and progress to more advanced stages.

However, analogous to what was described for RAD51-knockout mice (Lim and Hasty, 1996), p53 inactivation could not completely rescue the RAD51 depletion phenotype. Similar to what was found in the small population of RAD51-deficient pachytene cells that escaped early-prophase I apoptosis, p53 and RAD51 double-deficient pachytene spermatocytes exhibited a reduction in crossover formation, as well as increased sex-chromosome asynapsis (21%, N= 317 compared to the 9%, N=303 in wild type cells) (Figure 4B and Figure S5). However, as in RAD51 single-knockdown pachytene cells, normal autosomal synapsis was observed in these double-knockdown cells (Figure 4A and Figure S5A). Moreover, in double-knockdown samples collected 14 days after injection, about 50% of tubules showed metaphase I block of spermatogenic progression (data not shown), which is similar to what was observed in RAD51 single-knockdown samples. Thus, p53 depletion cannot fully rescue the phenotype of late prophase I spermatocyte depletion caused by RAD51 knockdown.

In contrast, p53 depletion in the DMC1-knockdown testis did not rescue the late-zygotene/early-pachytene arrest phenotype (Figure 5). However, we observed a suppression of apoptosis in p53 and DMC1 double-knockdown testes (Figure 5A and Figure S3). These findings were confirmed by examining Dmc1−/−p53−/− mice and DMC1 knockdown in p53−/− mice (Figure S3 and Figure S6).

The RAD51 Knockdown Phenotype is Rescued by Complementation with RAD51 Protein

To confirm the RAD51 dependence of the knockdown phenotype, we complemented siRNA-knockdown testes with purified RAD51 protein. We used 6xHis tag-fused human RAD51 that was active in ssDNA and dsDNA binding, D-loop formation, and DNA strand exchange in vitro (Figure S7). The protein-rescue experiments for RAD51 in-vivo knockdown were carried out by co-injection of the wild type RAD51 protein and RAD51 siRNAs. Ten days after injection, only about 20 % tubules (20.9 ± 12.2 % including 3.5 ± 3.2 % tissue damage in samples injected at 8 dpp, and 22.9 ± 14.1 % including 3.0 ± 2.4 % tissue damage in samples injected at 20 dpp) showed depletion of late prophase 1

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spermatocytes; in comparison, about 70% (68.4 ± 9.7% including 7 ± 4.4% tissue damage in samples injected at 8 dpp, and 68.0 ± 20.6% including 5.2 ± 3.2% tissue damage in samples injected at 20 dpp) tubules in the testes co-injected with RAD51 siRNAs and protein buffer showed late prophase I spermatocyte depletion (Figure 6, Figure 7A and Figure S1). In addition, the RAD51-protein complementation rescues the defect on crossover formation, and most of pachytene spermatocytes in knockdown samples with RAD51 protein complementation show normal MLH1 foci (Figure 7B).

The depletion of endogenous RAD51 in the co-injected testes was verified by RNA-FISH (Figure 6). Despite the fact that we did not incorporate a nuclear localization signal (Gildemeister et al., 2009) in the protein sequence, 6xHis tag signals were detected in the nucleus of spermatocytes in about 60% of tubules injected with His-tag fused proteins, but not in the controls (Figure 6). In order to rescue the RAD51 knockdown, the RAD51 protein has to be intact and fully functional. When the tubules were co-injected with RAD51 siRNA and the same amount of wild-type RAD51 protein subjected to comprehensive tryptic digestion, no rescue of the knockdown phenotype was observed (Figure 7A). We also tested a mutant 6xHis tag-fused RAD51-II3A protein that has three basic amino acids mutated to alanines in the putative secondary DNA binding site (Cloud et al., 2012). In our assays, this mutant RAD51-II3A protein retained some ss- and ds-DNA binding activity but was completely defective in D-loop formation and DNA strand exchange (Figure S7). We were unable to rescue the knockdown phenotypes with microinjection of the RAD51-II3A protein (Figure 6 and Figure 7). Taken together, the RNA FISH and 6xHis tag immunostaining results confirm that an exogenous injected protein rescues the knockdown phenotype in the RAD51 siRNA-injected tubules. Furthermore, complementation of siRNA knockdown with wild-type RAD51 protein injection rules out the possibility that the observed phenotype is due to off-target effects caused by RAD51-specific siRNA.

DISCUSSION

A common method used to study gene functions in mammalian systems is through the generation of knockout mice, which is time- and labor-consuming. The study of essential genes in mammals can be hindered by embryonic lethality resulting from the disruption of these genes. The difficulty increases when appropriate spatiotemporal promoters for creating conditional gene knockouts are not available. In the case of RAD51, its inactivation in mice causes early embryonic lethality, and fibroblasts derived from RAD51-knockout embryos fail to proliferate in tissue culture (Lim and Hasty, 1996). In addition to its indispensability in somatic cells, RAD51 plays a crucial role in meiosis as well, which is evident from the fact that the inactivation of the ubiquitously expressed RAD51 has a more severe meiotic phenotype than the disruption of the meiosis-specific recombinase Dmc1 in yeast (see introduction).

To overcome the restrictions imposed by the embryonic lethality of RAD51 knockout and to gain insight into the role of RAD51 in spermatogenesis, we developed an in-vivo meiotic knockdown and complementation system in mice. It allows us, to evaluate the effect of RAD51 inactivation during mammalian gametogenesis.

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Our findings show that RAD51 knockdown in mouse testis results in depletion of late prophase I spermatocytes, likely as a result of apoptosis. RAD51 functions in both mitosis and meiosis. Our results from assays using EdU incorporation indicate that no significant decrease in replication is observed in the RAD51-knockdown tubules. However, we cannot rule out the possibility that some defects in replication fork progression as a result of the RAD51 knockdown, which might escape the detection by EdU labeling, may delay the transition from the S phase to meiotic division. This may help to explain our observation of an increased population of spermatogonia in the RAD51-knockdown tubules. However, it is also possible that the increase of spermatogonia is just a consequence of the global reduction in cell number in knockdown tubules.

In RAD51-knockdown tubules, we observed a few cells escaping early-prophase I apoptosis and progressing beyond pachytene stage. In these spermatocytes, crossover formation was reduced and the sex-chromosome asynapsis was increased, however autosomal synapsis appeared to be normal. These cells are blocked at metaphase I probably due to the presence of multiple univalents resulting from incomplete HR. The trivial explanation for this observation would be that RAD51 is not completely knocked down in these cells; however, there may be another interpretation. In budding yeast rad51 mutants, cells start to lose viability right after DSBs form; 40–60% of these cells arrest before the first meiotic division and the remaining cells undergo a delayed meiosis I. Nevertheless, some Rad51-deficient cells still can complete the second division (Shinohara et al., 1997). Similarly, phenotypic heterogeneity of RAD51-knockdown cells in mammals may explain the progression of spermatocytes to the pachytene stage. In wild-type mouse testes, active p53 is downregulated during early meiosis, while in RAD51-knockdown testes, p53 is apparently activated and capable of inducing quick apoptosis (Figure 5A). Some cells with a lower level of p53 may escape early-prophase I apoptosis and reach later stages. However, these cells still cannot complete normal recombination, and this is manifested as a reduced crossover formation and an increased sex-chromosome asynapsis. In fact, this model is supported by our observation that p53 and RAD51 double-deficient spermatocytes show the same defects in meiotic HR as the RAD51 single-knockdown cells, however the apoptosis and the depletion of late prophase I spermatocytes are rescued. Thus, it might be that different to the indispensable role of DMC1 in homology search and synapsis, RAD51 may play a role in crossover formation as well as synapsis on sex chromosomes.

Why two homologous DNA recombinases, RAD51 and DMC1, are both required in mammalian meiosis remains unclear. However, the ability to inactivate these proteins in vivo in germline cells has allowed us to further our understanding of the role of RAD51 in meiosis. We are able to compare the consequences of the meiotic knockdown of RAD51 and DMC1 in the same mammalian system. Our results reveal that RAD51 and DMC1 depletions result in easily distinguishable phenotypes in mouse testes. DMC1-deficient spermatocytes cannot progress beyond the late-zygotene/early-pachytene stage, while in RAD51-knockdown tubules, the primary block of spermatogenesis occurs earlier at the leptotene stage, and deficient zygote spermatocytes lose viability and are eliminated quickly. Besides the observation of tubules lacking of spermatocytes in RAD51 knockdown tubules, the rapid elimination of RAD51 deficient cells is also inferred from the detection of more apoptotic cells by TUNEL assay in DMC1 knockdown tubules than in RAD51.
knockdown tubules. This is consistent with what was found in budding yeast—while the rad51-mutant cells exhibit a quick decrease in viability during sporulation, the dmc1-mutant cells arrest in meiotic prophase could still maintain an almost wild-type viability for a very long time (Dresser et al., 1997). These results suggest that apoptosis is a direct result of the depletion of RAD51 but not of DMC1, which is consistent with our findings indicating that p53 depletion could partially rescue the knockdown phenotype and allow spermatocytes progress to more advanced stages in RAD51-knockdown testes but not in DMC1-knockdown testes. With the use of our system, studies on protein structure-function relationships will provide further insights into the distinct roles of RAD51 and DMC1 during mammalian meiotic HR.

In conclusion, our gene knockdown system provides a unique technical platform for in-vivo studies of genetic interactions in mammalian meiosis. This system is flexible and allows combinations of different approaches to be used—from utilizing gene-knockout mouse strains to in vivo gene inactivation via siRNA-mediated knockdown and complementation of genetic defects with purified proteins. This opens endless new possibilities for studying the roles of essential genes in meiosis, and for carrying out in vivo biochemical studies of meiosis-specific gene products. Using this system, we address the long-standing question about the role of RAD51 in meiosis to reveal that RAD51 is essential for normal progression of mammalian meiosis. Our findings provide a framework for the further molecular study of RAD51 in mammalian meiosis.

EXPERIMENTAL PROCEDURES

Mouse Strains

The mice used in this study were as follows: C57BL/6 (Charles Rivers), p53-knockout (Jackson labs), and DMC1-knockout (Pittman et al., 1998). DMC1 and p53 double-knockout mice were generated by cross breeding p53 heterozygous mice with DMC1 heterozygous mice. All the animal protocols were approved by the National Institutes of Health (NIH) Institutional Animal Care and Use Committee.

siRNAs and Antibodies

The following Mouse Accell SMART Pools of siRNAs (Dharmacon) were used for the in-vivo knockdown experiments: RAD51 siRNAs (target sequences): GCGAUGUCUAGAUAAUGU, UGCUUGUUCUCUAAAUUAC, CAAUGAUGUGAAGAAAUUA, CUGAUUUCUUGGUGUUUA (E-062730-00-0010); DMC1 siRNAs (target sequences): GGUGAAAGCUUGGAGUAUA, CCAACAAGCUUUAUGGAACC, CUACCAUGGCGAAAUUAUCA, GAAGCACUGCUUGAGAAAUC (E-043236-00-0010); Trp53 siRNAs (target sequences): GGAAUAGGUUGAUUGUUGU, CCAGGAUGUGAGGAGGUUU, CUUUGAGGUUCGGGUGGUUU, GAAGAGGCCUCAGAGGUAUA (E-040642-00-0010); non-targeting siRNAs (target sequences): UGGUUACAUUGUCGACUA, UGGUUUCAUGUUUCUGA, UGGUUUCAUGUUUCUGA, UGGUUUCAUGUUGUGUGA (D-001910-10-05); Red non-targeting siRNA (target sequence): UGGUUUCAUGUUCGACUA (D-001960-01-05).
The following primary antibodies were used: mouse anti-SYCP3 at 1:100 (Santa Cruz Biotechnology, SC-74569), rabbit anti-SYCP3 at 1:100 (Novus Biologicals, NB300-231), rabbit anti-RAD51 at 1:50 (Santa Cruz Biotechnology, SC-8349), rabbit anti-DMC1 at 1:50 (Santa Cruz Biotechnology, SC-22768), mouse anti-p53 at 1:50 (Cell Signaling Technology, 2524S), mouse anti-MLH1 at 1:50 (BD Biosciences, 551091), mouse anti-γH2AX at 1:1000 (Millipore, 05-636), mouse anti-γH2AX with biotin conjugation at 1:1000 (Millipore, 16-193), rabbit anti-SYCP1 at 1:100 (Novus Biologicals, NB300-229), guinea pig anti-H1t at 1:200 (gifted by Mary Ann Handel from The Jackson Laboratory), rabbit anti-c-kit at 1:200 (abcam, ab5506), goat anti-GATA-4 at 1:200 (Santa Cruz Biotechnology, SC-1237), rabbit anti-RPA at 1:100 (abcam, ab10359), mouse anti-His-tag at 1:100 (abcam, ab18184), and mouse anti-Digoxigenin at 1 μg/ml (Roche, 11333062910).

**In vivo Knockdown and Protein Complementation**

Solutions containing siRNAs (diluted in 1 X siRNA buffer, Dharmacon), proteins (diluted in buffer containing 50 mM Tris acetate pH 7.5, 200 mM KCl, 0.1 mM EDTA, 14.2 mM β-mercaptoethanol, 10% Glycerol) and the tracer Trypan blue were microinjected into the seminiferous tubules of live mouse pups, according to the method described by Ogawa et al. (see Supplemental Experimental Procedures for the details). For preparing paraffin-embedded tissue sections, the testes were extracted and fixed with 10% formalin for 24 h at 4°C. The sections were prepared and H&E staining was performed by American HistoLabs, Inc. (Gaithersburg, MD), and the slides were then kept at 4°C until use. For the preparation of frozen tissue sections, the testes were extracted, embedded with O.C.T. Compound (Sakura Tissue-Tek), and kept under dry ice. The sections were prepared by Histoserv, Inc. (Germantown, MD), and the slides were then stored at −80°C until use.

**Immunofluorescence**

Spermatocytes from fresh testes were prepared for chromosome spreads and processed using established methods (Bellani et al., 2005). Paraffin sections were de-waxed, antigen retrieved, and processed the immunostaining steps (see Supplemental Experimental Procedures for the details). Apoptosis assay was performed using the In Situ Cell Death Detection Kit (Roche). mRNA FISH was performed according to a previously published protocol (Mahadevaiah et al., 2009) with modifications (see Supplemental Experimental Procedures for the details). All images were captured with an upright epi-fluorescence microscope, Leica DM5000 B (Leica Microsystems Inc., Buffalo Grove, IL, USA). Images were acquired and analyzed using Velocity software (Perkin-Elmer).

**EdU Labeling of Replicating Cells**

EdU (20 μg/g body weight, Invitrogen) was intraperitoneally injected into mice 2 h before testis extraction, according to established methods (Chehrehasa et al., 2009; Salic and Mitchison, 2008). EdU detection was carried out using the Click-iT Plus EdU Imaging kit (Life Technologies) according to the manufacturer’s instruction.
**Protein Expression and Purification**

Wild-type and mutant human RAD51 proteins were expressed as N-terminal 6xHis-tagged fusions in BL21 recA *Escherichia coli* cells (Novagen) from a pET15b-based vector kindly provided by Dr. A. Mazin and purified as described previously (Bugreev et al., 2014). Trypsin digested RAD51 was prepared using TPCK Treated Trypsin Immobilized on Agarose Resin (Thermo Fisher) as recommended by manufacturer. Mutant RAD51-II3A was prepared by introducing R130A, R303A, and K313A point substitutions into the wild-type RAD51 DNA sequence using the QuickChange Lightning Multi Site-Directed Mutagenesis kit (Agilent Technologies) according to the manufacturer’s instructions. Whole gene sequencing (Eurofins Genomics) confirmed the absence of additional mutations. Protein activities were examined using the *in vitro* D-loop assay, DNA strand exchange, and DNA binding assay steps (see Supplemental Experimental Procedures for the details).

**Statistics**

Statistical analyses were performed using the GraphPad Prism software. The t-test was used to determine the difference between two groups, and ANOVA was used to test differences between multiple groups. Statistical significance was presented as *p < 0.05; **p < 0.01; ***p < 0.005, ****p < 0.001, and NS: no significance.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Analysis of DMC1 Knockdowns Generated by the in vivo siRNA microinjection approach

Testes of 8 dpp pups were injected with control or DMC1 siRNAs and collected 10 days after microinjection. The samples were fixed, paraffin embedded, stained using appropriate techniques, and analyzed by microscopy. Scale bars represent 40 μm.

(A) Immunostaining of DMC1 in tubule cross sections. (B) Hematoxylin and eosin (H&E) staining was used for the morphological analysis of the DMC1 knockdown phenotype. Spermatogonia (Sg) and pre-leptotene (PL) and leptotene (L) spermatocytes are indicated with a green arrow, the zygotene (Z) cells are indicated with a blue arrow, and pachytene (P) and diplotene (Di) spermatocytes are indicated by a red arrow. Examples of magnified nuclei morphology of different germ cells are shown. (C) Apoptosis was detected using the TUNEL assay. See also Figure S2 for the quantification of cell populations in DMC1 knockdown tubules, and Figure S3 for the quantification of apoptosis.
Figure 2. Summary of Results from in vivo Knockdown Experiments

Testes of 8 dpp or 20 dpp pups were injected with siRNAs (control: 100 μM, RAD51: 50 μM, p53: 50 μM, DMC1: 100 μM). Samples were collected after 7, 10, or 14 days of injection, fixed and paraffin embedded. The proportion of tubules showing phenotypes was estimated after the evaluation of cell morphology on H&E stained tissue sections. In RAD51 knockdown and RAD51/p53 double-knockdown samples, the phenotype we scored was spermatocyte depletion. In DMC1 knockdown and DMC1/p53 double-knockdown samples, the phenotype we scored was zygotene arrest. Although tubules depleted of both somatic and germ cells were likely the result of tissue damage caused by microinjection, they were included in the quantification of knockdown phenotypes. See also Figure S1 for the quantification of the proportion of tissue damage in each sample. N – Number of testes evaluated. For each testis, at least 6 sections were checked, and more than 100 tubules were assessed for each section. Results are presented as means ± SD of the values. Statistical significance was determined using ANOVA. ****p < 0.001, NS: no significance.
Figure 3. Depletion of Primary Spermatocytes in RAD51 Seminiferous Tubules from in vivo Knockdown Tests

Testes of 8 dpp pups were injected with control or RAD51 siRNA. Collected samples were fixed and paraffin embedded for tissue sectioning (A, C and D), or prepared for chromosome spreads (B). Scale bars represent 40 μm.

(A) Samples were collected 10 days after injection. Immunostaining of RAD51 in the tubule cross sections show a reduction of RAD51 staining in the RAD51 siRNA injected tubules. (B) Spermatocyte chromosome spreads were prepared from the whole testis. RAD51 foci were quantified in zygotene spermatocytes from 3 experimental repeats. N – Number of cells evaluated. Results are presented as means ± SD of the values. Statistical significance was determined using t-tests. ****p < 0.001. (C) Samples were collected 10 days (upper two

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rows) and 14 days (lower two rows) after injection. Morphological analysis of the H&E stained seminiferous tubule cross sections reveals the depletion of primary spermatocytes in the knockdown testes. Spermatogonia and pre-leptotene and leptotene spermatocytes are indicated with green arrows, zygotene cells are indicated with blue arrows, pachytene and diplotene spermatocytes are indicated with red arrows, metaphase I cells are indicated with grey arrows, and round spermatids are indicated with orange arrows. (D) Samples were collected 10 days after injection. Apoptosis was detected using the TUNEL assay. The numbers of apoptotic cells per tubule cross section are plotted. N – Number of tubules evaluated. Results are presented as means ± SD of the values. Statistical significance was determined using t-test. ****p < 0.001. See also Figures S2, S3, and S4.
Figure 4. Normal Autosomal Synapsis but Reduced Crossover Formation in Rad51-Knockdown Spermatocytes

Testes of 8 dpp pups were injected with siRNAs and collected 10 days after injection. Samples were fixed and paraffin embedded for tissue sectioning (A), or prepared for chromosome spreads (B).

(A) Autosomal synapsis was determined by SYCP1 (red) and SYCP3 (green) staining in the spermatocytes. Over 600 tubule sections were examined for each sample. Scale bars represent 40 μm. (B) Crossover formation was analyzed by counting MLH1 foci numbers in the H1t-positive pachytene cells. Spermatocyte chromosome spreads were prepared from the whole testes. N – Number of cells evaluated. Results are presented as means ± SD of the values. Statistical significance was determined using ANOVA. ****p < 0.001. See also Figure S5.
Figure 5. p53 Knockdown Prevents Spermatocyte Depletion in RAD51-Knockdown Testes by Suppressing Apoptosis

Testes of 8 dpp pups were injected with siRNAs and collected 10 days after injection. Samples were fixed and paraffin embedded. Scale bars represent 40 μm.

(A) Morphological analysis of the H&E stained seminiferous tubule cross sections reveals that p53 depletion rescues the spermatocyte depletion phenotype in RAD51 knockdown testes, but not the zygotene arrest phenotype in DMC1 knockdown testes. Expression of p53 (red) was detected by immunofluorescence. Apoptosis was detected using the TUNEL (green) assay. (B) Immunostaining of RAD51 in the tubule cross sections. (C) Immunostaining of DMC1 in the tubule cross sections. See also Figures S3, S4, and S6.
Figure 6. Rescue of the RAD51-Knockdown Phenotype by Complementation with RAD51 Protein

Testes of 8dpp or 20 dpp pups were injected with siRNAs. Frozen tissue sections were prepared from samples collected 7 days after injection, and paraffin embedded tissue sections were prepared from samples collected 10 days after injection. The knockdown phenotype was determined by morphological analysis based on H&E staining (1st and 2nd rows). The RAD51 transcription level was analyzed using mRNA FISH (3rd row). Injected His-tag–fused proteins were detected using a His-tag antibody with a secondary signal amplification system (4th row). The boxed regions are displayed with magnification separately (5th row). Scale bars represent 40 μm. See also S7.
Figure 7. Summary of the Results from *in vivo* Protein-Complementation Experiments

Testes of 8 dpp or 20 dpp pups were injected with siRNAs (control: 100 μM, RAD51: 50 μM,) and proteins (6 μM). Samples were collected after 7, 10, or 14 days of injection, fixed and paraffin embedded.

(A) The proportion of tubules showing phenotypes was estimated after the evaluation of cell morphology on H&E stained tissue sections. The phenotype of RAD51 knockdown we scored was spermatocyte depletion. Although tubules depleted of both somatic and germ cells were likely the result of tissue damage caused by microinjection, they were included in the quantification of knockdown phenotypes. See also Figure S1 for the quantification of the proportion of tissue damage in each sample. N – Number of testes evaluated. For each testis, at least 6 sections were checked, and more than 100 tubules were assessed for each section.

(B) Spermatocyte chromosome spreads were prepared from whole testes. Crossover formation was analyzed by counting MLH1 foci number in H1t-positive pachytene cells. N -
Number of cells evaluated. Results are presented as means ± SD of the values. Statistical significance was determined using ANOVA. ***p < 0.005, ****p < 0.001, NS: no significance.