DNA double strand breaks repair pathways in mouse male germ cells

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DNA dubbelstrengs breuk reparatie routes in spermatogenetische cellen van de muis

(met een samenvatting in het Nederlands)

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INTRODUCTION

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Spermatogenesis

Spermatogenesis is a cyclic process taking place in the epithelium of the seminiferous tubules within the testis. During spermatogenesis various generations of germ cells undergo a series of developmental steps according to a fixed time schedule**.** There are three main types of male germ cells (Table 1). First, spermatogonia that include stem cells and who's differentiating descendants go through a series of about 10 mitotic divisions [1, 2]. Most generations of spermatogonia do not show heterochromatin in their nuclei and are called A spermatogonia, among which reside the spermatogonial stem cells called A-single spermatogonia (A_s). A_s spermatogonia can self renew or divide into a pair of interconnected spermatogonia, the A paired (A_{pr}). These A_{pr} divide further to form A aligned spermatogonia (A_{al}) which consist of chains of 4, 8 or 16 interconnected cells [3]. The A_{al} differentiate into differentiating type A_1 spermatogonia that divide into A_2 that divide further to subsequently form A_3 and A_4 spermatogonia. The last couple of spermatogonial generations increasingly show nuclear heterochromatin and are called intermediate (In) or B spermatogonia. The final spermatogonial division renders the second type of germ cells, the spermatocytes. The primary and secondary spermatocytes respectively engage in the first and second meiotic division. The primary spermatocyte traverses the G1 cell cycle phase followed by the premeiotic S-phase during which DNA replication occurs. After S phase, primary spermatocytes enter the prophase of the first meiotic division. The first meiotic prophase can be subdivided into the leptotene, zygotene, pachytene and diplotene stages. Before their entry into leptotene, primary spermatocytes are called preleptotene. During leptotene stage, meiotic recombination is initiated by the induction of double strand breaks (DSBs) throughout the genomic DNA, by the SPO11 protein, the meiosis specific topoisomerase II related inducer of DSBs [4]. Homologous chromosomes pair, synapse and undergo recombination. After two meiotic divisions haploid round spermatids are generated that, through a lengthy process, transform into spermatozoa via elongated spermatids.

Staging of mouse seminiferous tubule cross-sections

In 1956, Oakberg subdivided spermatid development, also called spermiogenesis, into 16 subsequent steps, the first 12 of which were used by Oakberg to subdivide the mouse epithelial cycle into 12 stages [5]. This cycle has been defined as "a complete series of the successive cellular associations appearing in any one given area of the tubule" [6]. Russell et al. [7] published a practical guide for the histological evaluation of the testis indicating 12 stages of the epithelial cycle and different germ cell types in the mouse (Fig. 1).

Cross-sectioned seminiferous tubules contain multiple germ cell types that proliferate, undergo meiosis and differentiate into spermatozoa (Fig. 2). Most studies describing the epithelial

stages are based on Periodic acid Schiff (PAS) and hematoxylin stained sections and primarily use the changes of the acrosome and the nuclear morphology of the younger generation of spermatids. Unfortunately, in a number of occasions PAS-hematoxylin stained sections are not available, as is the case with immunohistochemistry or in cases where particular germ cell types are missing, as in young mice and some mutant mice. This makes stage identification much more difficult. To determine the stage of the epithelial cycle in tubule cross-sections, we have recently summarized all stage identifying characteristics including ones not depending on PAS staining (Table 2).

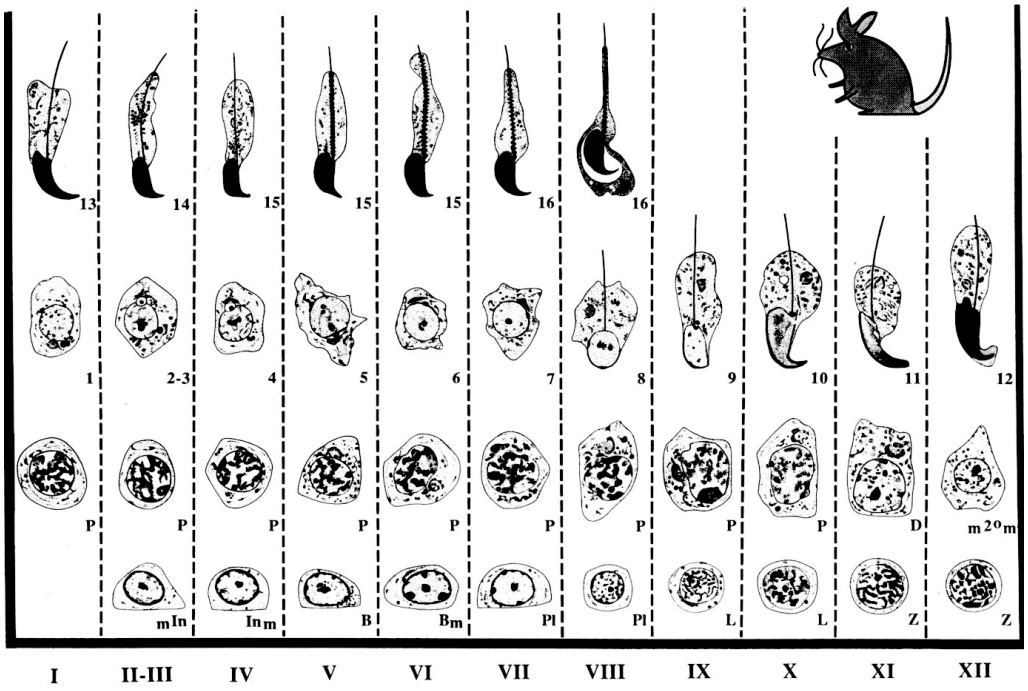

Fig. 1. *Diagram for the stages of the cycle of the seminiferous epithelium in the mouse showing the 12 stages and the various types of spermatogenic cells as depicted by Russell et al, 1990 [7].*

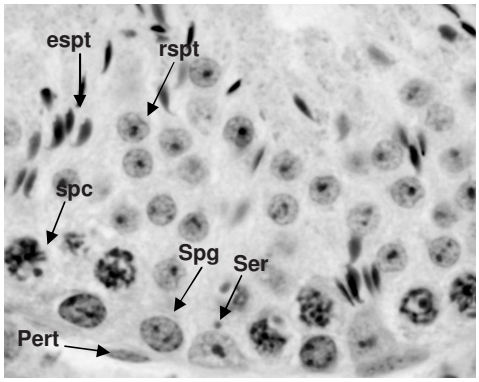

Fig. 2. *Cross-section of a seminiferous tubule showing multiple germ cell types. Spg – spermatogonia; spc – spermatocytes; rspt – round spermatids; espt – elongating spermatids; Pert – peritubular cells and Ser – Sertoli cells.*

Table 1. *Associations of germ cell types present in the various stages of the cycle of the seminiferous epithelium in the mouse. Abbreviations: preL - spermatocytes before the start of the meiotic prophase; L - spermatocytes in Leptotene phase of the meiotic prophase; Z - zygotene; P - pachytene; D - diplotene; numbers 1 through 16 - steps in the development of round spermatids to spermatozoa that are released into the tubule lumen during epithelial stage VIII. When two types of spermatogonia are mentioned in the same stage, this means that in that stage one generation of spermatogonia divides into the subsequent one. The A_{al} spermatogonia differentiate into A_1 spermatogonia without a concomitting division. de Rooij 1973 [2] and Oakberg, 1956 [4].*

Stages / cell types	**I**	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**	**IX**	**X**	**XI**	**XII**
spermatids	13	13	14	14	15	15	16	16				
spermatids	1	2	3	4	5	6	7	8	9	10	11	12
Spermatocytes	P	P	P	P	P	P	P	P	P	P/D	D	meiotic division I and II
spermatogonia	A_3 / A_4	A_4 / In	In	In / B	B	B / preL	preL	preL / L	L	L / Z	Z	Z / P
spermatogonia							A_1	A_1	A_1 / A_2	A_2	A_2 / A_3	A_3
spermatogonia	A_s ⇄ A_{pr} → A_{al}						A_{al} ↗					

DNA double strand breaks repair pathways

Of all types of DNA damage, DNA DSBs represent the greatest threat to cell viability and genome integrity. DNA DSBs are generated naturally in replication forks, immunoglobulin class-switch recombination and at meiotic recombination during meiosis [8]. DSBs are also induced by exogenous agents such as ionizing radiation (IR). The failure to repair DSBs, or misrepair, can result in cell death or large-scale chromosome changes including deletions, translocations, and chromosome fusions that decrease genome stability and are hallmarks of cancer cells. Before these definitive situations are reached, the cell can embark on a DNA repair attempt in virtually any stage of the cell cycle, depending on the accessibility of the chromatin for chromatin remodeling annex recruitment of repair complexes. Progress of repair is checked at the so called cell cycle checkpoints [9, 10] and cell faith, continuing repair and/or a choice for the apoptosis pathway likely originates from these checkpoints.

Eukaryotic cells repair DSBs primarily by two mechanisms: homologous recombination (HR) and non-homologous end-joining (NHEJ). HR in principle leads to accurate repair, because of the availability of a correct DNA template while NHEJ is more error prone and can therefore be mutagenic. Both DSB repair pathways play important roles in mammalian cells [11].

To date, six NHEJ factors have been identified: KU70, KU80, the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs), Artemis, XRCC4 and DNA ligase IV (LigIV). KU70 and KU80 form a heterodimer (KU70/80) that binds to DNA ends [12] and recruits DNA-PKcs, a Ser/Thr kinase of the PI3 family of kinases [13]. DNA-PKcs forms a complex with Artemis (DNA-PKcs/Artemis), a nuclease with intrinsic 5'-3'-exonuclease activity and phosphorylates Artemis thus activating its endonuclease activity which cleaves DNA structures containing single- to double-strand transitions (e.g. 5'- or 3'-overhangs) [14]. In this way, DNA ends can be processed to allow for subsequent ligation which is catalyzed by a complex formed by XRCC4 and LigIV (XRCC4/LigIV) [15, 16].

HR is a complex process requiring many proteins, that can be grouped [17] into proteins involved in DNA processing (including MRE11, RAD50, NBS1, γ-H2AX, MDC1, ATM and BRCA1) and proteins involved in homolog synapsis and strand exchange (including; RAD51, DMC1 “meiosis specific RAD51 paralog”, RAD51 paralogs, RAD52, RPA, RAD54, and RAD54B). Most models assume that after DSB induction, the first step of HR repair is the processing of DNA ends by 5' resection to produce molecules with 3' single-stranded tails, followed by nucleofilament formation catalyzed by RAD51, along with accessory proteins. The nucleofilament is formed when RAD51 polymerizes on single-stranded DNA which results from DNA damage processing [18]. RAD51 is a key player in this process and is also critical for homology recognition and strand exchange between recombining DNA molecules. Then, RAD54 stabilizes the RAD51 nucleoprotein filament in an early stage of recombination [19] and subsequently promotes chromatin remodeling [20, 21] and stimulates RAD51-mediated formation of a joint molecule between the broken DNA and the repair template. In later stages of the reaction it can displace RAD51 from DNA [22] (Fig. 3A).

The exact mechanism by which NHEJ and HR interact, and how the choice is made between the two pathways has still remained unclear. NHEJ and HR are not of equal importance during the course of the cell cycle. HR repair seemingly plays a more prominent role during late S and G2 stages of the cell cycle, when sister chromatids are available as repair templates, whereas NHEJ is operational during the whole cell cycle and is more important during G1 and early S [23-26]. Increasing evidence indicates that the shift from NHEJ toward HR as cells progress from G1 to S/G2 is actively regulated in lower and higher eukaryotes (Fig. 3). NHEJ and HR may compete for a DSB, or the choice may be determined by the structure of the broken ends [27]. Recently, an alternative route for NHEJ repair depending on a set of proteins other than those of the classical NHEJ pathway (XRCC1, PARP1 and DNA-Ligase III), was proposed to compete with the classical one or act as a backup pathway [28].

Table 2. Determination of the stages of the cycle of the seminiferous epithelium in tubule cross sections under different circumstances with respect to histological staining and germ cell types present. Spg - spermatogonia; spc - spermatocytes; spt - spermatids; rspt - round spermatids; espt - elongating spermatids.

Stages	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII
Normal epithelium *PAS-hematoxylin stained*	No PAS positive granules in rspt	2-3 PAS positive granules near the nucleus in rspt	PAS-positive granules coalesce to one round granule	PAS positive granule adheres to spt nuclear membrane and flattens	Acrosomal cap starts to form. Edges of cap still straight	Acrosomal cap covers up to 1/3 of spt nuclear surface. Espt line up at the tubule lumen.	Acrosomal cap covers more than 1/3 of spt nuclear surface. Random orientation of cap towards basal membrane	More than half of the acrosomal caps orientated towards basal membrane	Rspt have started the elongation process and are no longer completely round	Spt are now somewhat elongated but did not yet get flattened	Espt now also flattened	Spc in meiotic divisions. Some tubules show secundary spc
Normal epithelium *hematoxylin stained*	Rspt and espt present Few and only A type spg present		In spg present	Late or mitotic In or telopha-sic B spg present	B spg present, espt still embedded in epithelium	Late or mitotic B spg and espt at tubule lumen	preL present and espt not yet spermiated	Post spermi ation, rpst still round	As in PAS stained material	As in PAS stained material	As in PAS stained material	As in PAS stained material
No spermatids present	One generation of spc present Few and only A type spg present		In spg present	Late or mitotic In or telopha-sic B spg present	B spg present	Late or mitotic B spg	preL present		Leptotenes present Two generations of spermatocytes	Zygotene spc present Two generations of spermatocytes.		As in PAS stained material
Spc arrest after reaching pachytene stage	early pachytene		In spg present	Late or mitotic In or telopha-sic B spg present	B spg present	Late or mitotic B spg	preL present		Leptotenes present	Zygotenes / early pachytenes present		

Male germ cells and the repair of DNA DSBs

In male germ cells, the DNA repair pathway of choice might not only depend on the phase of cell cycle the cells are in, but also on the cell type. In spermatogonia both DNA DSBs pathways may be possible. Spermatocytes, after traversing the G1 and S phase of the cell cycle, enter the prophase I of meiosis and start meiotic recombination. Meiotic recombination is a DSBs repair mechanism, akin to somatic HR repair and occurs after the endogenous induction of DSBs by the protein [29]. During meiotic recombination DNA repair pathways other than HR may be suppressed [30]. Meiotic recombination is ended by mid-pachytene stage and although both HR and NHEJ contribute to the repair of IR-induced DSBs in spermatocytes from mid-pachytene stage up till late diplotene stage, the relative contribution of these two pathways is not yet clear. In the haploid spermatids, immunohistochemical evidence indicates DNA–PKcs not to be expressed in round spermatids [31] suggesting that classical NHEJ may not be functional in these cells. In addition, HR will be impossible in round spermatids because they are in a formal G1 stage (HR operates in S, G2 and needs another copy of the DNA to repair).

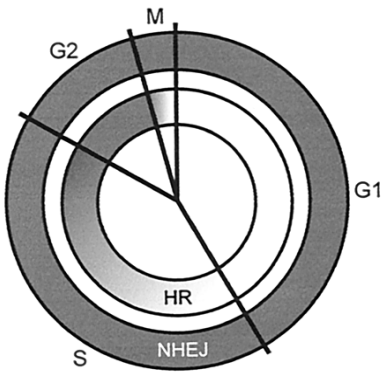

Fig. 3. *Model of the relative contributions of HR and NHEJ to the repair of IR-induced DSBs in different cell cycle phases, based on mutant phenotypes. Whereas NHEJ predominates in G1/early S, both HR and NHEJ contribute substantially to DSB repair during late. Rothkamm et al. [26].*

Proteins involved in the repair of DNA DSBs.

To activate DNA repair pathways, cells have evolved groups of proteins that function in signaling networks that sense and repair DSBs.

γ-H2AX

When DNA damage occurs, either caused by endogenous or exogenous factors, and DSBs are formed, phosphorylation of the histone H2A variant, H2AX, is initiated at serine 139 to generate γ-H2AX [32, 33]. Sites of chromatin with γ-H2AX can be conveniently detected immunohistochemically as irradiation induced immunofluorescent foci (IRIF), in case of DSB induction by irradiation and these foci represent the DSBs in a 1:1 manner and then, γ-H2AX is used as a marker for DSBs [34, 35]. The phosphorylation event of H2AX requires the activation

of the phosphatidylinositol-3-OH-kinase-like family of protein kinases, DNA-PKcs, ATM, and ATR. DNA DSBs are recognized by various proteins, including the MRN complex (a complex of three proteins, RAD50, MRE11 and NBS1), which recruits ATM to free DNA ends by a direct interaction between ATM and the NBS1 C-terminus [36]. When ATM becomes activated, it phosphorylates various target proteins at the site of DNA damage including H2AX. *H2AX*$^{-/-}$ mice are radiosensitive, growth retarded, immune deficient and mutant males are infertile. In mouse spermatocytes and after endogenous induction of breaks by SPO11 in leptotene spermatocytes, a general staining for γ-H2AX is obtained. When homologous synapsis is established during zygonema, γ-H2AX staining is lost over the synapsed areas and, by pachynema, it is undetectable on autosomal chromatin with the exception of likely stalled recombination events and autosomal segments that failed to synapse [29]. During pachynema and diplonema, γ-H2AX staining is very conspicuous in the sex body [29]. The sex body (or X-Y body) is a subnuclear domain during pachynema and diplonema, distinguishing the sex chromatin from the rest of chromatin. Mammals have adopted a strategy that involves seclusion of the sex chromosomes to a subnuclear compartment in order to deal with incomplete synapsis of the X-Y pair. Recently two types of foci have been found at the pachytene stage, small γ-H2AX foci (S-foci) specifically indicating sites of SPO11 induced-DSBs undergoing repair, and larger γ-H2AX signals on chromatin loops (L-foci) marking both unrepaired SPO11 induced-DSBs and SPO11-independent DSBs, for example caused by IR [37]. Several studies on somatic cells [26, 38] and germ cells [37, 39] have shown that the kinetics of γ-H2AX loss is related to DNA DSBs repair activity, especially at low doses of irradiation [40].

MDC1

At the site of DNA DSBs the mediator of DNA damage checkpoint protein 1 (MDC1) recognizes phosphorylated H2AX via its tandem BRCT domain and acts as a mediator/adaptor protein providing a “landing-platform” for the MRN complex to bind. Subsequently, MDC1 can attract ATM (increasing H2AX phosphorylation) and other proteins, such as 53BP1 (p53 binding protein 1) and BRCA1 [41-44] or DNA–PKcs and its interaction partner KU (in the case of NHEJ) to the site of damage which then in turn leads to the phosphorylation of even more H2AX [36]. Hence, MDC1 seems to fulfill a crucial role, even before the decision is made to repair via the NHEJ or the HR pathway. Also, MDC1 was found to regulate DNA–PK autophosphorylation in response to DNA damage [45]. A direct interaction between 53BP1 and MDC1 is required for the recruitment of 53PB1 to sites of damage [42]. In somatic cells, MDC1 forms foci directly after irradiation and MDC1 foci overlap completely with those of γ-H2AX and 53BP1 [42]. *Mdc1*$^{-/-}$ mice recapitulate many phenotypes of H2AX$^{-/-}$ mice, including growth retardation, male infertility, immune defects, chromosome instability, DNA repair defects, and radio-sensitivity [46]. Studying spermatogenesis in *Mdc1*$^{-/-}$ mice and MDC1 expression and also

interaction with other proteins such as 53BP1 and γ-H2AX in male germ cells and after irradiation is one of the goals of the present study.

53BP1

Within 5-15 min after exposure to ionizing radiation, 53BP1 forms discreet nuclear foci co-localizing with those of γ-H2AX and MDC1, indicating a role in the early response to DSBs for this protein [47]. *53BP1*-deficient mice are growth retarded, immune deficient, radiosensitive and cancer prone [48]. Increasing evidence indicates a major role in NHEJ but not during HR [49, 50].

DNA-PKcs

The defect in the "DNA-dependent protein kinase catalytic subunit" (DNA-PKcs) gene in mice is the main reason for a severe combined immunodeficiency (scid) syndrome and hypersensitivity to ionizing radiation. In these mice, some residual DNA-PKcs activity is left. The NHEJ pathway relies on a set of proteins that recognize, bind and repair the double-strand breaks. These proteins include DNA-PKcs which is recruited by the KU proteins to the site of damage and subsequently, both end-positioned KU and DNA-PKcs mediate the recruitment of the XRCC4-DNA ligase IV complex which is responsible for the ligation step (Fig. 3B) [51]. Scid mice are fertile with show some spontaneous apoptosis of spermatocytes in the testis. The majority of the apoptotic spermatocytes are found at stage IV of the cycle of the seminiferous epithelium [31] where a meiotic checkpoint has been suggested to exist [52].

KU proteins

NHEJ, or the KU-dependent repair process, appears to be the main DNA DSB repair mechanism in mammalian cells, KU itself is probably involved in stabilizing broken DNA ends, bringing them together and preparing them for ligation. KU also recruits DNA-PKcs to the DSB, activating its kinase function. Targeted disruption of the genes encoding KU70 and KU80 has identified significant differences between *Ku*-deficient mice and *DNA-PKcs*-deficient mice. Limited but statistically significant DNA rejoining was found in *Ku70*-deficient mice 3.5 days after irradiation, showing the presence of a DNA DSBs repair system other than the classical NHEJ [53]. In early spermatocytes during meiotic recombination, KU70 is not detectable and then NHEJ might be suppressed [30].

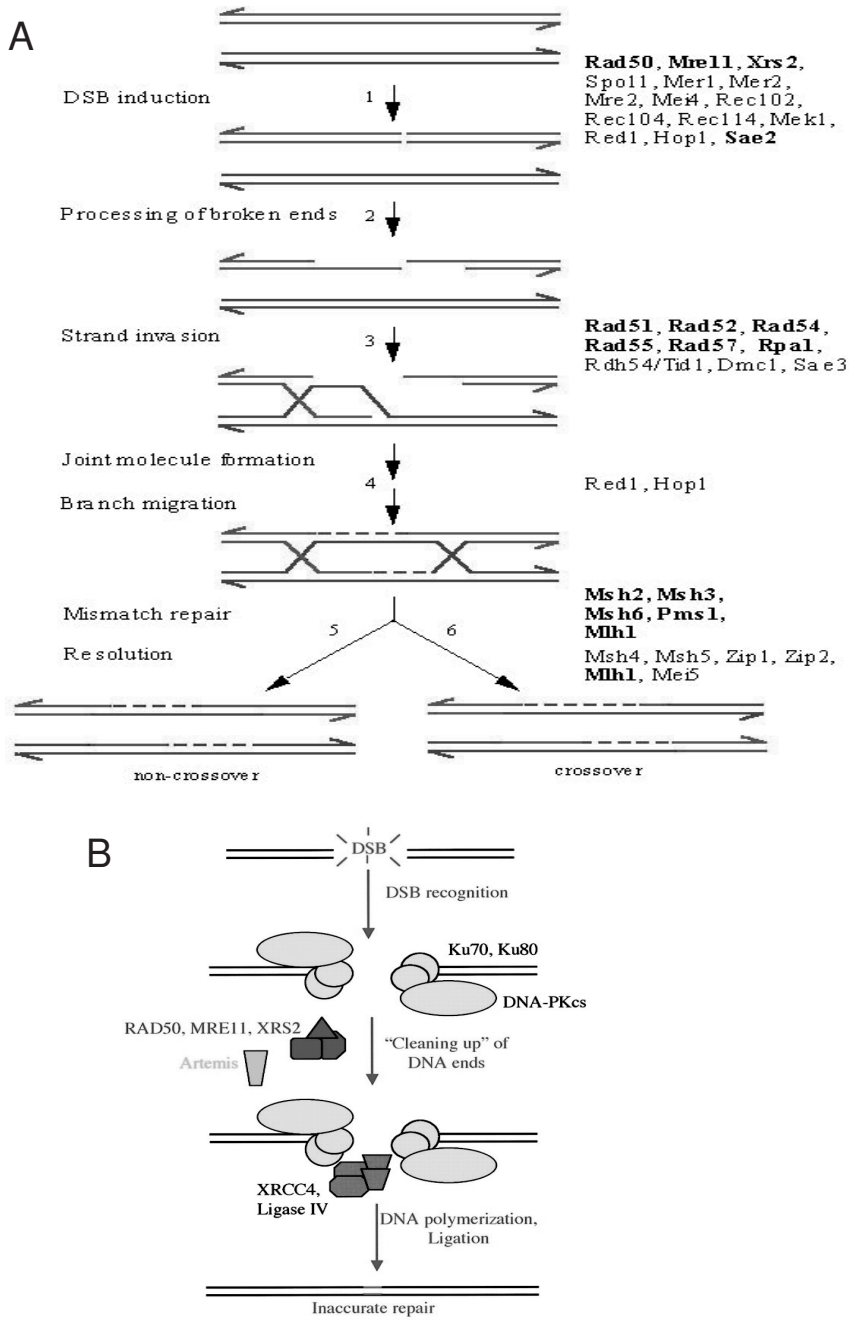

Fig. 3. *Model for DNA DSBs repair by HR (A) and classical NHEJ pathway (B). The DSB repair proteins in yeast and their mammals' homologs are shown.*

RAD51

HR is a complex process requiring a number of proteins of the RAD52 epistasis group, including RAD51 and RAD54. RAD51 is the key protein of HR and is also required for meiotic recombination, in combination with its meiotic counterpart DMC1. RAD51 is critical for homology recognition and performs strand exchange between recombining DNA molecules [18]. RAD51 deficient chicken DT40 cells accumulate chromosomal abnormalities prior to cell death [54]. Targeted disruption of mouse RAD51 leads to early embryonic lethality [55, 56].

RAD54 and RAD54B

RAD54 is a core constituent of the RAD52 epistasis group that encodes the proteins that are essential for HR in eukaryotes. RAD54 protein is a member of the Snf2-family of SF2 helicases. A novel human *RAD54* homologue, *RAD54B*, was also found to be associated with *RAD51* [57]. RAD54, but not RAD54B, is needed for a normal distribution of RAD51 on meiotic chromosomes. RAD54 possesses DNA-dependent ATPase which allows it to translocate on DNA, thereby affecting DNA topology. Biochemically, RAD54 has been implicated in multiple steps of HR. It stabilizes the RAD51 nucleoprotein filament in an early stage of recombination [19] and subsequently promotes chromatin remodeling [20, 21] and stimulates RAD51-mediated formation of a joint molecule between the broken DNA and the repair template. In later stages of the reaction it can displace RAD51 from DNA [22]. While ablation of RAD54 in mouse embryonic stem (ES) cells leads to a mild reduction in homologous recombination efficiency, the absence of RAD54B has little effect. However, the absence of both Rad54 and Rad54B dramatically reduces homologous recombination efficiency [58].

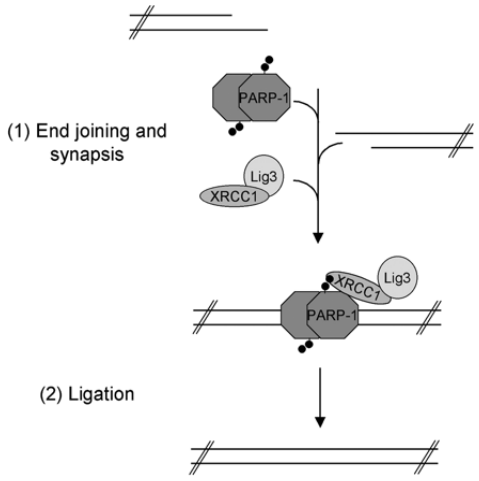

Fig. 4. *Model for NHEJ repair by alternative pathway including XRCC1-PARP1 and DNA ligase III (Lig3). Audebert et al., [28].*

PARP1

PARP1 is an abundant nuclear enzyme of higher eukaryotes that has been implicated in many cellular processes including DNA repair. It is a member of a superfamily of eighteen proteins containing PARP domains, of which only PARP-2 has also been implicated in the DNA damage response. *Parp1* knockout mice are fertile and viable but sensitive to ionizing radiation. *Parp-2*-deficient mice exhibit severely impaired spermatogenesis, with a defect in prophase of meiosis I characterized by massive apoptosis at pachytene and metaphase I stages [59]. Recently, Audebert et al [28] proposed an alternative route that has *in vitro* end-joining activity independent of the DNA-PK/XRCC4-ligase IV complex but required a novel synaptic activity of PARP1 and the ligation activity of the XRCC1-DNA ligase III complex, proteins otherwise involved in the base excision repair pathway (Fig. 4). This alternative pathway of NHEJ may function as a backup (B-NHEJ) to the DNA-PK dependent pathway (D-NHEJ) particularly in the absence of KU or essential components of the classical pathway and then PARP1 and KU compete for repair of DNA DSBs [60].

XRCC1

X-ray repair cross complementing group 1 (XRCC1) is involved in single strand repair (SSR) repair, base excision repair (BER) or together with PARP1, DNA ligase III may be involved in an alternative route to repair DSBs by NHEJ. PCR results indicate that *XRCC1* is highly expressed in the mouse testis [61] but there is not much information about its role in spermatogenesis and in DNA repair in germ cells.

Somatic cells in the testis and the repair of DNA DSBs

Cells that do not divide and do not change their phenotype are known as terminally differentiated cells, being very common in multicellular eukaryotes. Numerous cell types are considered to be terminally differentiated in the adult animal, including neurons, myocytes, auditory hair cells, epidermal cells, and Sertoli cells [62]. Terminally differentiated cells may not need to bother with repairing their genome after DNA damage has been inflicted as these cells only need relatively few genes: house-keeping genes and genes involved in the specific function of the cells. Some terminally differentiated cells, like neurons or adipocytes, are allocated at birth and cannot be replaced if lost [63]. Others, like Sertoli cells in mouse, proliferate until day 16 after birth [64], whereafter they terminally differentiate. Terminal differentiation of Sertoli cells involves loss of proliferative ability, formation of functional inter-Sertoli cell tight junctions, and expression of functions or proteins not present in immature Sertoli cells (for review [62]). While the DNA repair capacity of terminally differentiated cells was examined in several cell types, no attention has been paid to DNA damage repair in adult Sertoli cells or the other quiescent somatic cell types in testis like Leydig cells and myoid cells.

DNA-PKcs, KU70, KU86, P53 and phosphorylated ATM were found in Sertoli cells after IR [31, 65, 66]. Also non phosphorylated H2A is distinctively expressed in Sertoli cells [67], but not γ-H2AX. It will be interesting to study DNA damage response in Sertoli cells.

Aim and outline of this thesis

The purpose of the work presented in this thesis was to study the expression of proteins involved in the repair of DSBs in the various testicular cell types as can be achieved by immunohistochemisty and immunofluorescence. Combining these methods with mutants for DSB repair pathways and probing with enzyme inhibitors leads to an appraisal of the role of the various DSB repair pathways in different germ cell types and in Sertoli cells after irradiation. As we included repair kinetics after irradiation insult, comparisons with somatic cell systems became more insightful, leading to a biological view of DSB repair in the meiotic and post-meiotic phases of the male germline. As in the human, the mutational load derived from the male usually is bigger compared to the female [68], the results presented here may be useful in understanding this difference.

Chapter 2

In this chapter, we have asked if proteins such as MDC1, 53BP1 and RAD51, that are involved in the formation of IRIFs, upon the induction of a DSB in somatic cells, also play a role in the processing of recombination and irradiation induced DSBs in the various spermatogenic cell types. This because previous studies suggested that not all types of germ cells express all of the repair proteins normally seen in somatic cell lines. The kinetics of foci removal were studied in vivo, as well as the interplay between recombination and irradiation induced DNA double strand break processing.

Chapter 3

This chapter describes our studies into the *in vitro* ability of adult terminally differentiated quiescent Sertoli cells to divide and repair DNA damage. This because our studies described in chapter 2 revealed that in these cells the numbers of radiation induced 53BP1 foci diminishes with time while these cells express a rather limited number of repair proteins. In addition, while these cells in the adult testis are quiescent they do express a puzzling mixture of proliferation inducers and inhibitors.

Chapter 4

This chapter describes the expression of the PARP1 and XRCC1 proteins in the irradiated and non irradiated mouse testis and also the involvement of the alternative NHEJ pathway relying on PARP1/XRCC1, in the repair of DSBs in round spermatids. The immunocytochemical

approach allowed round spermatids to be differentiated as to DSB repair kinetics. Our data point to a sensitivity of round spermatids for the induction of chromosome aberrations that is not accompanied by an apoptosis response.

Chapter 5

In chapter 5, an attempt is made to assess the relative contribution of HR and NHEJ in early pachytene spermatocytes (during crossing-over) and at the end of pachytene, early diplotene (after crossing-over). We have followed the kinetics of γ-H2AX foci loss, after irradiation, in nucleus spreads from scid mice (deficient in NHEJ), *Rad54/Rad54B* double knockout mice (potentially deficient for HR) and wild type controls. Results point at communication between these two pathways, also during spermatogenesis.

References

1. Tegelenbosch RA, de Rooij DG. A quantitative study of spermatogonial multiplication and stem cell renewal in the C3H/101 F1 hybrid mouse. Mutat Res 1993; 290: 193-200.
2. de Rooij DG. Spermatogonial stem cell renewal in the mouse. I. Normal situation. Cell Tissue Kinet 1973; 6: 281-287.
3. de Rooij DG, Russell LD. All you wanted to know about spermatogonia but were afraid to ask. J Androl 2000; 21: 776-798.
4. Keeney S. Mechanism and control of meiotic recombination initiation. Curr Top Dev Biol 2001; 52: 1-53.
5. Oakberg EF. A description of spermiogenesis in the mouse and its use in analysis of the cycle of the seminiferous epithelium and germ cell renewal. Am J Anat 1956; 99: 391-413.
6. Leblond CP, Clermont Y. Definition of the stages of the cycle of the seminiferous epithelium in the rat. Ann N Y Acad Sci 1952; 55: 548-573.
7. Russell LD, Ettlin RA, Hikim APS, and Clegg ED. Histological and histopathological evaluation of the testis, Cache River Press 1990, Clearwater, Fl. USA.
8. Szostak JW, Orr-Weaver TL, Rothstein RJ, Stahl FW. The double-strand-break repair model for recombination. Cell 1983; 33: 25-35.
9. Lukas J, Lukas C, Bartek J. Mammalian cell cycle checkpoints: signalling pathways and their organization in space and time. DNA Repair 2004; 3: 997-1007.
10. Iliakis G, Wang Y, Guan J, Wang H. DNA damage checkpoint control in cells exposed to ionizing radiation. Oncogene 2003; 22: 5834-5847.
11. Shrivastav M, De Haro LP, Nickoloff JA. Regulation of DNA double-strand break repair pathway choice. Cell Res 2008; 18: 134-147.
12. Featherstone C, Jackson SP. Ku, a DNA repair protein with multiple cellular functions? Mutat Res 1999; 434: 3-15.
13. Meek K, Gupta S, Ramsden DA, Lees-Miller SP. The DNA-dependent protein kinase: the director at the end. Immunol Rev 2004; 200: 132-141.
14. Ma Y, Pannicke U, Lu H, Niewolik D, Schwarz K, Lieber MR. The DNA-dependent protein kinase catalytic subunit phosphorylation sites in human Artemis. J Biol Chem 2005; 280: 33839-33846.
15. Ma Y, Lu H, Schwarz K, Lieber MR. Repair of double-strand DNA breaks by the human nonhomologous DNA end joining pathway: the iterative processing model. Cell Cycle 2005; 4: 1193-1200.
16. Ma Y, Lu H, Tippin B, Goodman MF, Shimazaki N, Koiwai O, Hsieh CL, Schwarz K, Lieber MR. A biochemically defined system for mammalian nonhomologous DNA end joining. Mol Cell 2004; 16: 701-713.
17. Bannister LA, Schimenti JC. Homologous recombinational repair proteins in mouse meiosis. Cytogenet Genome Res 2004; 107: 191-200.
18. Symington LS. Role of RAD52 epistasis group genes in homologous recombination and double-strand break repair. Microbiol Mol Biol Rev 2002; 66: 630-670
19. Mazin AV, Alexeev AA, Kowalczykowski SC. A novel function of Rad54 protein. Stabilization of the Rad51 nucleoprotein filament. J Biol Chem 2003; 278: 14029-14036.

20. Alexeev A, Mazin A, Kowalczykowski SC. Rad54 protein possesses chromatin-remodeling activity stimulated by the Rad51-ssDNA nucleoprotein filament. Nat Struct Biol 2003; 10: 182-186.
21. Alexiadis V, Lusser A, Kadonaga JT. A conserved N-terminal motif in Rad54 is important for chromatin remodeling and homologous strand pairing. J Biol Chem 2004; 279: 27824-27829.
22. Bugreev DV, Hanaoka F, Mazin AV. Rad54 dissociates homologous recombination intermediates by branch migration. Nat Struct Mol Biol 2007; 14: 746-753.
23. Lee SE, Mitchell RA, Cheng A, Hendrickson EA. Evidence for DNA-PK-dependent and -independent DNA double-strand break repair pathways in mammalian cells as a function of the cell cycle. Mol Cell Biol 1997; 17: 1425-1433.
24. Takata M, Sasaki MS, Sonoda E, Morrison C, Hashimoto M, Utsumi H, Yamaguchi-Iwai Y, Shinohara A, Takeda S. Homologous recombination and non-homologous end-joining pathways of DNA double-strand break repair have overlapping roles in the maintenance of chromosomal integrity in vertebrate cells. Embo J 1998; 17: 5497-5508.
25. Fukushima T, Takata M, Morrison C, Araki R, Fujimori A, Abe M, Tatsumi K, Jasin M, Dhar PK, Sonoda E, Chiba T, Takeda S. Genetic analysis of the DNA-dependent protein kinase reveals an inhibitory role of Ku in late S-G2 phase DNA double-strand break repair. J Biol Chem 2001; 276: 44413-44418.
26. Rothkamm K, Kruger I, Thompson LH, Lobrich M. Pathways of DNA double-strand break repair during the mammalian cell cycle. Mol Cell Biol 2003; 23: 5706-5715.
27. Frank-Vaillant M, Marcand S. Transient stability of DNA ends allows nonhomologous end joining to precede homologous recombination. Mol Cell 2002; 10: 1189-1199.
28. Audebert M, Salles B, Calsou P. Involvement of poly(ADP-ribose) polymerase-1 and XRCC1/DNA ligase III in an alternative route for DNA double-strand breaks rejoining. J Biol Chem 2004; 279: 55117-55126.
29. Mahadevaiah SK, Turner JM, Baudat F, Rogakou EP, de Boer P, Blanco-Rodriguez J, Jasin M, Keeney S, Bonner WM, Burgoyne PS. Recombinational DNA double-strand breaks in mice precede synapsis. Nat Genet 2001; 27: 271-276.
30. Goedecke W, Eijpe M, Offenberg HH, van Aalderen M, Heyting C. Mre11 and Ku70 interact in somatic cells, but are differentially expressed in early meiosis. Nat Genet 1999; 23: 194-198.
31. Hamer G, Roepers-Gajadien HL, van Duyn-Goedhart A, Gademan IS, Kal HB, van Buul PP, Ashley T, de Rooij DG. Function of DNA-protein kinase catalytic subunit during the early meiotic prophase without Ku70 and Ku86. Biol Reprod 2003; 68: 717-721.
32. Rogakou EP, Pilch DR, Orr AH, Ivanova VS, Bonner WM. DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. J Biol Chem 1998; 273: 5858-5868.
33. Srivastava N, Gochhait S, de Boer P, Bamezai RN. Role of H2AX in DNA damage response and human cancers. Mutat Res 2009; 681: 180-188.
34. Kuo LJ, Yang LX. Gamma-H2AX - a novel biomarker for DNA double-strand breaks. In Vivo 2008; 22: 305-309.
35. Celeste A, Difilippantonio S, Difilippantonio MJ, Fernandez-Capetillo O, Pilch DR, Sedelnikova OA, Eckhaus M, Ried T, Bonner WM, Nussenzweig A. H2AX haploinsufficiency modifies genomic stability and tumor susceptibility. Cell 2003; 114: 371-383.

36. Stucki M, Jackson SP. gammaH2AX and MDC1: anchoring the DNA-damage-response machinery to broken chromosomes. DNA Repair 2006; 5: 534-543.
37. Chicheportiche A, Bernardino-Sgherri J, de Massy B, Dutrillaux B. Characterization of Spo11-dependent and independent phospho-H2AX foci during meiotic prophase I in the male mouse. J Cell Sci 2007; 120: 1733-1742.
38. Nazarov IB, Smirnova AN, Krutilina RI, Svetlova MP, Solovjeva LV, Nikiforov AA, Oei SL, Zalenskaya IA, Yau PM, Bradbury EM, Tomilin NV. Dephosphorylation of histone gamma-H2AX during repair of DNA double-strand breaks in mammalian cells and its inhibition by calyculin A. Radiat Res 2003; 160: 309-317.
39. Derijck A, van der Heijden G, Giele M, Philippens M, de Boer P. DNA double-strand break repair in parental chromatin of mouse zygotes, the first cell cycle as an origin of de novo mutation. Hum Mol Genet 2008; 17: 1922-1937.
40. Bouquet F, Muller C, Salles B. The loss of gammaH2AX signal is a marker of DNA double strand breaks repair only at low levels of DNA damage. Cell Cycle 2006; 5: 1116-1122.
41. Wang B, Matsuoka S, Carpenter PB, Elledge SJ. 53BP1, a mediator of the DNA damage checkpoint. Science 2002; 298: 1435-1438.
42. Lou Z, Chini CC, Minter-Dykhouse K, Chen J. Mediator of DNA damage checkpoint protein 1 regulates BRCA1 localization and phosphorylation in DNA damage checkpoint control. J Biol Chem 2003; 278: 13599-13602.
43. Stewart GS, Wang B, Bignell CR, Taylor AM, Elledge SJ. MDC1 is a mediator of the mammalian DNA damage checkpoint. Nature 2003; 421: 961-966.
43. Xu X, Stern DF. NFBD1/MDC1 regulates ionizing radiation-induced focus formation by DNA checkpoint signaling and repair factors. FASEB J 2003; 17: 1842-1848.
45. Lou Z, Chen BP, Asaithamby A, Minter-Dykhouse K, Chen DJ, Chen J. MDC1 regulates DNA-PK autophosphorylation in response to DNA damage. J Biol Chem 2004; 279: 46359-46362.
46. Lou Z, Minter-Dykhouse K, Franco S, Gostissa M, Rivera MA, Celeste A, Manis JP, van Deursen J, Nussenzweig A, Paull TT, Alt FW, Chen J. MDC1 maintains genomic stability by participating in the amplification of ATM-dependent DNA damage signals. Mol Cell 2006; 21: 187-200.
47. Schultz LB, Chehab NH, Malikzay A, Halazonetis TD. p53 binding protein 1 (53BP1) is an early participant in the cellular response to DNA double-strand breaks. J Cell Biol 2000; 151: 1381-1390.
48. Ward IM, Minn K, van Deursen J, Chen J. p53 Binding protein 53BP1 is required for DNA damage responses and tumor suppression in mice. Mol Cell Biol 2003; 23: 2556-2563.
49. Adams MM, Carpenter PB. Tying the loose ends together in DNA double strand break repair with 53BP1. Cell Div 2006; 1: 19.
50. Nakamura K, Sakai W, Kawamoto T, Bree RT, Lowndes NF, Takeda S, Taniguchi Y. Genetic dissection of vertebrate 53BP1: a major role in non-homologous end joining of DNA double strand breaks. DNA Repair 2006; 5: 741-749.
51. Calsou P, Delteil C, Frit P, Drouet J, Salles B. Coordinated assembly of Ku and p460 subunits of the DNA-dependent protein kinase on DNA ends is necessary for XRCC4-ligase IV recruitment. J Mol Biol 2003; 326: 93-103.

52. de Rooij DG, de Boer P. Specific arrests of spermatogenesis in genetically modified and mutant mice. Cytogenet Genome Res 2003; 103: 267-276.
53. Uehara Y, Ikehata H, Komura J, Ito A, Ogata M, Itoh T, Hirayama R, Furusawa Y, Ando K, Paunesku T, Woloschak GE, Komatsu K, Matsuura S, Ikura T, Kamiya K, Ono T. Absence of Ku70 gene obliterates X-ray-induced lacZ mutagenesis of small deletions in mouse tissues. Radiat Res 2008; 170: 216-223.
54. Sonoda E, Sasaki MS, Buerstedde JM, Bezzubova O, Shinohara A, Ogawa H, Takata M, Yamaguchi-Iwai Y, Takeda S. Rad51-deficient vertebrate cells accumulate chromosomal breaks prior to cell death. EMBO J 1998; 17: 598-608.
55. Lim DS, Hasty P. A mutation in mouse rad51 results in an early embryonic lethal that is suppressed by a mutation in p53. Mol Cell Biol 1996; 16: 7133-7143.
56. Tsuzuki T, Fujii Y, Sakumi K, Tominaga Y, Nakao K, Sekiguchi M, Matsushiro A, Yoshimura Y, MoritaT. Targeted disruption of the Rad51 gene leads to lethality in embryonic mice. Proc Natl Acad Sci U S A 1996; 93: 6236-6240.
57. Tanaka K, Hiramoto T, Fukuda T, Miyagawa K. A novel human rad54 homologue, Rad54B, associates with Rad51. J Biol Chem 2000; 275: 26316-26321.
58. Wesoly J, Agarwal S, Sigurdsson S, Bussen W, Van Komen S, Qin J, van Steeg H, van Benthem J, Wassenaar E, Baarends WM, Ghazvini M, Tafel AA, Heath H, Galjart N, Essers J, Grootegoed JA, Arnheim N, Bezzubova O, Buerstedde JM, Sung P, Kanaar R. Differential contributions of mammalian Rad54 paralogs to recombination, DNA damage repair, and meiosis. Mol Cell Biol 2006; 26: 976-989.
59. Dantzer F, Mark M, Quenet D, Scherthan H, Huber A, Liebe B, Monaco L, Chicheportiche A, Sassone-Corsi P, de Murcia G, Menissier-de Murcia J. Poly(ADP-ribose) polymerase-2 contributes to the fidelity of male meiosis I and spermiogenesis. Proc Natl Acad Sci U S A 2006; 103: 14854-14859.
60. Wang M, Wu W, Wu W, Rosidi B, Zhang L, Wang H, Iliakis G. PARP-1 and Ku compete for repair of DNA double strand breaks by distinct NHEJ pathways. Nucleic Acids Res 2006; 34: 6170-6182.
61. Walter CA, Trolian DA, McFarland MB, Street KA, Gurram GR, McCarrey JR. Xrcc-1 expression during male meiosis in the mouse. Biol Reprod 1996; 55: 630-635.
62. Sharpe RM, McKinnell C, Kivlin C, Fisher JS. Proliferation and functional maturation of Sertoli cells, and their relevance to disorders of testis function in adulthood. Reproduction 2003; 125: 769-784.
63. Nouspikel T, Hanawalt PC. DNA repair in terminally differentiated cells. DNA Repair 2002; 1: 59-75.
64. Van Haaster LH, De Jong FH, Docter R, De Rooij DG. The effect of hypothyroidism on Sertoli cell proliferation and differentiation and hormone levels during testicular development in the rat. Endocrinology 1992; 131: 1574-1576.
65. Hamer G, Roepers-Gajadien HL, van Duyn-Goedhart A, Gademan IS, Kal HB, van Buul PP, de Rooij DG. DNA double-strand breaks and gamma-H2AX signaling in the testis. Biol Reprod 2003; 68: 628-634.

66. Hamer G, Kal HB, Westphal CH, Ashley T, de Rooij DG. Ataxia telangiectasia mutated expression and activation in the testis. Biol Reprod 2004; 70: 1206-1212.
67. Bhatnagar YM. Immunocytochemical localization of an H2A variant in the spermatogenic cells of the mouse. Biol Reprod 1985; 32: 957-968.
68. Crow JF. The origins, patterns and implications of human spontaneous mutation. Nat Rev Genet 2000; 1: 40-47.

Chapter 2

Differences in DNA double stand breaks repair in male germ cell types: Lessons learned from a differential expression of Mdc1 and 53BP1

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Differences in DNA double strand breaks repair in male germ cell types: Lessons learned from a differential expression of Mdc1 and 53BP1

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ABSTRACT

In male germ cells the repair of DNA double strand breaks (DSBs) differs from that described for somatic cell lines. Irradiation induced immunofluorescent foci (IRIF's) signifying a double strand DNA breaks, were followed in spermatogenic cells up to 16 h after the insult. Foci were characterised for Mdc1, 53BP1 and Rad51 that always were expressed in conjecture with γ-H2AX. Subsequent spermatogenic cell types were found to have different repair proteins. In early germ cells up to the start of meiotic prophase, i.e. in spermatogonia and preleptotene spermatocytes, 53BP1 and Rad51 are available but no Mdc1 is expressed in these cells before and after irradiation. The latter might explain the radiosensitivity of spermatogonia. Spermatocytes from shortly after premeiotic S-phase till pachytene in epithelial stage IV/V express Mdc1 and Rad51 but no 53BP1 which has no role in recombination involved repair during the early meiotic prophase. Mdc1 is required during this period as in Mdc1 deficient mice all spermatocytes enter apoptosis in epithelial stage IV when they should start mid-pachytene phase of the meiotic prophase. From stage IV mid pachytene spermatocytes to round spermatids, Mdc1 and 53BP1 are expressed while Rad51 is no longer expressed in the haploid round spermatids. Quantifying foci numbers of γ-H2AX, Mdc1 and 53BP1 at various time points after irradiation revealed a 70% reduction after 16 h in pachytene and diplotene spermatocytes and round spermatids. Although the DSB repair efficiency is higher then in spermatogonia where only a 40% reduction was found, it still does not compare to somatic cell lines where a 70% reduction occurs in 2 h. Taken together, DNA DSBs repair proteins differ for the various types of spermatogenic cells, no germ cell type possessing the complete set. This likely leads to a compromised efficiency relative to somatic cell lines. From the evolutionary point of view it may be an advantage when germ cells die from DNA damage rather than risk the acquisition of transmittable errors made during the repair process.

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1. Introduction

Of all types of DNA damage, DNA double strand breaks (DSBs) represent the greatest threat to the integrity of the genome. DNA DSBs can be induced by exogenous agents such as ionizing radiation (IR), or by replication stress and occur naturally in meiotic recombination and V(D)J recombination [1]. Cells have complicated mechanisms to maintain genomic integrity using cell-cycle checkpoints [2] and DNA repair pathways [3] in response to DNA damage. Considerable progress is being made in our understanding of the DNA damage response. The identity of a number of key proteins involved in the repair of DNA was established. According to a recent model for the recruitment and retention of DNA repair proteins at the sites of DNA double strand breaks, DNA DSBs are recognized by various proteins, including the MRN complex, which recruit Atm to free DNA ends by a direct interaction between Atm and the Nbs1 C-terminus [4]. Atm thus becomes activated and phosphorylates various target proteins at the site of DNA damage including H2AX [5]. Next, Mdc1 (mediator of DNA damage checkpoint protein 1) recognizes the phosphorylated H2AX via its tandem BRCT domain and acts as a mediator/adaptor protein providing a "landing-platform" for the MRN complex to bind [6–8]. Subsequently, Mdc1 can attract Atm and other proteins, such as 53BP1 (p53 binding protein 1) and BRCA1 [6,9–11] or DNA–PKcs and its interaction partner Ku (in the case of non homologous end joining repair; NHEJ) to the site of damage which then in turn leads to the phosphorylation of even more H2AX [4]. Hence, Mdc1 seems to fulfil a crucial role at a time point even before the decision is made to repair via the NHEJ or the HR pathway. Recently, Mdc1 was found to regulate DNA–PK autophosphorylation in response to DNA damage [12] and to act as a key upstream determinant of 53BP1's interaction with DSBs [13]. Nakamura et al. [14] proposed a major role for 53BP1 in non-homologous end joining of DNA double strand breaks.

The above data were obtained in somatic cells but for germ cells the situation may well be different due to differences in the expression or even the absence of some DNA repair proteins in particular male germ cell types. Male germ cells can be subdivided into three main types. First, spermatogonia that include stem cells and go through a series of many mitotic divisions. Most generations of spermatogonia do not show heterochromatin and are called A spermatogonia. The last two generations in the mouse do develop increasing amounts of heterochromatin and are called intermediate (In) and B spermatogonia, respectively. The division of the B spermatogonia renders the second type of germ cells, the spermatocytes. The spermatocytes pass through G1 and S phase and subsequently meiotic prophase is initiated which starts with chromosome contraction (leptotene), followed by initiation of synapsis between homologous chromosomes (zygotene). At the completion of this process, pachytene is reached during which stage homologous recombination intermediates are processed. The results of reciprocal exchanges between non-sister chromatids are visible as chaismata from the diplotene stage on. After renewed chromosome contraction, diakinesis and metaphase are reached at stage XII of the cycle of the seminiferous epithelium, during which the first and second meiotic division are completed, leading to the production of spermatids, the third type of male germ cells. After some 6 days, spermatids, that are round at first, start the elongation process and DNA repair processes essentially come to a halt.

Interestingly it was found that Ku70, a key protein in NHEJ, is not expressed in early spermatocytes [15,16] and another key protein involved in NEHJ, DNA–PKcs is absent in round spermatids [16]. On the other hand, the ATM-Chk2 module and γ-H2AX are physiologically active in human spermatocytes where DNA DSBs occur during meiotic recombination [17], demonstrating that the upstream signaling from DSB lesions is functional in at least some stages of spermatogenesis. Clearly, DSB repair in male germ cells does not necessarily follow the principles established for somatic cells, making it important to find out more about the possibilities of male germ cells to repair DNA damage, especially since failure to do so properly, may lead to the formation of mutated spermatozoa.

In the present investigation, we have asked ourselves if proteins such as Mdc1, 53BP1 and Rad51, that are involved in the formation of irradiation induced immunofluorescent foci (IRIF) [18], also play a role in the processing of recombination and irradiation induced DNA DSBs of the various spermatogenic cell types. The virtue of the seminiferous epithelium is that spermatogenic cell types can be unequivocally recognized [19], and that fixation protocols are compatible with epitope detection by both light microscopy and confocal laser-microscopy. Chromatin areas that were suspect for containing a DSB were identified by γ-H2AX staining. Also the kinetics of foci removal were studied in vivo, as well as the interplay between recombination and irradiation induced DNA double strand break processing.

2. Materials and methods

2.1. *Animals, irradiation and fixation*

Testes of male FvB mice, between 8 weeks and 10 weeks of age, were given a dose of 4 Gy of X-rays (local irradiation, 200 kV, 20 mA, 0.5-mm Cu filter; Philips, Eindhoven, The Netherlands). Mice were divided into groups of four, and 5 min, 10 min, 20 min, 1 h, 2 h, 4 h, 8 h, and 16 h after irradiation, a group was killed. Control mice were sham irradiated. Mice were killed by cervical dislocation and testes were fixed in three different fixatives/procedures: 4% phosphate-buffered formaldehyde (pH 7–7.4, Klinipath, The Netherlands, 4078.9001) for 24 h at room temperature, 4% paraformaldehyde in PBS for 24 h at 4 °C and 4% phosphate-buffered formaldehyde (pH 7–7.4) for 4 h at room temperature and post-fixed in a diluted Bouin solution [20,21] for 16 h at 4 °C. Tissues were washed in 70% EtOH prior to embedding in paraffin (Stemcowax; Adamas Instruments, Amerongen, The Netherlands). The animals were used and maintained according to regulations provided by the animal ethical committee of the Utrecht University that also approved the experiments.

The Mdc1 gene was disrupted by gene trapping to generate Mdc1−/− mice [8]. Testes from control and knockout mice were fixed in Bouin to be stained with PAS-hematoxylin for the analysis of spermatogenesis in Mdc1−/− testes.

2.2. Immunohistochemistry

Sections (5 μm) of testes of control mice and of mice killed at different time intervals after irradiation were mounted together on a TESPA (3-aminoproyl-tri-ethoxysilane)-coated glass slide and dried overnight at 37 °C. Sections were dewaxed in xylene and hydrated in a graded series of alcohols. Between each step, sections were washed in PBS. For Mdc1 staining, the sections were boiled once for 10 min in 0.01 M sodium citrate using a microwave oven (H2500; Bio-Rad, Hercules, USA). For 53BP1 and Rad51 sections were boiled twice for 10 min in 0.01 M sodium citrate. Sections were incubated in 0.35% H_2O_2 in PBS for 10 min. Blocking was done in 5% BSA (Sigma, St. Louis, USA, A-7906)/5% goat serum (Aurion, Wageningen, The Netherlands) in PBS. The slides were then incubated with mouse monoclonal antibody against Mdc1 (clone DCS-380-1) or rabbit polyclonal against γ-H2AX (antiphospho-H2AX [Ser139]; Upstate Biotechnology, Lake Placid, NY, IHC-00059). The hybridoma DCS-380 was made from splenocytes of a Balb/c mouse immunized by recombinant human MDC1 protein, and the antibody validated for its specificity and performance in both Western blotting and immunostaining applications. For 53BP1, we used rabbit polyclonal antibody (Alexis Biochemicals, 210419R050 CH 4415 Lausen/Switzerland). The antibodies were diluted 1:200 in PBS including 1% BSA in a humidified chamber overnight at 4 °C. For Rad51, we used polyclonal rabbit antibody (2307) anti hRAD51 (a gift from R Kanaar Erasmus MC Rotterdam) diluted in 1:300 in PBS including 10% BSA in a humidified chamber for 60 min at room temperature. Incubations were carried out with secondary PowerVision Poly Hrp-anti mouse/rabbit (ImmunoVision Technologies, Co. Brisbane, CA 94005, USA) diluted 1:100 in PBS including 1% BSA, in a humidified chamber for 60 min at room temperature. Bound antibodies were visualized using 0.3 μg/μl 3, 3′-diaminobenzidine (DAB; Sigma) in PBS, then the solution was activated by 0.03% H_2O_2. Sections were counterstained with Mayer hematoxylin. For negative controls, primary antibodies were replaced by rabbit or mouse IgGs. Sections were dehydrated in a series of graded alcohols and xylene and mounted with Pertex (Cellpath Ltd., Hemel Hempstead, UK).

2.3. Immunofluorescence

For confocal microscopy, a protocol similar to that for immunohistochemistry was used. For antigen retrieval, the sections were boiled for 10 min in 0.01 M sodium citrate using an H2500 microwave oven. For double labeling, slides were incubated whether in a mixture of mouse monoclonal antibody against Mdc1 and rabbit polyclonal antibody against γ-H2AX or in a mixture of Mdc1 and Rad51. For co-localization of γ-H2AX and 53BP1, we used mouse monoclonal antibody against γ-H2AX (antiphospho-H2AX [Ser139]; Upstate Biotechnology, Lake Placid, NY JBW301). Slides were incubated in 50 mM glycine in PBS for 30 min. Fluorescein isothiocyanate-labeled goat anti-rabbit (Alexa flour 488, A-11008), goat anti-mouse (Alexa flour 488, A-21121 and 594, A-21125) and Texas Red-labeled goat anti-mouse were obtained from Jackson ImmunoResearch West Grove, USA, T-2767). The sections were mounted in VECTAshield (Vector) and viewed with a 63× planapo objective on a Leitz DMIRB fluorescence microscope (Leica, Voorburg, The Netherlands) interfaced with a Leica TCS4D confocal laser scanning microscope (Leica, Heidelberg, Germany). Images were recorded digitally. Negative control sections were treated in the same way, except that the primary antibodies were replaced by rabbit or mouse IgGs.

2.4. Pachytene substages

The cycle of the seminiferous epithelium in the mouse can be subdivided into 12 stages, I to XII, that can be distinguished by the types of germ cells present [19]. Zygotene spermatocytes develop into pachytenes in epithelial stage XII and these pachytenes enter diplotene phase in stage X. In view of the long duration of pachytene phase of the meiotic prophase, pachytene spermatocytes were divided into early (epithelial stages XII to III), mid (epithelial stages IV to VII) and late (epithelial stages VIII to X), adapted and extended from the pachytene substages proposed by Moses [22].

3. Results

3.1. Mdc1 is expressed during early meiotic prophase but not during pre-meiosis in control testis

In the leptotene stage of meiosis (epithelial stage IX) and after the induction of meiotic DSBs, the immunohistochemical staining for Mdc1 revealed focus formation (Fig. 1A) and 2–8 foci/cell were seen. As synapsis between homologous chromosomes was initiated during zygotene (stage X), the staining for Mdc1 was less well circumscript (Fig. 1B). By late zygotene (stage XI to XII), the staining for Mdc1 became concentrated in few foci (Fig. 1C), which were bigger in size than those in leptotene spermatocytes. In early pachytene (late XII to early I) Mdc1 staining was restricted to a tadpole-shaped structure (Fig. 1D), which in view of the co-localization with γ-H2AX (see below) probably represents the sex chromosomes. A dense staining for Mdc1 was found in the sex vesicles of pachytene spermatocytes as well as a weak general nuclear staining. In late pachytene spermatocytes the nuclear staining was dense. In addition, occasionally some foci were seen in pachytene spermatocytes (Fig. 1B). A weak staining for Mdc1 was found in diplotene spermatocytes and round spermatids. No Mdc1 staining was found in spermatogonia, preleptotene spermatocytes and testicular somatic cells (Fig. 1C, E and F).

3.2. Irradiation induced Mdc1 foci in late spermatocytes and round spermatids but not in spermatogonia and early spermatocytes

Within 5 min after IR, foci for Mdc1 were seen in mid (stages IV to VII) to late pachytene (stages VIII to early X) and diplotene spermatocytes (stages X to XI), and in round spermatids (Fig. 1G and H). In the normal testis, Mdc1 foci are present in leptotene spermatocytes but these foci disappeared shortly after irradiation and remained absent up till 16 h post-irradiation (Fig. 1I and K). However, we observed foci in some leptotene spermatocytes at longer time points post-irradiation

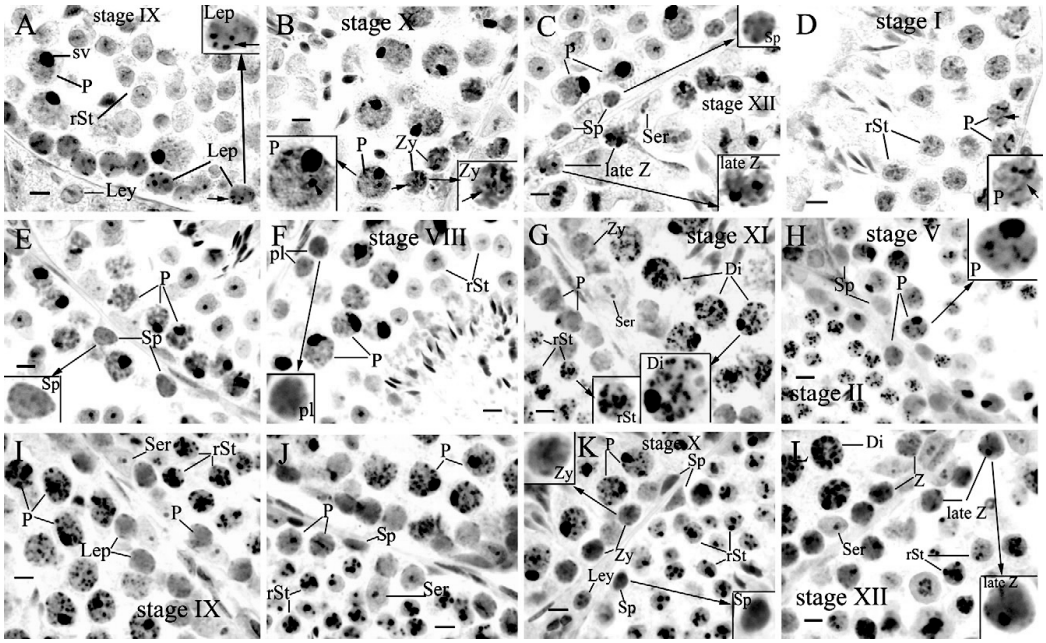


Fig. 1 – Mdc1 localization in the control and irradiated testis. (A) As the DSBs are introduced during early meiotic prophase Mdc1 showed small foci (arrows) in leptotene spermatocytes "Lep" (stage IX) followed by (B) more diffuse staining in stage X in zygotene spermatocytes "Zy". (C) By late zygotene (stage XI), Mdc1 became concentrated in two or more big foci. (D) Mdc1 localized in tadpole-shaped structure (arrows) in early pachytenes "P" (stage I). As pachytene phase progressed, Mdc1 became restricted to the sex body "sv". No Mdc1 was found in spermatogonia "Sp" (C and E), preleptotene spermatocytes "pl" (F), Leydig cells "Ley" (A) and Sertoli cells "Ser" (C). After IR, within 5 min (G), Mdc1 formed foci in pachytene spermatocytes and round spermatids. (G, K and L) Sertoli and Leydig cells were negative. Zygotene spermatocytes showed some staining (G and L) or not (K). (H) No foci were observed in early pachytene spermatocytes. Large foci were present in some round spermatids 8 h (J) and 16 h (K and L) post-irradiation. Late pachytene and diplotene spermatocytes "Di" showed many foci in stages IX and X even after 8 and 16 h of IR (J and L). Bar represents 20 μm.

(data not shown) and also a weak staining for Mdc1 in most of zygotene spermatocytes at 5 min and up till 16 h after IR was seen (Fig. 1G and L). The Mdc1 foci in pachytene spermatocytes and round spermatids were small in size at the early time points after irradiation (Fig. 1G and H) but large foci were observed in some round spermatids at 4 h after IR (Fig. 1I) and continued to be seen in most round spermatids and at 8 and 16 h post-irradiation (Fig. 1J–L). Late pachytene spermatocytes and diplotene spermatocytes showed a higher number of Mdc1 foci than mid pachytene spermatocytes (Fig. 1).

3.3. *Spermatogenesis is arrested at stage IV in Mdc1−/− mice*

We analyzed the spermatogenic defect in Mdc1−/− mice (Fig. 2A and B). An almost complete spermatogenic arrest was found in stage IV of the cycle of the seminiferous epithelium at the level of pachytene spermatocytes. The formation of spermatocytes by spermatogonia was apparently normal and the spermatocytes formed entered the meiotic prophase at the appropriate time. However, when the epithelium reached stage IV, when the spermatocytes normally would start mid pachytene, there was a massive apoptosis of virtually all spermatocytes present. Only a few surviving spermatocytes were seen a short time after stage IV, but these cells entered apoptosis or were sloughed off through the tubule lumen soon thereafter. As a result no further advanced spermatocytes could be found and no spermatids were formed in the testes of Mdc1−/− mice.

3.4. *Co-localization of Mdc1 and γ-H2AX before and after irradiation*

In order to study the interaction between Mdc1 and γ-H2AX during meiotic recombination and after IR we performed a Mdc1/γ-H2AX double staining. Mdc1 and γ-H2AX foci were found to be co-localized in leptotene (Fig. 3C) and zygotene (Fig. 3F and I) spermatocytes. Furthermore, staining was completely overlapping in the tadpole structure (sex chromosomes) of early pachytene spermatocytes (Fig. 3L) and in the sex vesicle of pachytene spermatocytes. Mdc1 foci in leptotene spermatocytes (stage IX) appeared smaller than those

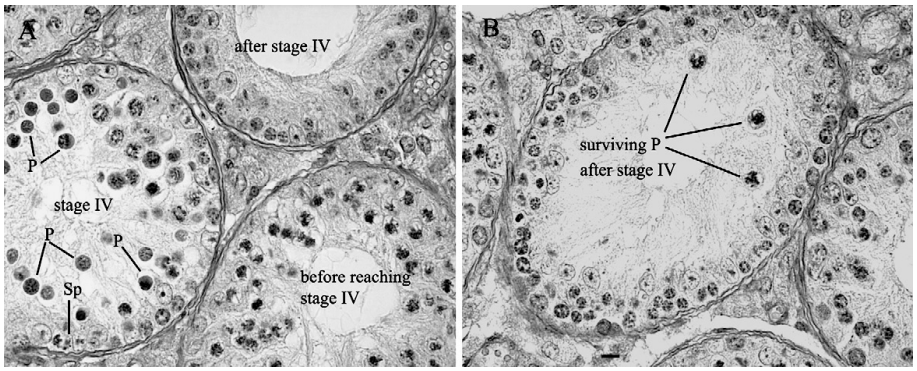


Fig. 2 – Spermatogenesis in Mdc1−/− mice. (A) Spermatogenic arrest occurred at stage IV of the cycle of the seminiferous epithelium. In this stage virtually all spermatocytes entered apoptosis and disappeared. (B) Only a few surviving spermatocytes were seen till shortly after stage IV. Spermatogonia in G2 or division (Sp), apoptotic pachytene spermatocytes (P).

of γ-H2AX and were also more numerous (Fig. 3C). After exposure to IR, Mdc1 foci of pachytene spermatocytes and round spermatids extensively overlapped with γ-H2AX foci and continued to do so up till at least 16 h after IR (Fig. 3O).

3.5. 53BP1 is expressed in spermatogonia and preleptotene spermatocytes but not during meiotic recombination in control and irradiated testes

In the control testis, a dense nuclear staining for 53BP1 was detected in Sertoli cells, the somatic cells present in the seminiferous epithelium that support spermatogenesis, and in all types of spermatogonia and preleptotene spermatocytes. Interestingly, some foci were found in the most advanced type of spermatogonia, the B type spermatogonia and in preleptotene spermatocytes (Fig. 4A and C). Subsequently, leptotene and zygotene spermatocytes were negative for 53BP1 (Fig. 4A and B). However, the sex vesicles of mid to late pachytene spermatocytes were positive while these cells as well as diplotene spermatocytes also showed some general nuclear staining (Fig. 4A–C). The nuclei of round spermatids also stained for 53BP1 (Fig. 4). After irradiation, 53BP1 very rapidly (within 5 min) formed nuclear foci in Sertoli cells, all types of spermatogonia and preleptotene spermatocytes (Fig. 4D–G). Few foci were observed in pachytene spermatocytes and round spermatids at longer time intervals post-irradiation (Fig. 4E). No 53BP1 was detected in leptotene, zygotene and early pachytene spermatocytes after IR (Fig. 4E and F).

3.6. Co-localization of 53BP1 and γ-H2AX before and after irradiation

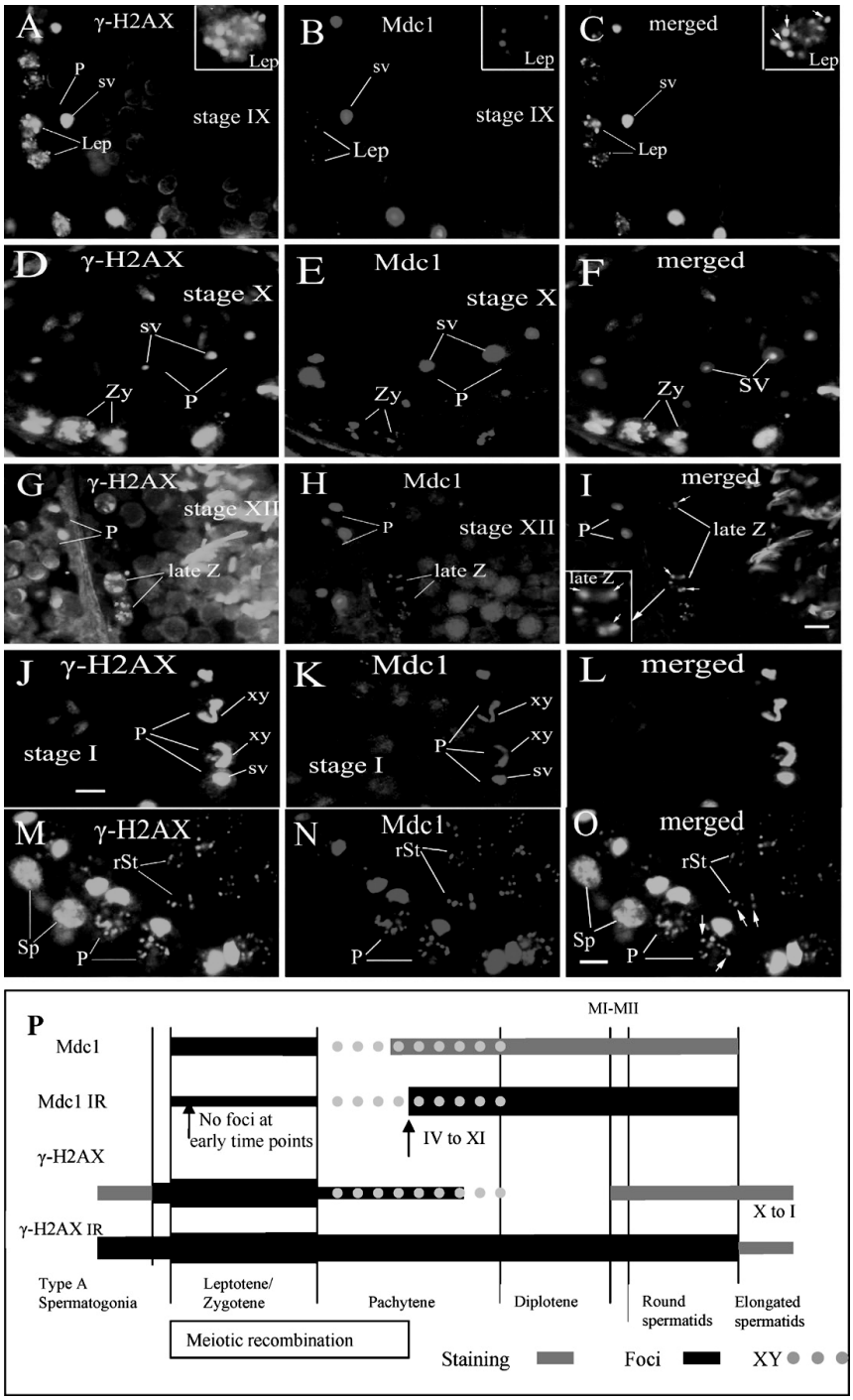
53BP1 foci were found to overlap with γ-H2AX foci in spermatogonia and preleptotene spermatocytes before and after IR (Fig. 5). In mid to late pachytene spermatocytes and round spermatids 53BP1 showed diffuse staining at early time points, however, at longer time intervals (after 8 h of IR) 53BP1 foci were seen to co-localize with γ-H2AX foci in pachytene spermatocytes and round spermatids (Fig. 5F).

3.7. Localization of Rad51 in control and irradiated testes

In the control testis, a diffuse cytoplasmic and nuclear staining was seen in all types of spermatogonia while in addition a few Rad51 nuclear foci were observed in B spermatogonia (Fig. 6A). In spermatogonia more foci were seen after IR. The early spermatocytes showed numerous small foci for Rad51 before and after IR (Figs. 6 and 7). Very few Rad51 foci were seen in late-pachytene spermatocytes before IR, but more foci were observed in these cells and even diplotene spermatocytes after IR. These foci co-localized with Mdc1 foci but they did not overlap completely (Fig. 7F). No Rad51 foci were seen in Sertoli cells before and after IR (Fig. 6A and B).

3.8. The kinetics of IRIF removal

To estimate the duration (kinetics) of the presence of Mdc1 foci we counted numbers of foci per cell in pachytene spermatocytes and round spermatids (in 50 cells) at different time points after irradiation and compared these numbers with those of γ-H2AX. Both Mdc1 and γ-H2AX foci in pachytene spermatocytes and round spermatids reached their highest numbers within a few minutes after IR. A significant reduction in foci numbers of γ-H2AX and Mdc1 was found within 4 h after IR, and the foci numbers were reduced to 50% and became bigger in size at 8 h after IR. At 16 h after IR, Mdc1 and γ-H2AX foci in pachytene spermatocytes and round spermatids showed a 70% reduction in number compared to 5 min after IR (Fig. 8). We also counted 53BP1 and γ-H2AX foci per time point in spermatogonia and preleptotene spermatocytes. These foci showed a reduction of 40% and 50% in numbers at 16 h after IR (Fig. 8). 53BP1 foci were counted in Sertoli cells at different time points after IR. The numbers of these foci showed a reduction of 60% at 16 h after IR.


A γ-H2AX
Lep
P
sv
stage IX
Lep
B Mdc1
Lep
sv
stage IX
Lep
C merged
Lep
sv
Lep
D γ-H2AX
stage X
sv
Zy
P
E Mdc1
stage X
sv
Zy
P
F merged
Zy
SV
G γ-H2AX
stage XII
P
late Z
H Mdc1
P
stage XII
late Z
I merged
P
late Z
late Z
J γ-H2AX
xy
P
xy
stage I
sv
K Mdc1
xy
P
xy
stage I
sv
L merged
M γ-H2AX
rSt
Sp
P
N Mdc1
rSt
P
O merged
rSt
Sp
P
P
MI-MII
Mdc1
Mdc1 IR
No foci at early time points
IV to XI
γ-H2AX
X to I
γ-H2AX IR
Type A Spermatogonia
Leptotene/ Zygotene
Pachytene
Diplotene
Round spermatids
Elongated spermatids
Meiotic recombination
Staining
Foci
XY

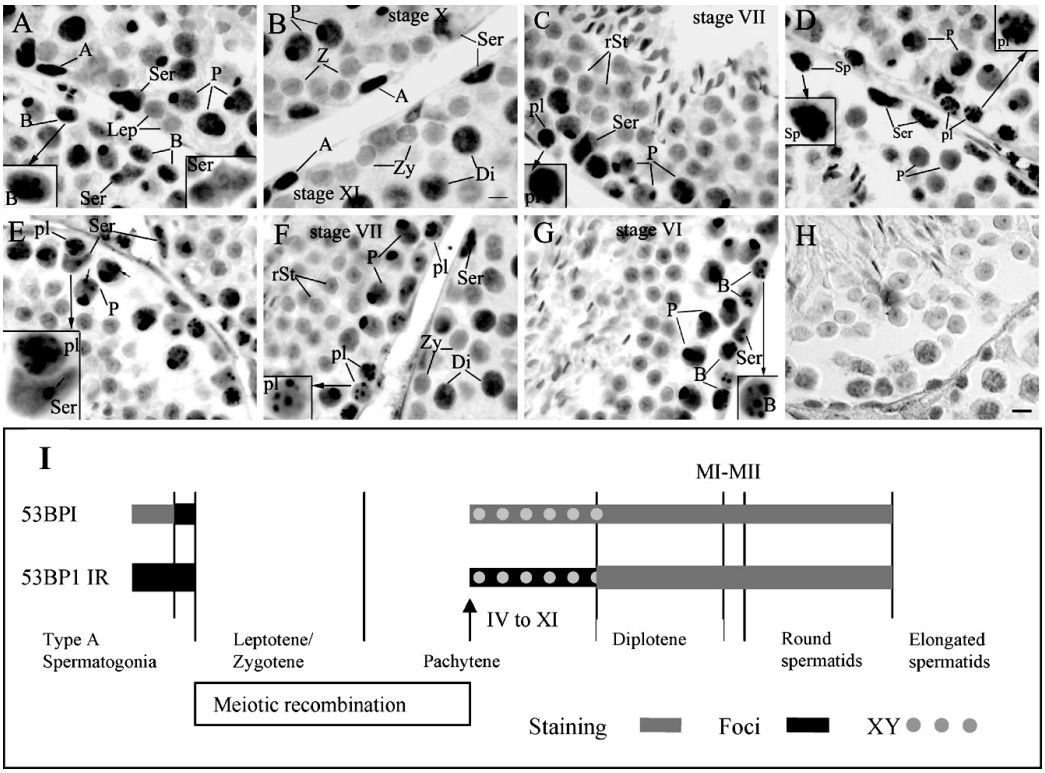


Fig. 4 – Localization of 53BP1 in control and irradiated testis. (A, B and C) There was a dense staining for 53BP1 in A spermatogonia "A", sex body "sv" and Sertoli cells "Ser", (A and C) Furthermore, there was staining in pachytene "P" and diplotene "Di" spermatocytes and round spermatids "rSt" and (A) staining or even foci in B spermatogonia "B" and preleptotene spermatocytes "pl" (C). (A and B) Leptotene "Lep" and zygotene "Zy" spermatocytes were negative. After IR, (D, E, F and G) foci were present in spermatogonia "Sp", preleptotene spermatocytes and Sertoli cells. During meiotic recombination, there was no 53BP1 in leptotene, zygotene and early pachytene spermatocytes (D and E). Large foci for 53BP1 were observed after 8 h (E) and 16 h (F and G) of IR. No staining was found in the negative control (H). (I) Schematic representation of immunohistochemical expression of 53BP1 before and after IR. The widths of the lines represent the relative intensity of the staining and foci numbers.

4. Discussion

Our results confirm and greatly extend the notion that DNA repair potential differs in different types of germ cells. It can be concluded that DNA repair in male germ cells is generally less powerful than in somatic cell lines. On basis of the present results, germ cells could be subdivided into three groups with roughly similar potentials, spermatogonia and spermatocytes that did not yet enter the meiotic prophase (preleptotene), spermatocytes in meiotic prophase up till early pachytene and germ cells from mid-pachytene onwards till round spermatids.

4.1. *Spermatogonia and preleptotene spermatocytes*

In the mitotically dividing spermatogonia and in preleptotene spermatocytes, no Mdc1 IRIFs as typified by the co-localization

Fig. 3 – Co-localization of γ-H2AX and Mdc1, in control and irradiated testis. Mdc1 and γ-H2AX foci ((arrows) overlapped in leptotene "lep" (A, B and C), early zygotene "Zy" (D, E and F) and late zygotene "late Z" spermatocytes (G, H and I). Mdc1 and γ-H2AX co-localized in tadpole-shaped structures (sex chromosomes) in early pachytene (J, K and L) and sex bodies (sv) in pachytene spermatocytes. After IR, foci in pachytene spermatocytes and round spermatids (arrows) showed a complete overlapping (M, N and O). (P) Schematic representation of immunohistochemical expression of Mdc1, and γ-H2AX before and after IR. (Adapted and extended from Goedecke et al. [15].) The widths of the lines represent the relative intensity of the staining and foci numbers.

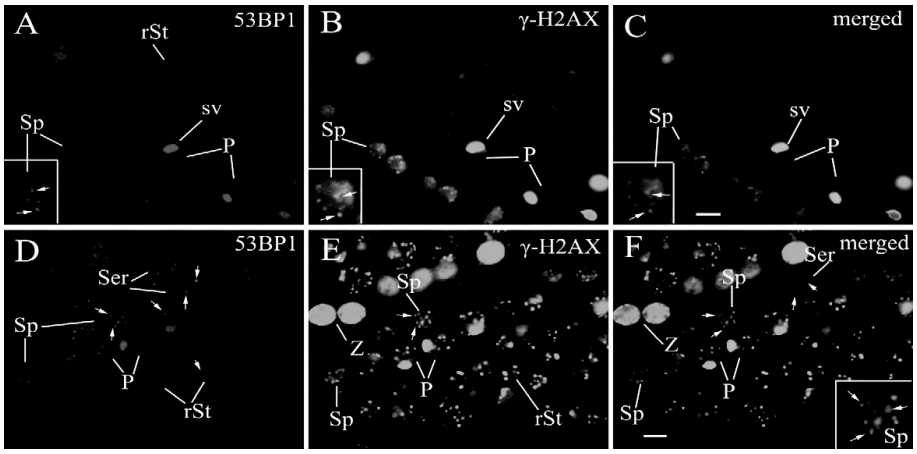


Fig. 5 – Co-localization of 53BP1 and γ-H2AX, in control (A, B and C) and irradiated testis (D, E and F). (C and F) An overlapping in spermatogonia "Sp" before and after IR (see arrows). (F) 53BP1 foci in pachytene spermatocytes "P" were seen after 8 h and overlapped with γ-H2AX foci.

with γ-H2AX and 53BP1 were found in control and irradiated testis. In somatic cells, Mdc1 is supposed to play an important role in the initiation of DNA DSB repair at a step before the decision is made for homologous recombination or NHEJ [4]. In accordance with this, Mdc1 deficient cells are radiosensitive [6]. Taken together, this suggests that the high radiosensitivity of spermatogonia as determined by cell killing assays [23] may be related to the absence of Mdc1.

The question then is: are spermatogonia and preleptotene spermatocytes capable of any repair of DNA DSBs in the absence of Mdc1? We therefore studied the presence of two other important repair proteins in these cells, 53BP1, a marker protein for NHEJ [14], and Rad51, a marker for HR. Already in the unirradiated testis, staining for 53BP1 and Rad51 was observed in spermatogonia and preleptotene spermatocytes. B spermatogonia and preleptotene spermatocytes even showed

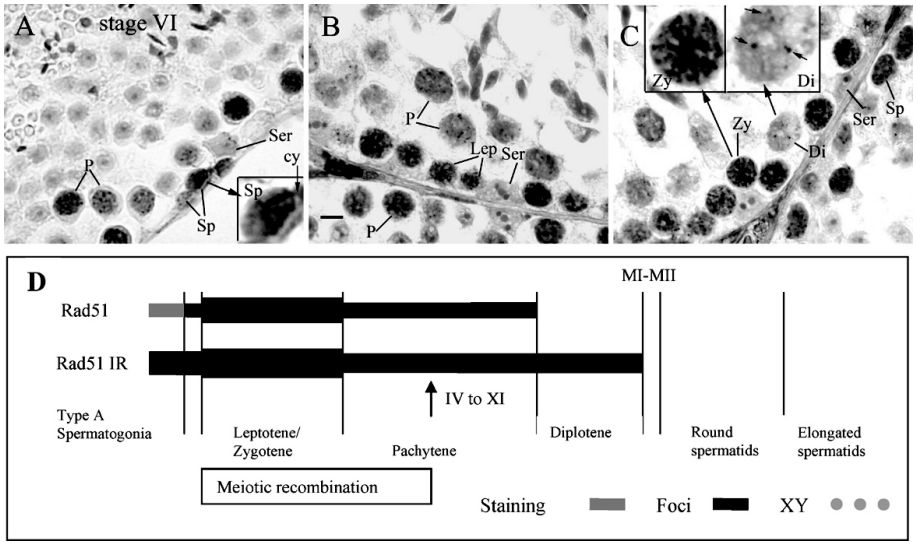


Fig. 6 – Localization of Rad51 in control (A) and irradiated testis (B and C). Rad51 in spermatogonia "Sp" (A and B) and early spermatocytes and even in late pachytene and diplotene spermatocytes "Di" after IR (B and C). No Rad51 was found in Sertoli cells "Ser" before or after IR. (D) Schematic representation of immunohistochemical expression of Rad51 before and after IR. The widths of the lines represent the relative intensity of the staining and foci numbers.

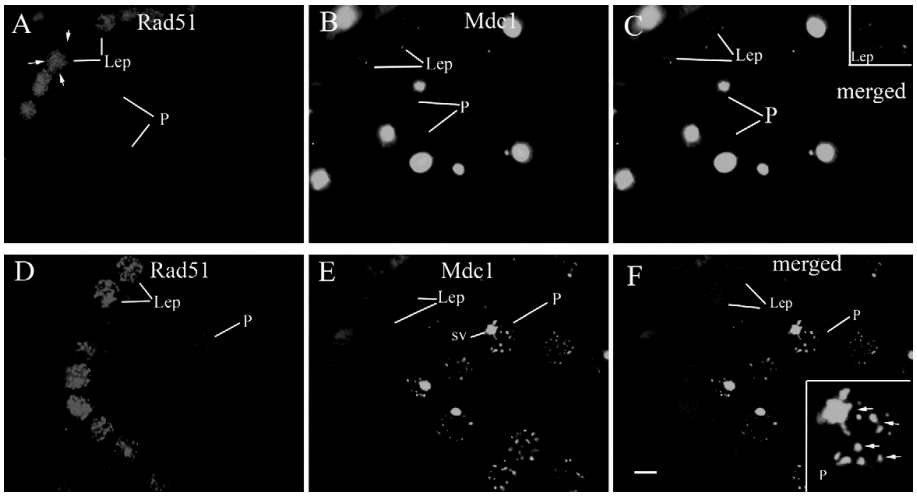


Fig. 7 – Co-localization of Rad51 and Mdc1 in control and irradiated testis. (C) The few Mdc1 foci in leptotene "Lep" co-localized with Rad51 foci. (E) No Mdc1 foci were found in leptotene spermatocytes after IR. (F) Rad51 foci partially overlapped with Mdc1 foci in late pachytene after IR.

some foci for 53BP1 and for Rad51, in accordance with the γH2AX foci found in these cells in the unirradiated testis [16] (and this study). The not-irradiation-related focus formation by 53BP1 and Rad51 in these cells may be due to a function during S-phase [9,24]. After irradiation, foci for 53BP1 and Rad51 were found in all spermatogonia and preleptotene spermatocytes. In previous studies these cells were shown to also express DNA–PKcs and the Ku proteins [15,25]. Apparently in spermatogonia and preleptotene spermatocytes proteins active in both DNA repair pathways are present. In order to determine whether repair does indeed take place and if so, at which efficiency, we studied foci numbers at various intervals after irradiation. A 40% reduction in foci numbers for both γH2AX and 53BP1 was found at 16 h after irradiation (Fig. 8). This indicates that some 40% of the DSBs are repaired within this time interval, which, compared to somatic cells suggests

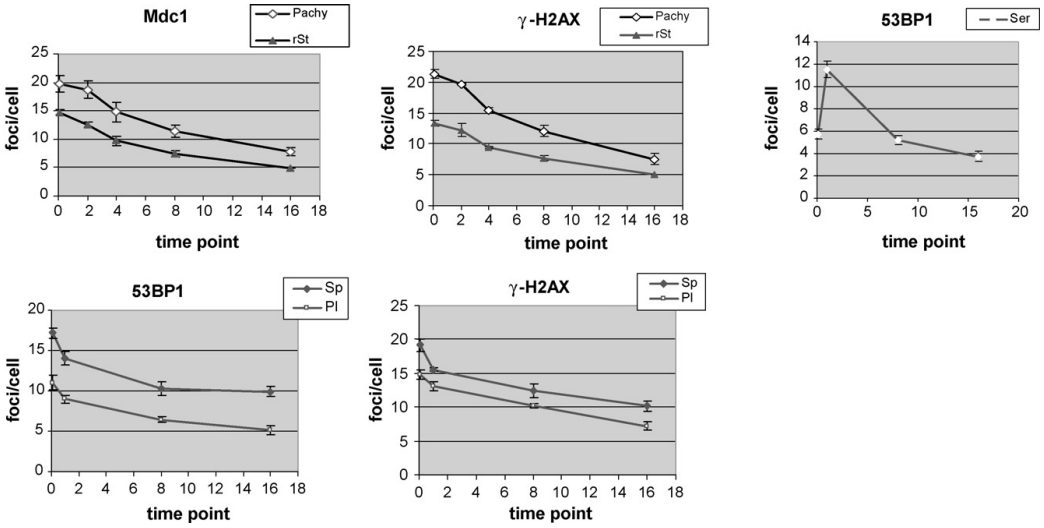


Fig. 8 – Foci counts in pachytene spermatocytes and round spermatids (upper panel), spermatogonia (Sp) and preleptotene spermatocytes (lower panel) and in Sertoli cell (Ser, right panel) at different time points after IR. Time points are in hours after IR. The error bar represents the standard error of results from three mice.

a much slower and incomplete repair in spermatogonia and preleptotene spermatocytes [26]. At 8 h after IR the foci became large and intensely bright and may represent slowly repaired or even irreparable DSB since at longer time intervals (12 h) a dose of 4 Gy of X-rays was found to cause a 10-fold increase in the number of apoptotic spermatogonia [27]. Our data suggest the absence of Mdc1 to be an important aspect of the inefficiency of the DSB repair process in spermatogonia and preleptotene spermatocytes, probably because of a reduced accumulation of repair proteins at the sites of DSBs. [4]. This also explains the high radiosensitivity of differentiating spermatogonia for killing by apoptosis [23,28].

4.2. *Spermatocytes from the start of meiotic prophase till mid-pachytene (epithelial stage IV)*

In the control testis, as soon as the meiotic prophase starts, i.e. in leptotene and zygotene spermatocytes, Mdc1 foci were observed. Apparently, Mdc1 plays a role in the formation of early homologous recombination intermediates, co-localizing with γ-H2AX (Fig. 3) and Rad51. These foci are too few in number to represent processing of all recombination involved DNA DSBs, because γ-H2AX and Rad51 foci in leptotene and zygotene spermatocytes appeared much more numerous than those of Mdc1. By late zygotene/early pachytene, Mdc1 became concentrated in the tadpole-shaped structure of the sex chromosomes in a way identical to what has been shown earlier for γ-H2AX [29].

To further elucidate the role of Mdc1 during meiotic recombination, we can compare spermatogenesis in Mdc1−/− and Msh5−/− testes. Both genotypes show an arrest in epithelial stage IV spermatocytes [30] (and this study). Chromosome structure is more compact for Mdc1−/− than for Msh5−/−, suggesting more advanced synapsis. However, this interpretation awaits a more detailed cytological study into the morphology of homologous synapsis in Mdc1−/− males. A similar arrest was previously seen in Atm, Spo11 and Dmc1 deficient mice [31,32]. The stage IV spermatocyte arrest in Mdc1−/− mice is a strong indication for a role of Mdc1 in processing recombination involved DSBs. Clearly the expression of Mdc1 in early spermatocytes is required for a satisfactory processing of meiotic DSBs and the low numbers of foci (Fig. 1A) suggest the presence of Mdc1 to be required in a short meiotic prophase window. Surprisingly, irradiation, especially at early time points, destroyed the assumed recombination repair involved Mdc1 foci, but not those of γ-H2AX or Rad51, as if in the presence of irradiation induced breaks, these breaks compete for Mdc1 which is at a limited supply. However, no longer lasting consequences of early meiotic prophase irradiation for chiasma formation are known to the authors [33].

Leptotene, zygotene and early pachytene spermatocytes do not express 53BP1 before and after irradiation indicating that 53BP1 has no role in recombination involved repair during the early meiotic prophase. This result also explains why 53BP1−/− mice are fertile [34]. In previous studies it was found that early spermatocytes do not express the Ku proteins [15,25] making it impossible for these cells to carry out NHEJ. Hence, the absence of 53BP1 that has been shown to function in the NHEJ pathway [14], would not be of a disadvantage for early primary spermatocytes.

4.3. *Spermatocytes from mid-pachytene onwards and round spermatids*

In the unirradiated testis, a nuclear staining for 53BP1 and Mdc1 was found in mid to late pachytene spermatocytes, diplotene spermatocytes and in round spermatids. In addition, one or two γ-H2AX and Mdc1 foci were seen in some pachytene spermatocytes. Rad51 showed a few foci at mid-pachytene and more rarely so in late pachytene spermatocytes. Apparently, in the normal testis a few DSBs occur in mid to late pachytene spermatocytes that are solved by HR.

Within 5 min after ionizing radiation, focus formation of Mdc1 occurred in mid to late pachytene and diplotene spermatocytes and in round spermatids. The kinetics of Mdc1 focus formation in these germ cells seems comparable to that in somatic cell lines in which Mdc1 foci were found within a minute after IR [6,10]. The radiation induced Mdc1 foci in late spermatocytes and spermatids overlapped completely with the γ-H2AX foci in these cells (Fig. 3, l). Such a full overlap is consistent also with the spatial redistribution of Mdc1 in real time in live human cells [13,18]. Initially, these foci were devoid of 53BP1, but at longer time interval post-irradiation, i.e. 8 h or more, 53BP1 was also present. Intriguingly, the rate of the appearance of 53BP1 after irradiation in late spermatocytes and round spermatids is much slower than in spermatogonia where 53BP1 was present in the foci within 5 min after irradiation. This temporal delay could indicate a distinct kinetics of unmasking the methylated histone sites that serve as docking sites for the TUDOR domain of 53BP1 [18,35]. However, few Rad51 foci co-localized with Mdc1 foci in late pachytene and diplotene spermatocytes, but they did not overlap completely (Fig. 7F). Such partial overlap is fully consistent with the dynamic spatial redistribution of Mdc1 and Rad51 in real time in live human cells [18]. Taken together late spermatocytes express Mdc1, Rad51, 53BP1 and all components of the DNA–PK complex [25] and should be able to carry out both HR and NHEJ. Indeed, quantifying foci numbers at various time points after irradiation revealed a 70% reduction after 16 h. Although the DSB repair efficiency is higher then in spermatogonia where only a 40% reduction was found, it still does not compare with somatic cell lines where a 70% reduction in 2 h occurred [26]. The NHEJ pathway for somatic cells has been shown to be active in each part of the cell cycle, but to be more dominant in G1 phase [36]. Therefore, the observed slow recruitment of 53BP1 to the sites of DSBs in late spermatocytes may be related to a lower efficiency for NHEJ this late in the cell cycle, these cells being close to the metaphase of the first meiotic division.

At the single chromatid level in haploid round spermatids, HR is not possible and indeed after irradiation, Rad51 was not expressed. However, round spermatids express Mdc1 and 53BP1 and after irradiation foci are formed, that for 53BP1 are "delayed". The efficiency of the DSB repair as determined by the disappearance of the foci in round spermatids was comparable to that in late spermatocytes, about a 70% reduction in 16 h. Numbers of foci were lower in round spermatids compared to Mdc1 positive meiotic prophase stages, but not at the expected 25%, as based on DNA content (Fig. 8). Another point of note is that the number of foci that we counted per Gy is much lower than the 40 Gy^{-1} counted for G1 stage cells [26].

We cannot at present solve these discrepancies, but clustering of foci could play a role. In conjuncture with slow repair, a change in cell cycle kinetics could also be envisaged [37], the time span of our observations being too short to be able to observe such an effect.

4.4. *Somatic cells*

In this study no expression of Mdc1, 53BP1, Rad51 and γH2AX was seen in interstitial somatic cells, peritubular cells and myoid cells before and after irradiation. This suggests that quiescent, mostly terminally differentiated somatic cells do not sense and repair DNA DSBs. This is in sharp contrast with the proliferating somatic cells usually used to perform DNA repair studies. However, there was one unexpected exception, Sertoli cells did express 53BP1 and after irradiation 53BP1 foci were rapidly formed. This is an intriguing finding, even more so because with time the numbers of 53BP1 foci showed a reduction of 60% at 16 h after IR (Fig. 8). This suggests the presence of a DNA DSBs repair system in Sertoli cells. Further, DNA damage repair proteins present in Sertoli cells are DNA–PKcs, ku70, ku86, p53 and phosphorylated ATM [16,25,38]. However, Sertoli cells do not express γH2AX which is supposed to have a rather crucial role. Clearly, the DNA damage repair pathway in Sertoli cells will be interesting to study in further detail. Especially, since Sertoli cells are terminally differentiated and do not proliferate anymore.

4.5. *General aspects*

When we compare our IRIF kinetics data with irradiation mutation induction as measured by dominant visible and specific locus mutations [39], the stages exemplified by Mdc1 IRIFs and hence slow repair kinetics coincide with high mutation rates. For dominant visible mutations the post-recombination spermatocytes are more vulnerable than round spermatids. For recessive specific locus mutations, the difference is smaller. Specific locus mutations are often caused by deletions of several kb in size [39]. In a cytogenetic study that measured radiation-induced fragments and exchanges in doses up to 3 Gy, Matsuda and Tobari [33] found rather similar dose-response curves for both endpoints for leptotene, zygotene, pachytene and diplotene spermatocytes, with the exception of the induction of exchanges at the leptotene stage, that did not occur (parallel with loss of recombination Mdc1 foci). In a transmission experiment [40], zygotes did not show male derived chromosome abnormalities after spermatocyte irradiation, suggesting a selection pressure possibly at the meiotic divisions. When these authors followed the descendants of germ cells irradiated while being round spermatids, the abnormalities found, were in agreement with incomplete repair in persisting Mdc1 positive IRIF's in round spermatids. Hence, these abnormalities are transmitted to the zygote, leading to dominant prenatal lethality [41].

The present data indicate that DNA DSB repair in germ cells is different from that described for somatic cell lines. Different germ cell types have different repair proteins at their disposal to carry out repair. As judged by the rate of disappearance of IRIFs and their growth up to 16 h, repair does not reach the efficiency of somatic cell lines. In response to irradiation, HR does occur in germ cells, and NHEJ is indicated but either Mdc1 or 53BP1 and maybe other repair proteins are missing in the quick radiation response. One can speculate that from the evolutionary point of view, this may be advantageous because in that way, germ cells with DNA damage will most often die instead of carrying out a repair process that might involve the introduction of a mutation. However, we will have to await further research in this field to see whether or not there are as yet unknown, perhaps germ cell specific, repair proteins that take over from the presently studied ones.

Acknowledgement

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REFERENCES

[1] D.C. van Gent, J.H. Hoeijmakers, R. Kanaar, Chromosomal stability and the DNA double-stranded break connection, Nat. Rev. Genet. 2 (2001) 196–206.
[2] J. Lukas, C. Lukas, J. Bartek, Mammalian cell cycle checkpoints: signalling pathways and their organization in space and time, DNA Rep. 3 (2004) 997–1007.
[3] J.H. Hoeijmakers, Genome maintenance mechanisms for preventing cancer, Nature 411 (2001) 366–374.
[4] M. Stucki, S.P. Jackson, GammaH2AX and MDC1: anchoring the DNA-damage-response machinery to broken chromosomes, DNA Rep. 5 (2006) 534–543.
[5] M.B. Kastan, J. Bartek, Cell cycle checkpoints, Nature 432 (2004) 316–323.
[6] G.S. Stewart, B. Wang, R.C. Bignell, A.M. Taylor, S.J. Elledge, MDC1 is a mediator of the mammalian DNA damage checkpoint, Nature 421 (2003) 961–966.
[7] M. Goldberg, M. Stucki, J. Falck, D. D'Amours, D. Rahman, D. Pappin, J. Bartek, S.P. Jackson, MDC1 is required for the intra-S-phase DNA damage checkpoint, Nature 421 (2003) 952–956.
[8] Z. Lou, K. Minter-Dykhouse, S. Franco, M. Gostissa, M.A. Rivera, A. Celeste, J.P. Manis, J. van Deursen, A. Nussenzweig, T.T. Paull, F.W. Alt, J. Chen, MDC1 maintains genomic stability by participating in the amplification of ATM-dependent DNA damage signals, Mol. Cell. 21 (2006) 87–200.
[9] B. Wang, S. Matsuoka, P.B. Carpenter, S.J. Elledge, 53BP1, a mediator of the DNA damage checkpoint, Science 298 (2002) 1435–1438.
[10] X. Xu, D.F. Stern, NFBD1/MDC1 regulates ionizing radiation-induced focus formation by DNA checkpoint signaling and repair factors, FASEB J. 17 (2003) 1842–1848.
[11] Z. Lou, C.C. Chini, K. Minter-Dykhouse, J. Chen, Mediator of DNA damage checkpoint protein 1 regulates BRCA1 localization and phosphorylation in DNA damage checkpoint control, J. Biol. Chem. 278 (2003) 13599–13602.
[12] Z. Lou, B.P. Chen, A. Asaithamby, K. Minter-Dykhouse, D.J. Chen, J. Chen, MDC1 regulates DNA-PK autophosphorylation in response to DNA damage, J. Biol. Chem. 279 (2004) 46359–46362.
[13] S. Bekker-Jensen, C. Lukas, F. Melander, J. Bartek, J. Lukas, Dynamic assembly and sustained retention of 53BP1 at the sites of DNA damage are controlled by Mdc1/NFBD, J. Cell Biol. 170 (2005) 201–211.
[14] K. Nakamura, W. Sakai, T. Kawamoto, R.T. Bree, N.F. Lowndes, S. Takeda, Y. Taniguchi, Genetic dissection of

vertebrate 53BP1: a major role in non-homologous end joining of DNA double strand breaks, DNA Rep. 6 (2006) 741–749.
[15] W. Goedecke, M. Eijpe, H.H. Offenberg, M. van Aalderen, C. Heyting, Mre11 and Ku70 interact in somatic cells, but are differentially expressed in early meiosis, Nat. Genet. 23 (1999) 194–198.
[16] G. Hamer, H.L. Roepers-Gajadien, A. van Duyn-Goedhart, I.S. Gademan, H.B. Kal, P.P. van Buul, T. Ashley, D.G. de Rooij, Function of DNA-protein kinase catalytic subunit during the early meiotic prophase without Ku70 and Ku86, Biol. Reprod. 68 (2003) 717–721.
[17] J. Bartkova, C.J. Bakkenist, E. Rajpert-De Meyts, N.E. Skakkebaek, M. Sehested, J. Lukas, M.B. Kastan, J. Bartek, ATM activation in normal human tissues and testicular Cancer, Cell Cycle 4 (2005) 838–845.
[18] S. Bekker-Jensen, C. Lukas, R. Kitagawa, F. Melander, M.B. Kastan, J. Bartek, J. Lukas, Spatial organization of the mammalian genome surveillance machinery in response to DNA strand breaks, J. Cell Biol. 173 (2006) 195–206.
[19] L.D. Russel, R.A. Ettlin, A.P. Hikim, E.D. Clegg, Histological and Histopathological Evaluation of the Testis, Cache River Press, Clearwater, FL, USA, 1990.
[20] T.L. Beumer, H.L. Roepers-Gajadien, I.S. Gademan, P.P. van Buul, G. Gil-Gomez, D.H. Rutgers, D.G. de Rooij, The role of the tumor suppressor p53 in spermatogenesis, Cell Death Diff. 5 (1998) 669–677.
[21] B. Xu, A.H. O'Donnell, S.T. Kim, M. Kastan, Phosphorylation of serine 1387 in Brca1 is specifically required for the Atm-mediated S-phase checkpoint after ionizing radiation, Cancer Res. 62 (2002) 4588–4591.
[22] M.J. Moses, New cytogenetic studies on mammalian meiosis, in: M. Serio, L. Martini (Eds.), Animal Model in Human Reproduction, Raven, New York, 1980, pp. 169–190.
[23] Y. Van der Meer, R. Huiskamp, J. Davids, I. van der Tweel, D.G. de Rooij, The sensitivity of quiescent and proliferating mouse spermatogonial stem cells to X-irradiation, Radiat. Res. 130 (1992) 289–295.
[24] T. Yamamoto, T. Hikino, Y. Nakayama, S. Abe, Newt RAD51: cloning of cDNA and analysis of gene expression during spermatogenesis, Dev. Growth Differ. 41 (1999) 401–406.
[25] G. Hamer, H.L. Roepers-Gajadien, A. van Duyn-Goedhart, I.S. Gademan, H.B. Kal, P.P. van Buul, D.G. de Rooij, DNA double-strand breaks & γ-H2AX signaling in the testis, Biol. Reprod. 68 (2003) 628–634.
[26] E. Riballo, M. Kuhne, N. Rief, A. Doherty, G.C. Smith, M.J. Recio, C. Reis, K. Dahm, A. Fricke, A. Krempler, A.R. Parker, S.P. Jackson, A. Gennery, P.A. Jeggo, M. Lobrich, A pathway of double-strand break rejoining dependent upon ATM, Artemis, and proteins locating to gamma-H2AX foci, Mol. Cell. 13 (2004) 715–724.
[27] T.L. Beumer, H.L. Roepers-Gajadien, L.S. Gademan, D.H. Rutgers, D.G. de Rooij, P21(Cip1/WAF1) expression in the mouse testis before and after X irradiation, Mol. Reprod. Dev. 47 (1997) 240–247.
[28] Y. Van der Meer, R. Huiskamp, J.A. Davids, D.G. de Rooij, Differential effects of fractionated X irradiation on mouse spermatogonial stem cells, Radiat. Res. 135 (1993) 222–228.
[29] S.K. Mahadevaiah, J.M. Turner, F. Baudat, E.P. Rogakou, P. de Boer, J. Blanco-Rodriguez, M. Jasin, S. Keeney, W.M. Bonner, P.S. Burgoyne, Recombinational DNA double-strand breaks in mice precede synapsis, Nat. Genet. 27 (2001) 271–276.
[30] S.S. de Vries, E.B. Baart, M. Dekker, A. Siezen, D.G. de Rooij, P. de Boer, H. te Riele, Mouse MutS-like protein Msh5 is required for proper chromosome synapsis in male and female meiosis, Genes Dev. 13 (1999) 523–531.
[31] D.G. de Rooij, P. de Boer, Specific arrests of spermatogenesis in genetically modified and mutant mice, Cytogenet. Genome Res. 103 (2003) 267–276.
[32] M. Barchi, S. Mahadevaiah, M. Di Giacomo, F. Baudat, D.G. de Rooij, P.S. Burgoyne, M. Jasin, S. Keeney, Surveillance of different recombination defects in mouse spermatocytes yields distinct responses despite elimination at an identical developmental stage, Mol. Cell Biol. 25 (2005) 7203–7215.
[33] Y. Mutsuda, I. Tobari, Studies on radiation-induced chromosome aberrations in mouse spermatocytes I Stage specificity and dose-response relationships of chromosome aberrations induced in mouse primary spermatocytes following X-irradiation, Mut. Res. 176 (1987) 243–250.
[34] I.M. Ward, K. Minn, J. van Deursen, J. Chen, p53 binding protein 53BP1 is required for DNA damage responses and tumor suppression in mice, Mol. Cell Biol. 23 (2003) 2556–2563.
[35] Y. Huyen, O. Zgheib, R.A. Ditullio Jr., V.G. Gorgoulis, P. Zacharatos, T.J. Petty, E.A. Sheston, H.S. Mellert, E.S. Stavridi, T.D. Halazonetis, Methylated lysine 79 of histone H3 targets 53BP1 to DNA double-strand breaks, Nature 432 (2004) 406–411.
[36] M. Takata, M.S. Sasaki, E. Sonoda, C. Morrison, M. Hashimoto, H. Utsumi, Y. Yamaguchi-Iwai, A. Shinohara, S. Takeda, Homologous recombination and non-homologous end-joining pathways of DNA double-strand break repair have overlapping roles in the maintenance of chromosomal integrity in vertebrate cells, EMBO J. 17 (1998) 5497–5508.
[37] S. Matulis, M.A. Handel, Spermatocyte responses in vitro to induced DNA, Mol. Reprod. Dev. 73 (2005) 1061–1072.
[38] G. Hamer, H.B. Kal, C.H. Westphal, T. Ashley, D.G. de Rooij, Ataxia telangiectasia mutated expression and activation in the testis, Biol. Reprod. 70 (2004) 1206–1212.
[39] W.L. Russell, J.W. Bangham, L.B. Russell, Differential response of mouse male germ-cell stages to radiation-induced specific-locus and dominant mutations, Genetics 148 (1998) 1567–1578.
[40] L. Tusell, L. Latre, I. Ponsa, R. Miro, J. Egozcue, A. Genesca, Capping of radiation-induced DNA breaks in mouse early embryos, J. Radiat. Res. (Tokyo) 45 (2004) 415–422.
[41] F. Marchetti, A.J. Wyrobek, Mechanisms and consequences of paternally transmitted chromosomal abnormalities, Birth Defects Res. C. Embryo Today 75 (2005) 112–129.

Chapter 3
Proliferative Activity *In Vitro* and DNA Repair Indicate that Adult Mouse and Human Sertoli Cells Are Not Terminally Differentiated, Quiescent Cells

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ABSTRACT

Sertoli cells, isolated from the adult mouse and human testis, resume proliferation in culture. After 20 days of culture in Dulbecco's Modified Eagle Medium (DMEM/F12) medium containing 5% FCS, about 36% of the mouse Sertoli cells, identified by their immunohistochemical staining for the Sertoli cell marker vimentin, incorporated BrdU. The renewed proliferation was associated with a 70% decrease in the expression of the cell cycle inhibitor DKN1B ($P27^{kip1}$) and a 2-fold increase in the levels of the proliferation inducer ID2. Probably in vivo, the balance between cell cycle inhibitors and inducers is such that the cells remain quiescent, while in culture the balance is disturbed and Sertoli cells start to proliferate again. The renewed proliferative activity of Sertoli cells in culture could be further confirmed by double staining for BrdU and the Sertoli cell marker clusterin (CLU), showing about 25% of the CLU positive to be also positive for BrdU after 13 days of culture. Radiobiologically, Sertoli cells are also different from other quiescent somatic cells in the testis as they express several DNA repair proteins (XRCC1, PARP1 and others). Indeed, a comet assay on irradiated Sertoli cells revealed a 70% reduction in tail length and moment at 20 hr after irradiation. Hence, Sertoli cells repair DNA damage while other quiescent somatic testicular cells do not. This repair may be carried out by non homologous end joining (NHEJ) via XRCC1 and PARP1. In conclusion, both cell kinetic and radiobiological data indicate that Sertoli cells more resemble arrested proliferating cells than the classical postmitotic and terminally differentiated somatic cells they have always been supposed to be.

INTRODUCTION

Cells that do not divide, no longer show changes in phenotype and carry out a specific function, are known as terminally differentiated cells. Many cell types are considered to be terminally differentiated in the adult animal, including neurons, myocytes, auditory hair cells, epidermal cells, and Sertoli cells [1, 2]. In mammals, Sertoli cells are formed before puberty and after puberty these cells are considered to be unable to proliferate, except in seasonal breeders in which season dependent variations in Sertoli cell numbers per testis occur [3-5]. The latter implicates a residual capacity of adult Sertoli cells to proliferate or the long-term presence of some Sertoli cell precursors in the testis of adult seasonal breeders. The terminal differentiation of Sertoli cells involves loss of proliferative activity, formation of inter-Sertoli cell tight junctions and establishment of the Sertoli cell barrier, and the acquisition of the ability to fully sustain the spermatogenic process (for review, see, [1]. Sertoli cell differentiation is also accompanied by the expression of many gene products that are not present in immature cells [6, 7].

In the mouse, Sertoli cells proliferate until day 16 after birth [8], whereafter they terminally differentiate. However, the period of Sertoli cell differentiation can be postponed in case of

hypothyroidism [9] or deficiency for the testicular gap junction protein GJA1 [10]. Some growth factors and FSH increase proliferation of Sertoli cells in neonatal and prepubertal testes but adult Sertoli cells fail to respond [11-13]. Intriguingly, while adult Sertoli cells are quiescent they do express genes that are involved in the regulation of the cell cycle, for example DKN1B ($P27^{kip1}$), a cell cycle inhibitor which arrests cells in G1 phase of the cell cycle [14]. Furthermore, after infliction of DNA damage by irradiation, Sertoli cells express P53 (TP53) that causes cell cycle arrest [15]. Besides cell cycle inhibitors, adult Sertoli cells also express ID proteins, which are helix-loop-helix proteins, that inhibit differentiation and promote proliferation, which is in contrast with other quiescent and differentiated cells in which ID proteins are either expressed at very low levels or not at all [1, 16, 17]. Taken together, unlike other terminally differentiated cells, Sertoli cells express a puzzling mixture of proliferation inducers and inhibitors.

In addition to the presence of cell cycle regulatory proteins, Sertoli cells differ from most other quiescent somatic cells in that they express a number of DNA damage repair proteins. Adult Sertoli cells express XRCC6 (KU70), XRCC5 (KU86) and TP53 already before irradiation and phosphorylated ATM in response to irradiation [15, 18, 19]. Furthermore, after irradiation, TP53BP1 (53BP1) foci are formed in Sertoli cells that diminish in number with time suggesting that in contrast to other quiescent somatic cells in the testis, Sertoli cells might repair DNA double strand breaks (DSBs) [20].

Hence, both with respect to the expression of cell cycle genes and to radiobiological aspects, Sertoli cells differ from other supposedly terminally differentiated cells that carry out a specific task in an adult tissue. We now show that adult mouse and human Sertoli cells resume proliferation in culture and that adult Sertoli cells actually repair DNA double strand breaks (DSBs). Our results indicate that Sertoli cells should no longer be regarded as one of the prime examples of terminally differentiated quiescent cells.

MATERIALS AND METHODS

Animals, Irradiation and Fixation

Testes of male FvB mice, 8 to 12 weeks of age, were given a dose of 4 Gy of X-rays (local irradiation, 200 kV, 20 mA, 0.5-mm Cu filter; Philips, Eindhoven, The Netherlands). Mice were killed by cervical dislocation and testes were fixed in 4% paraformaldehyde in PBS for 24 hr at 4 °C and embedded in paraffin (Stemcowax; Adamas Instruments, Amerongen, The Netherlands). The animals were used and maintained according to regulations provided by the animal ethical committee of the Utrecht University that also approved the experiments.

Immunohistochemistry

Sections (5 µm) of testes of control and irradiated mice were mounted together on TESPA (3-aminoproyl-tri-ethoxysilane)-coated glass slides and dried overnight at 37 °C. Sections were dewaxed in xylene and hydrated in a graded series of alcohols. The sections were boiled once or twice (each for 10 min) in 0.01 M sodium citrate using a microwave oven (H2500; Bio-Rad, Hercules, USA and incubated in 0.35% H_2O_2 in PBS for 10 min. Blocking was done in 5% BSA (Sigma, St Louis, MO, USA) /5% goat serum (Aurion, Wageningen, The Netherlands) in PBS. The slides were incubated with the primary antibody for 1 hr at room temperature. The primary antibodies used were: mouse monoclonal antibody against DKN1B (BD Bioscience, San José, CA, USA), rabbit anti-ID2 (C-20, Santa Cruz Biotechnology, Santa Cruz, CA, USA), polyclonal rabbit anti-phospho-XRCC1 (Bethyl Laboratories, Montgomery, USA), pre-diluted mouse monoclonal anti-XRCC1 (Abcam, Cambridge, UK), rabbit polyclonal anti-PARP1 (Abcam) mouse monoclonal anti-vimentin (Mu163-uc, Biogenex, San Ramon, CA, USA), goat polyclonal anti-clusterin (CLU, Santa Cruz Biotechnology) and α-smooth muscle actin antibody (Biogenex, San Ramon, CA, USA). The slides were washed in PBS and then incubated with secondary antibody, PowerVision Poly Hrp-anti mouse/rabbit/rat (ImmunoVision Technologies, Co. Brisbane, CA 94005, USA), ready to use or biotinylated rabbit anti goat or goat anti mouse IgM from Vector Laboratories, Burlingame, CA, USA) for 40 min at room temperature. Sources, concentrations and dilutions of the used antibodies are provided in Table 1. Bound antibodies were visualized using 0.3 µg/µl 3,3'-diaminobenzidine (DAB, Sigma) in PBS, to which 0.03% H_2O_2 was added. Sections were counterstained with Mayer hematoxylin.

Sertoli Cell Isolation and Culture

Testes of male FVB mice, 8 to 12 weeks of age, were decapsulated and Sertoli cells were isolated as described [21, 22], with some modifications. Briefly, only two testes per isolation were used and the isolation was done in a 15ml centrifuge tube. Testes were decapsulated with forceps in 1x Hanks fluid, washed twice in PBS (calcium/magnesium-free, pH 7.4) and dispersed, but not fragmented, in 0.1% collagenase (type IV) and 0.04% DNase I (all from Sigma) in 1x Hanks and pH 7.4 at 34°C for 10–15 min, shakingat 100 oscillations/min. The tubules were allowed to settle and after being washed two times by sedimentation with PBS, they were incubated in a 1x Hanks (pH 7.4) containing 0.04% DNase I, 0.05% hyaluronidase and 0.5% trypsin (all Sigma) for at least 10 min at 34°C with agitation. The fragmented tubules were allowed to settle and then washed two times in PBS. After repeated pipetting in DMEM/F12, cells were centrifuged twice (each for 3 min) at 900 rpm and 700 rpm, respectively. Cells in the supernatant were collected and cultured (in MEM/F12 medium containing 5% FCS), after 3 hours in culture, the medium was removed very carefully and the culture was washed and fresh medium was added. During overnight culturing, Sertoli cells attached to the bottom and acquired an irregular shape, while the germ cells did not attach and could easily be

removed by repeated washing. Hypotonic shock (with Tris-Hcl, 0.02 M, PH 7.2, for 2 min.) was used when needed. Following this protocol the purity of the Sertoli cells in culture was above 90% and could reach 99% after 20 days.

Human testicular cells were enzymatically (Trypsine, Hyaluronidase and collagenase) isolated from testes donated by patients undergoing bilateral castration as part of prostate cancer treatment. Cells were cultured in Stempro medium (Invitrogen, Carlsbad, CA, USA) according to Kanatsu-Shinohara et al (2003) [23].

Cell Identification

Sertoli cells in culture were identified immunocytochemically by mouse monoclonal IgM anti-vimentin (Mu163-uc, Biogenex) or by goat polyclonal anti-clusterin (CLU, Santa Cruz Biotechnology). Peritubular cells were identified using α-smooth muscle actin antibody (Biogenex).

Immunocytochemistry and Cell Counts

After fixation in methanol, cells were incubated in 0.05% triton X in PBS for 15 min. For vimentin detection, after blocking with goat serum (0.5%), cells were incubated for 60 min with mouse monoclonal anti-vimentin antibody followed by incubation with secondary biotinylated goat anti-mouse IgMs (Vector Laboratories) in a humidified chamber for 30 min at room temperature. The horseradish peroxidase avidin-biotin complex reaction was performed according to the manufacturer's protocol (Vector Laboratories). Bound antibodies were visualized in red color by 3-Amino-9-ethyl carbazol in AEC buffer, activated with 0.03% H_2O_2 or in brown color using 0.3 µg/µl DAB (Sigma) in PBS, to which 0.03% H_2O_2 was added. When ABC-alkaline phosphatase standard kit was used (according to the manufacturer's protocol, Vector Laboratories), vimentin labeled cytoplasm was blue after incubation with an alkaline phosphatase substrate.

For peritubular cell staining, the same protocol was used except the primary antibody was mouse monoclonal IgG anti α-smooth muscle actin (Biogenex) and the secondary antibody was biotinylated horse antimouse (Vector Laboratories).

For CLU detection, cells were incubated with rabbit serum (0.5%) as a blocking agent, the primary antibody was goat polyclonal (Santa Cruz Biotechnology) and the secondary antibody was rabbit anti goat (HRP, from DAKO, Glostrup, Denmark or biotinylated from Vector Laboratories).

For BrdU staining, Sertoli cells in culture were incubated with BrdU (25 µg/ml, for 15h). Before incubation with mouse monoclonal IgG anti-BrdU (BD Bioscience) antibody, cells were incubated in periodic acid for 30 min at 60 ^{0}C and blocked in goat serum (0.5%) or horse serum (0.5%). Then cells were incubated with secondary antibody conjugated HRP, PowerVision Poly Hrp-anti mouse (ImmunoVision Technologies) or with biotinylated horse anti mouse in case of

double labeling with CLU. After incubation with the biotinylated secondary antibody the horseradish peroxidase avidin-biotin complex reaction was performed according to the manufacturer's protocol. BrdU positive nuclei were visualized (in dark blue color) by using 0.3 µg/µl DAB (Sigma) in PBS, to which cobalt (2.5%), nickel (2%) and H_2O_2 (0.03%) were added. Cells that incorporated BrdU could also be stained in red or brown color as described above.

To quantify the proliferative activity of Sertoli cells, the relative numbers of anti-BrdU/anti-vimentin double stained Sertoli cells to the numbers of vimentin positive Sertoli cells, from 4 different isolations, were calculated after 1, 3, 7, 13 and 20 days of culture.

To study the ability of adult human Sertoli cells to proliferate in vitro, BrdU (11 µg/ml for 3 hrs) was added to 3 weeks old cultures of cells from a human testicular biopsy before fixation in methacarn (60% methanol, 30% chloroform, 10% acetic acid) for 10 minutes. For double staining of adult human Sertoli cells, BrdU/vimentin or BrdU/CLU we used the same protocol as for mouse Sertoli cells.

In order to see whether the increasing proliferative activity of Sertoli cells with time in culture was associated with changes in the expression of DKN1B, or ID2, the expression of DKN1B and ID2 was studied by immunocytochemistry and Western analysis. For immunocytochemistry of DKN1B, we used the same double staining protocol as for vimentin/BrdU staining except that the cells were incubated with a mouse monoclonal antibody against DKN1B (BD Bioscience) instead of one against BrdU. The secondary antibody was PowerVision Poly Hrp-anti mouse/rabbit/rat (ImmunoVision Technologies), ready to use, in a humidified chamber for 30 min at room temperature. Bound antibodies were visualized, in brown nuclei, using 0.3 DAB (Sigma) in PBS, and 0.03% H_2O_2 or in dark blue nuclei, when cobalt and nickel were added. For ID2 immunostaining, the same protocol as for DKN1B/vimentin staining was used except that the primary antibody, rabbit anti-ID2 (C-20**,** Santa Cruz Biotechnology), was used instead of DKN1B. All experiments were carried out four times.

Irradiation and Comet Assay of Adult Mouse Sertoli Cells In Vitro

Directly after purification and attachment to a culture dish, cells were given a dose of 4 Gy of X-rays (200 kV, 20 mA, 0.5-mm Cu filter; Philips, Eindhoven, The Netherlands). At 1hr, 5hr and 20hr post-irradiation), cells were washed and carefully detached with trypsin (0.005%) and then cell pellets were collected by centrifugation. Viability was tested with trypan blue dye and more than 95% of the cells appeared to be viable.

The alkaline Comet Assay was performed according to Gobbel et al (1998) [24] and Karran et al (1973) [25]. Briefly, cells (suspended in 70 µL of low melting point agarose) were smeared over a slide covered (a day before) with a thin layer of normal melting point agarose. The slide was covered with a coverslip and then left for 10 min at 4 ^{0}C to harden the agarose. The coverslip was gently removed and the slide was slowly lowered in freshly made lysing solution (2.5M NaCl, 100mM EDTA, 10mM Tris–HCl, pH 10–12, 1% Triton X-100 and 10% DMSO) for

90 min. After washing in distilled water, slides were placed close together, side by side on the horizontal gel including freshly made electrophoresis buffer (pH>13) for 20 min. Electrophoresis was conducted for 20 min at 25 V and 300 mA. The slides were then washed 3 times with Tris–HCl buffer (0.3M, pH 7.5) and dehydrated in ethanol. For analysis, slides were stained with ethidium bromide (2 µg/ml in distilled water), scoring was done in a blinded manner. Image analysis was performed using the comet software IV program (Perceptive Instruments Ltd, UK) on 70 randomly selected cells (three experiments). The DNA lesions were quantified by measuring the increase in Tail length and Tail moment. Tail Length is the distance of DNA migration from the body of the nuclear core, the more DNA damage is inflicted the longer the tail will be. Tail moment is the product of the tail length and the fraction of total DNA in the tail and both are used to evaluate the extent of DNA damage.

Table 1. *Sources, concentrations and dilutions of the used primary and secondary antibodies.*

The antibody	Raised in	Source and concentration	Dilution
monoclonal anti-XRCC1	mouse	Abcam, 0.2 mg/ml	1- 10
polyclonal anti-phospho-XRCC1	rabbit	Bethyl Laboratories, 0.2 mg/ml	1- 200
polyclonal anti-PARP1	rabbit	Abcam, 0.1mg/ml	1- 200
monoclonal IgM anti vimentin	mouse	Biogenex, 0.1 mg/ml	1- 100
monoclonal anti-BrdU	mouse	BD Bioscience, 0.025 mg/ml	1- 80
monoclonal anti DKN1B	mouse	BD Bioscience, 0.025 mg/ml	1- 100
polyclonal anti-ID2 (C-20)	rabbit	Santa Cruz Biotechnology, 0.2 mg/ml	1- 100
Polyclonal anti CLU	goat	Santa Cruz Biotechnology, 0.2 mg/ml	1- 100
monoclonal anti smooth muscle actin	mouse	Biogenex, 0.1 mg/ml	1- 200
monoclonal anti alpha tubulin	mouse	Biogenex, 0.1 mg/ml	1- 100
poly Hrp-anti mouse	goat	ImmunoVision Technologies	Ready to use
poly Hrp-anti mouse/rabbit/rat	goat	ImmunoVision Technologies	Ready to use
biotinylated anti-mouse IgMs	goat	Vector laboratories., 0.5 mg/ml	1- 1000
HRP, anti mouse	rabbit	DAKO A/S, 0.2 mg/ml	1- 1000
biotinylated anti-mouse	goat	Vector laboratories., 0.5 mg/ml	1- 1000
biotinylated anti-mouse	horse	Vector laboratories., 0.5 mg/ml	1- 1000
biotinylated anti-goat	rabbit	Vector laboratories., 0.5 mg/ml	1- 1000

Western Blot Analysis

Total protein lysates from cultured Sertoli cells at 1, 3, 13 and 20 days were scraped by a teflon scraper after the addition of RIPA buffer. Lysates were sonicated on ice and cleared by centrifugation. Protein levels were measured using bicinchoninic acid analysis (Pierce Chemical Co., Rockford, IL). SDS-PAGE was performed as described by Laemmli, 1970 [26]. Proteins were blotted onto a polyvinylidene difluoride (PVDF) membrane (MilliPore, Bedford, MA, USA). Western blots were blocked using Blotto-A containing 5% Protifar (Nutricia, Zoetermeer, The Netherlands) in Tris-buffered saline (10 mM Tris-HCl, pH 8.0, 150 mM NaCl) including 0.05%

Tween-20 (TBST) and were washed in TBST between each step. The first antibodies, mouse monoclonal anti-DKN1B (BD Bioscience) and rabbit anti-ID2 (C-20, Santa Cruz Biotechnology) were diluted 1:1000 in blotto A. After incubation with secondary antibodies conjugated HRP, rabbit anti mouse (DAKO) or goat anti rabbit (Santa Cruz Biotechnology) the antigens were visualized using chemiluminescence (ECL; Amersham Pharmacia Biotech Benelux, Roosendaal, The Netherlands) and exposure to X-ray film (RX; Fuji Photo Film Co., Tokyo, Japan). The blots were analyzed using a mouse polyclonal antibody against α-tubulin (InnoGenex, San Ramon, CA, USA) after incubation with secondary antibodies conjugated HRP, rabbit anti mouse (DAKO). The experiments were carried four times. Quantification of the signal was performed using ImageJ software (http://rsb.info.nih.gov/ij/index.html).

Statistics

Statistical analysis between groups from four different experiments was done by one way analysis of variance (Dunnett's Multiple Comparison Test) using GraphPad software (GraphPad Software, Inc., CA 92037 USA).

RESULTS

Sertoli Cells Can Proliferate In Vitro

After 16 hr in culture followed by repeated washing and hypotonic shock, 89% or more of the cells were positive for the Sertoli cell marker vimentin (Fig. 1A). The specificity of the vimentin antibody was confirmed by using a mouse testis section as a positive control. In this control section only the cytoplasm of Sertoli cells became stained, while all other cell types in the testis were negative (Fig. S1A). In culture, Sertoli cells formed a monolayer, had an irregular shape (Fig. 1B-F) and stretched out at early time points (Fig. 1F). After a few days in culture and repeated washings the purity of the vimentin positive cells in culture increased to more than 98% (Fig. 1A). The few other cells were round shaped germ cells or irregular-shaped cells with less cytoplasm than Sertoli cells that were negative for vimentin and possibly were peritubular cells [27]. With time in culture, the irregular-shaped vimentin positive Sertoli cells enlarged in size (Fig. 1BC). However, besides these large cells there were also groups of smaller cells, positive for vimentin after 20 days in culture (Fig. 1D).

To determine whether Sertoli cells were able to proliferate in vitro, BrdU was added to the culture for 15 hr and double staining was performed for BrdU and vimentin. The specificity of the anti-BrdU antibody was confirmed by studying staining in testes of mice that received a dose of BrdU. Only cells capable of proliferation, spermatogonia and preleptotene spermatocytes, were found stained (data not shown). After 3 days in culture, 10% of the vimentin positive Sertoli cells were positive for BrdU and this number increased to 15% after 6

days. The BrdU-labeled Sertoli cells were dispersed over the culture (Fig. 1B), suggesting that more and more Sertoli cells resumed proliferation and that the labelled Sertoli cells did not derive from colonies formed by rare Sertoli cells that had retained their proliferative capacity. After one week in culture, Sertoli cells were flattened and larger in size and the number of BrdU/vimentin double-positive cells reached 36% after 20 days (Fig. 1A). Sertoli cells remained positive for vimentin during the full culture period. After 16 to 20 days of culture, both the enlarged as well as the smaller Sertoli cells were found to be capable of incorporating BrdU (Fig. 1CD).

It has been reported that serum can induce ID2 in cells, which stimulates proliferation [28, 29]. In order to see whether, after the initiation of the culture during which 5% serum is needed, Sertoli cells were induced to proliferate by the serum in the culture medium, Sertoli cells were isolated and cultured for 24 hr in 5% serum containing medium and then left in serum free medium for 5 days. At the end of the culture, 15% of the Sertoli cells were positive for BrdU similar to Sertoli cells continuously exposed to 5% serum during culture (Fig. S1B).

To investigate whether mouse Sertoli cells behaved differently from those in other species not showing season dependent spermatogenesis, BrdU was added to cultures of cells from a human testicular biopsy. In 3 weeks old cultures, double staining for vimentin and BrdU revealed many double positive cells (Fig. 1E) indicating that human Sertoli cells also resume proliferation in vitro.

In order to further confirm the identity and the purity of the cultured Sertoli cells, cultures were stained for an additional Sertoli cell marker,clusterin (CLU), and the peritubular cell marker, α-smooth muscle actin (Fig. 2A). After one week, around 91% and 6.3% of cells in culture were positive for CLU and α-smooth muscle actin, respectively (Fig. 2B). Importantly, after 20 days, 92.4% of the cells was positive for CLU and 7% for smooth muscle actin, showing that the purity of the Sertoli cells did not change much during culture.

When triple staining was done (Fig. 2C-G) for CLU, anti BrdU and α-smooth muscle actin after 13 days (Fig. 2C, D) of culture, 91.2% of cells were positive for CLU and 25.9% of the CLU positive cells were also for positive for BrdU (Fig. 2G). Only 6.3% of the cells were positive for smooth muscle actin and 27% of these were also positive for BrdU, suggesting that the proliferative activity of the Sertoli cells and peritubular cells in culture was comparable (Fig. 2G). Large-sized (Fig. 2E) and smaller-sized (Fig. 2F) cells positive for CLU or CLU and BrdU were seen after 20 days in culture. These smaller sized CLU positive cells were mostly seen after 20 days in culture.

Expression of DKN1B Decreases With Time In Culture

In order to see whether the proliferative activity of the Sertoli cells in culture was accompanied by changes in the expression of the cell cycle inhibitor DKN1B, we studied the expression of this protein in normal testis sections and after culture for 1 to 20 days. Immunohistochemistry revealed that the numbers of vimentin positive Sertoli cells that stained for DKN1B, decreased with time in culture (Fig. 3A-C). Most Sertoli cell nuclei were positive for DKN1B after 20 hr in culture (Fig. 2A). However, after 2 weeks most of these nuclei were negative or only lightly stained for DKN1B. Western blot analysis confirmed the decreasing expression levels of DKN1B protein (Fig. 3E). After 3 days in culture, DKN1B protein levels were reduced to 30% of those found after 20 hr. Western blots showed that the antibody only recognized one band at 27 KD. In testis sections, DKN1B stained the nuclei of Sertoli cells while a very weak staining was seen in Leydig cells (Fig. 3D).

Expression of ID2 Increases With Time In Culture

To find out whether the renewed proliferative activity of the Sertoli cells was associated with increased levels of the proliferation inducer ID2, western analysis was carried out on testis lysates and on Sertoli cell protein extracts from 1, 3, 13 and 20 day cultures. The antibody recognized two bands, ID2 at 15 KD and ID2 fusion proteins at 75 KD. An increased expression of ID2 (15 KD) was found with time in culture. After 3 days in culture, the expression of ID2 showed a 2-fold increase compared to after 20 hr and more than three fold after 13 days (Fig. 4A). Immunohistochemistry showed a clear ID2 staining of the Sertoli cell nuclei and a weak cytoplasmic staining, in 1 day and 1 week cultures (please see Supplemental Figure. S2 AB).

Adult Sertoli Cells Express DNA Repair Proteins

In testis sections, before and after irradiation, Sertoli cells were found to express XRCC1, phosphorylated-XRCC1 and PARP1 proteins (Fig. 5A-C). This expression did not clearly vary between the stages of the cycle of the seminiferous epithelium. Other somatic cells in the testis were negative for these proteins (Fig. 5). In Supplemental Table 1, we summarized the expression of proteins, involved in DNA DSB repair, in Sertoli cells in vivo before and after ionizing radiation, as reported by our and other groups. None of these proteins were found in Leydig cells or other somatic cells.

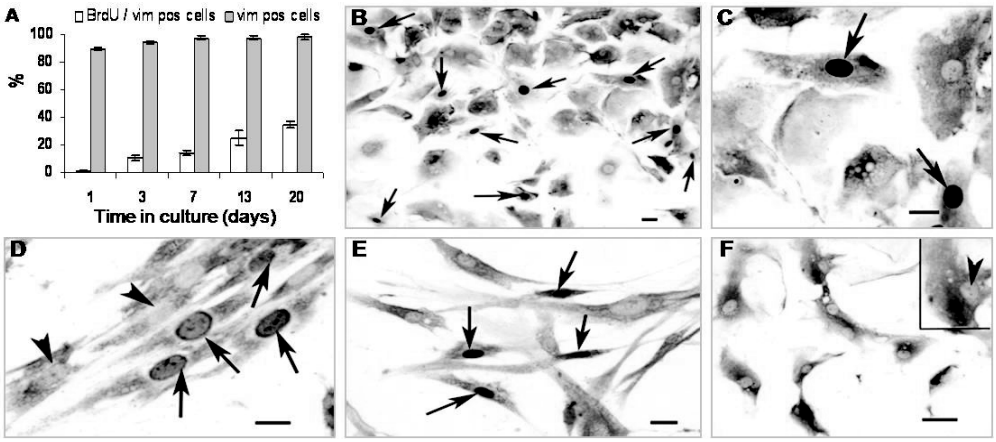

Figure 1. *Sertoli cells are able to proliferate in vitro. (A) The percentages of cells in culture positive for the Sertoli cell marker vimentin and the percentages of mouse Sertoli cells that incorporated BrdU, after various times in culture. The error bar represents the standard error of the mean (SEM) of results from four different experiments. vim - vimentin; pos - positive B-D. Adult mouse Sertoli cells that were positive for BrdU,after 6 (BC) and 16 days (D) in culture. Red - vimentin positive cells; dark blue - BrdU positive cells (arrows). Arrowheads - BrdU negative Sertoli cells (E) Adult human Sertoli cells isolated from a biopsy and cultured for several weeks. Several Sertoli cells were positive for vimentin (red) and BrdU (dark blue) (arrowheads) (F). Vimentin positive mouse Sertoli cells with large nuclei and three nucleoli (see head arrows) in a 4 day culture. Bar - 20 µm.*

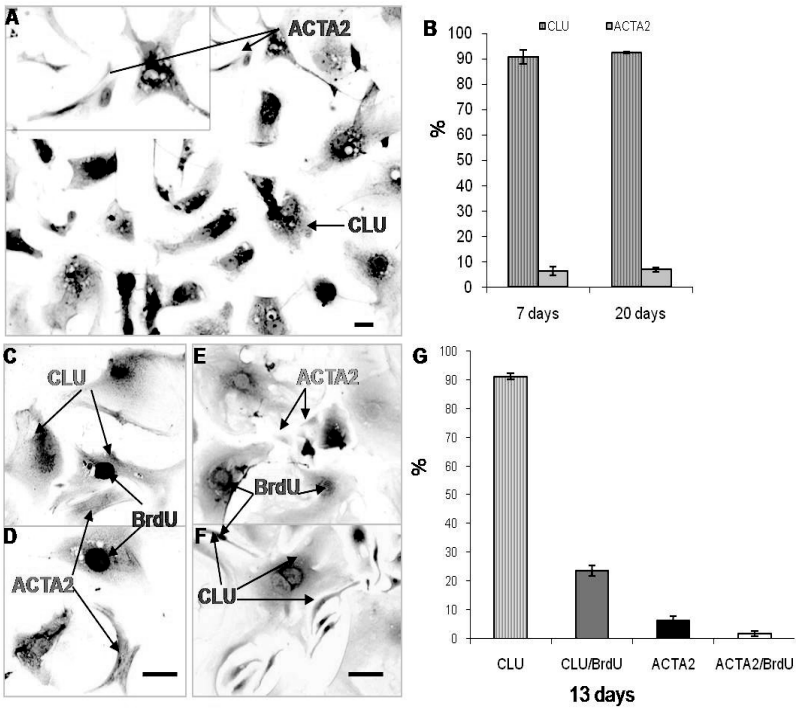

Figure 2. *Discrimination between mouse peritubular cells and Sertoli cells in culture. (A) Immunohistochemical staining for CLU (dark) and α-smooth muscle actin (ACTA2, red) after 7 days in culture. (B) Percentages of CLU and α-smooth muscle actin positive cells after 7 and 20 days in culture. Triple staining for CLU, anti BrdU and ACTA2 after 13 days (C,D) and 20 days (E,F). Smaller-sized CLU positive cells, positive for BrdU, were seen after 20 days in culture (F). (G) Percentages of CLU/BrdU and ACTA2/BrdU positive cells after 13 days in culture. The error bar represents the standard error of the mean (SEM) of results from three different experiments. Bar - 20 µm.*

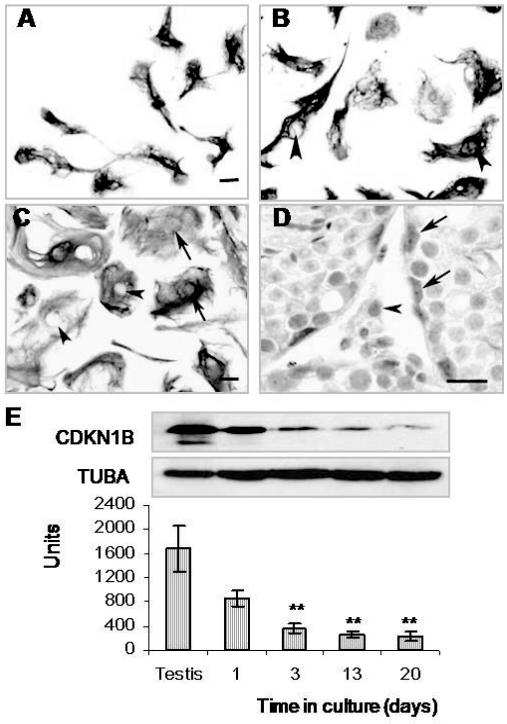

Figure 3. *DKN1B expression in mouse Sertoli cells (arrows) is differentially regulated with time in culture. A-D Immunohistochemical detection of DKN1B (brown) and vimentin as a Sertoli cell marker (blue) after 20 hrs (A), 4 days (B) and 16 days (C) of culture. (D) DKN1B expression in the adult mouse testis (arrows - Sertoli cells; arrowhead - Leydig cell). (E) Western analysis of DKN1B levels after various times in culture (1, 3, 13, 20 days). Expression levels significantly decrease with time in culture. The error bars represent the standard error of the mean (SEM) of results from three experiments, ($p < 0.05$ * or $p < 0.01$ **). Bar - 20 µm.*

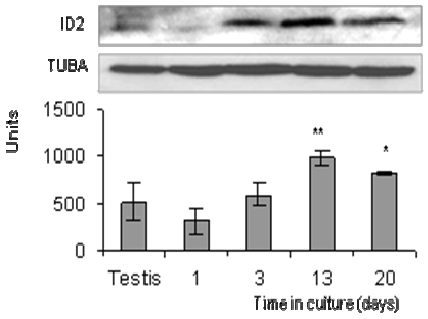

Figure 4. *The expression of ID2 in mouse Sertoli cell cultures significantly increases with time. Results were analyzed by ImageJ and the error bar represents the standard error of the mean (SEM) of results from four different experiments (p < 0.05 * or p < 0.01 **).*

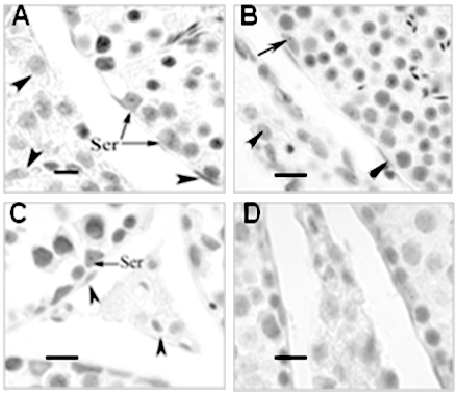

Figure 5. *Immunohistochemical detection of the DNA repair proteins XRCC1 (A), phosphorylated XRCC1 (B) and PARP1(C) in the mouse testis. Sertoli cells were positive but peritubular and other somatic cells (arrow heads) were negative. (D). negative control. Bar - 10 µm.*

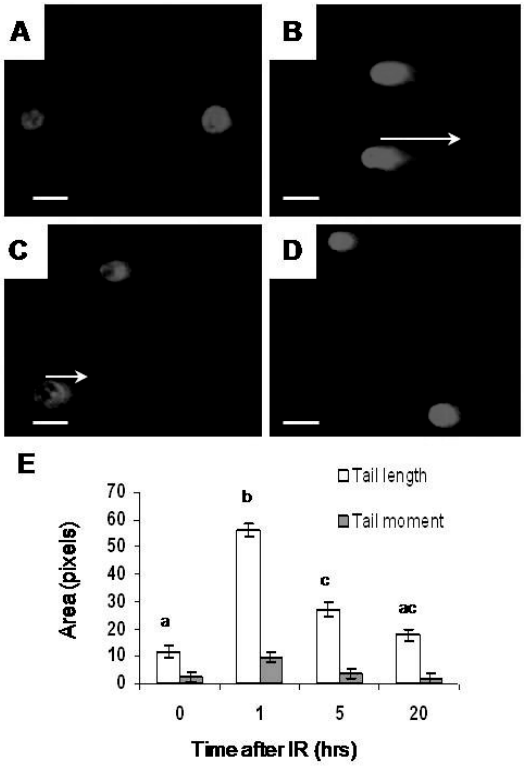

Figure 6. *Comet assay on cultured mouse Sertoli cells at various times after ionizing radiation induced DNA damage. A. Control Sertoli cells; B, C and D 1hr, 5hr and 20hr after irradiation, respectively. E. Analysis of comet assay data. The DNA lesions were quantified by measuring tail length (see arrows) and moment, illustrating DNA damage and repair in Sertoli nuclei. The error bar represents the standard error of the mean (SEM) of results from three experiments, 70 nuclei were studied in each experiment. Different characters above the tail length bars indicate significant differences (p < 0.01 or less). For tail moment 1 hr was significantly higher than all other bars (p < 0.05). Bar - 10 µm.*

Adult Sertoli Cells Are Able To Repair Ionizing Radiation Induced DNA Damage

In the comet assay, during electrophoresis, damaged DNA migrates from the nucleus towards the anode, forming the shape of a “comet” with a head (cell nucleus with intact DNA) and a tail (relaxed and broken DNA). The DNA damage and repair were quantified by measuring changes in the length of the nuclear tail and tail moment. Shortly after irradiation the changes in the shape of the Sertoli cell nuclear comets (Fig. 6A-D) indicated a 5-fold increase in tail length and tail moment compared to the control. Thereafter these parameters decreased by 50% and 70% at 5 hr and 20 hr, respectively, compared to 30 min after irradiation. At 20 hr a less than one fold increase was left compared to the control (Fig. 6E). These results clearly show that Sertoli cells are capable of repair of DNA DSBs.

DISCUSSION

Our results show that mouse and human Sertoli cells reenter the cell cycle in vitro. In addition, unlike the other somatic cells in the testis, Sertoli cells express DNA repair genes, suggesting their ability to actually repair DNA DSBs. The latter was proven using the comet assay. These findings completely change the traditional view on the nature of Sertoli cells in adult, non seasonal breeding mammals.

Adult mouse Sertoli cells were isolated and put into culture. The purity of the isolated Sertoli cells was more than 90% and this remained so throughout the culture, as determined by staining for the Sertoli cell markers vimentin and CLU. After 3 days in culture and as characterized by double immunohistochemical staining for the Sertoli cell marker vimentin, and for incorporated BrdU, 10% of the Sertoli cells were positive for BrdU and with time this percentage increased. After 20 days in culture, 36% of the Sertoli cells incorporated BrdU when exposed to precursor for 15 hr. Apparently, after the transition of the Sertoli cells from their in vivo situation in the seminiferous tubules to the culture conditions, these cells were gradually released from their cell cycle arrest and started to proliferate. These cell-kinetic results could be confirmed by similar culture experiments that were studied using CLU as a Sertoli cell marker and in which comparable data were obtained. Immunohistochemical experiments with the peritubular cell marker α-smooth muscle actin revealed that these cells remained rare (less than 7%) throughout the cultures and did not overgrow the Sertoli cells. Additional experiments were carried out in cultures of a cell suspension of a human testis, to which BrdU was added. Here too, vimentin / BrdU and CLU / BrdU double positive cells were found, indicating that adult human Sertoli cells can also resume proliferation in vitro. Apparently, the potential proliferative capacity of adult Sertoli cells is not specific for mice.

The increased BrdU incorporation in mouse Sertoli cells was accompanied by a decrease in the numbers of Sertoli cells expressing the cell cycle inhibitor DKN1B and by decreasing levels of the DKN1B protein with time in culture. DKN1B has an important role in the regulation of

Sertoli cell proliferation during development and *DKN1B*-/- mice show an increased testis size [14, 30], testis weight (+42%) and Sertoli cell numbers (+26%) [31]. The concomitant increase in the proliferative activity of Sertoli cells and their decreased expression of DKN1B suggest that this protein is indeed actively suppressing Sertoli cell proliferation in vivo. Nevertheless, DKN1B cannot be the only factor involved in regulating Sertoli cell proliferation in the adult mouse testis as in *DKN1B-/-* mice, Sertoli cells do not continue proliferation beyond a certain age [14].

Our results also show that the decrease in DKN1B expression in culture and the onset of Sertoli cell proliferation are associated with an increased expression of ID2. The ID proteins are considered dominant negative regulators of cellular differentiation pathways and act as positive regulators of cellular proliferation [31]. Adult Sertoli cells express the four ID proteins, but only ID2 is present in Sertoli cell nuclei [16, 17]. In adult mice, Sertoli cell nuclei at all stages exhibit immunoreactivity for ID2, indicating that ID2 protein is present throughout the cycle, irrespective of the germ cell types with which the Sertoli cells are associated [17]. Interestingly, ID2 null mice display defective spermatogenesis [33] in addition to retarded growth and neonatal morbidity [34]. Serum has been found to be an inducer of ID2 in other cells [28, 29]. However, the increased ID2 expression with time in our culture (containing 5% serum) may be independent of the effects of serum, because ID2 is induced in primary Sertoli cells cultured in serum-free medium as well as in MSC-1 cells maintained in 10% serum [35] Also in our culture, the rate of Sertoli cells proliferation in serum-free cultures is similar to that in cultures containing 5% serum.

Previously, Steinberger and Steinberger [36] found no labeled Sertoli cells after injection of ^{3}H-thymidine, in rats of 16 days of age and older, illustrating the absence of Sertoli cell proliferation after puberty. In addition, in rat testis organ cultures also no ^{3}H-thymidine incorporation was found in Sertoli cells [36]. Probably, in this organ culture the continued presence of the Sertoli cells in a seminiferous tubule structure inhibited these cells from reentering the cell cycle. Recently, Chaudhary et al. [1] studied the proliferative activity of Sertoli cells from 20 and 60 days old rats, cultured for a few days in F12-Ham medium with 10% bovine calf serum by incubating the cells with ^{3}H-thymidine. The incorporation of ^{3}H-thymidine was found to be within the background level of the assay. Possibly, autoradiographical studies on these cultures would have rendered a few labeled Sertoli cells that had incorporated 3H-thymidine. In studies using the cell proliferation marker MKI67, Sertoli cell proliferation also was found to be absent in the adult mouse testis [10]. To our knowledge, except in seasonal breeders [3-5], in mammals in vivo or in vitro, proliferation of adult Sertoli cells has never been described [14, 36]. However, some reports already indicated that the quiescence of Sertoli cells might not be irreversible. Sertoli cell nuclei injected within an enucleated oocyte did start to proliferate again but failed to produce full-term embryos [37] and in culture, adult rat post-mitotic Sertoli cells could be forced to re-enter the cell cycle and to

proliferate, by way of transfection with ID1 or ID2 [1]. We now have shown that already normal cell culture conditions can release Sertoli cells from their cell cycle arrest.

Second, unlike the other somatic cells in the testis, e.g. endothelial cells, Leydig cells, peritubular cells and macrophages, Sertoli cells express proteins that function in DNA damage repair. These repair proteins include PARP1, phosphorylated-XRCC1 (present results), pATM [18], XRCC6/XRCC5 (KU70/KU80) [19, 38] and TP53BP1 [20]. For the studied DNA repair proteins, XRCC1 and PARP1, and also for TP53BP1 (unpublished data), the staining and even the focus formation was not stage specific. Interestingly, while Sertoli cells express repair proteins, they do not express several proteins that are considered crucial for proper repair in somatic cell lines. For example, Sertoli cells lack MDC1 and RAD51, and in Sertoli cells H2AX is not phosphorylated in response to irradiation. One way to monitor DNA damage repair is by following the decrease in the number of γH2AX foci that are formed at the sites of DSBs, with time after irradiation [39]. However, γH2AX not being present in Sertoli cells, this was not feasible in this cell type. Previously, we followed TP53BP1 foci in Sertoli cells with time after irradiation and suggested that the observed 70% decrease in foci number was a sign that DSB repair might take place [20]. To see whether this reduction in foci number was indeed related to the repair of DSBs and not to the confluence of smaller foci into a few big foci at later time points after irradiation, a comet assay was done on freshly isolated Sertoli cells in culture. After a period of 20 hr a decrease of 70% in comet tail and moment was found which was comparable to the decrease in TP53BP1 foci with time [20]. Hence, adult Sertoli cells do indeed repair DNA damage.

Which DNA repair pathway is operational in Sertoli cells? As Sertoli cells in vivo are in G1 phase, homologous recombination (HR) is not possible and for canonical endjoining repair DNA-PKcs is needed and Sertoli cells do not express this protein [19]. Recently it was suggested that NHEJ repair may take place via PARP1 and XRCC1, using a recently discovered synapsis activity of PARP1, and the ligation activity of the XRCC1-DNA ligase III complex, proteins otherwise involved in base excision repair pathway [40]. Sertoli cells do express PARP1 and XRCC1 and might use these proteins to repair DNA DSBs.

In conclusion, our results indicate that postpubertal Sertoli cells cannot be regarded as terminally differentiated cells that have lost the capacity to proliferate. In vivo, the testis barrier and the formation of tight junction after maturation may prevent Sertoli cells from reentering cell cycle. Also, there seems to exist a balance between cell cycle inhibitors and inducers that in the normal in vivo situation keeps the cells quiescent. Probably the residual proliferation capacity of Sertoli cells in vivo is the underlying reason for the rare Sertoli cells-tumors seen in humans (41) and more often in the dog (42). Also in nodules in testis of infertile human patients mitoticaly active Sertoli cells have been found [43]. Clearly, putting Sertoli cells in culture disturbs the in vivo balance in such a way that these cells gradually start to proliferate again. Sertoli cells also do not behave as terminally differentiated somatic cells in that they are still

able to repair DSBs. DSBs when not repaired will be fatal during cell division. Quiescent cells will generally not be bothered by the presence of DSBs, unless some vital gene is involved in the break and indeed the other types of quiescent somatic cells in the testis do not express DNA repair proteins. Our finding that Sertoli cells do repair DSBs is compatible with the notion that these cells still have the capacity to proliferate, making it necessary to remove DSBs.

These findings in non seasonal breeding mammals completely change the present views on the nature of Sertoli cells and it will be interesting to study the regulatory mechanisms governing Sertoli cell proliferation in further detail. In this respect, seasonal breeders may provide a clue to investigate these regulatory mechanisms as probably the same mechanisms may trigger Sertoli cell proliferation in these animals at the start of each breeding season.

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REFERENCES

1. Chaudhary J, Sadler-Riggleman I, Ague JM, Skinner MK. The helix-loop-helix inhibitor of differentiation (ID) proteins induce post-mitotic terminally differentiated Sertoli cells to re-enter the cell cycle and proliferate. Biol Reprod 2005; 72:1205-1217.

2. Sharpe RM, McKinnell C, Kivlin C, Fisher JS. Proliferation and functional maturation of Sertoli cells, and their relevance to disorders of testis function in adulthood. Reproduction 2003; 125:769-784.

3. Hochereau-de Reviers MT, Lincoln GA. Seasonal variation in the histology of the testis of the red deer, Cervus elaphus. J Reprod Fertil 1978; 54:209-213.

4. Johnson L, Varner DD, Tatum ME, Scrutchfield WL. Season but not age affects Sertoli cell number in adult stallions. Biol Reprod 1991; 45:404-410.

5. Tarulli GA, Stanton PG, Lerchl A, Meachem SJ. Adult sertoli cells are not terminally differentiated in the Djungarian hamster: effect of FSH on proliferation and junction protein organization. Biol Reprod 2006; 74:798-806.

6. Holsberger DR, Jirawatnotai S, Kiyokawa H, Cooke PS. Thyroid hormone regulates the cell cycle inhibitor $P27^{KIP1}$ in postnatal murine Sertoli cells. Endocrinology 2003; 144:3732-3738.

7. Norton JN, Skinner MK. Regulation of Sertoli cell differentiation by the testicular paracrine factor PModS: potential role of immediate-early genes. Mol Endocrinol 1992; 6:2018-2026.

8. Vergouwen RP, Jacobs SG, Huiskamp R, Davids JA, de Rooij DG. Proliferative activity of gonocytes, Sertoli cells and interstitial cells during testicular development in mice. J Reprod Fertil 1991; 93:233-243.

9. van Haaster LH, de Jong FH, Docter R, de Rooij. DG, The effect of hypothyroidism on Sertoli cell proliferation and differentiation and hormone levels during testicular development in the rat. Endocrinology 1992; 131:1574-1576.

10. Sridharan S, Simon L, Meling DD, Cyr DG, Gutstein DE, Fishman GI, Guillou F, Cooke PS. Proliferation of adult sertoli cells following conditional knockout of the Gap junctional protein GJA1 (connexin 43) in mice. Biol Reprod 2007; 76:804-812.

11. Buzzard JJ, Wreford NG, Morrison JR. Thyroid hormone, retinoic acid, and testosterone suppress proliferation and induce markers of differentiation in cultured rat sertoli cells. Endocrinology 2003; 144:3722-3731.

12. Meachem SJ, McLachlan RI, de Kretser DM, Robertson DM, Wreford NG. Neonatal exposure of rats to recombinant follicle stimulating hormone increases adult Sertoli and spermatogenic cell numbers. Biol Reprod 1996; 54:36-44.

13. Petersen C, Boitani C, Froysa B, Soder O. Interleukin-1 is a potent growth factor for immature rat sertoli cells. Mol Cell Endocrinol 2002; 186:37-47.

14. Beumer TL, Kiyokawa H, Roepers-Gajadien HL, van den Bos LA, Lock TM, Gademan IS, Rutgers DH, Koff A, de Rooij DG. Regulatory role of $P27^{KIP1}$ in the mouse and human testis. Endocrinology 1999; 140:1834-1840.

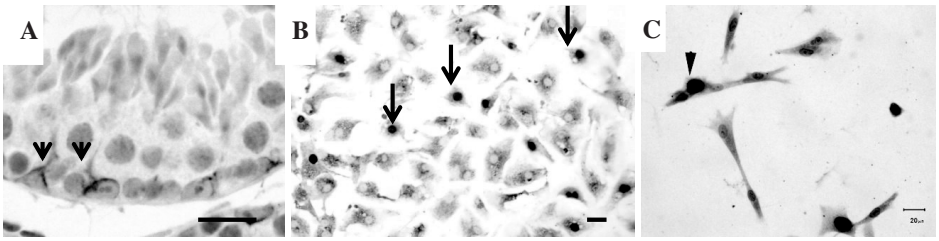
15. Beumer TL, Roepers-Gajadien HL, Gademan IS, van Buul PP, Gil-Gomez G, Rutgers DH, de Rooij DG. The role of the tumor suppressor p53 in spermatogenesis. Cell Death Differ 1998; 5:669-677.

16. Chaudhary J, Johnson J, Kim G, Skinner MK. Hormonal regulation and differential actions of the helix-loop-helix transcriptional inhibitors of differentiation (Id1, Id2, Id3, and Id4) in Sertoli cells. Endocrinology 2001; 142:1727-1736.

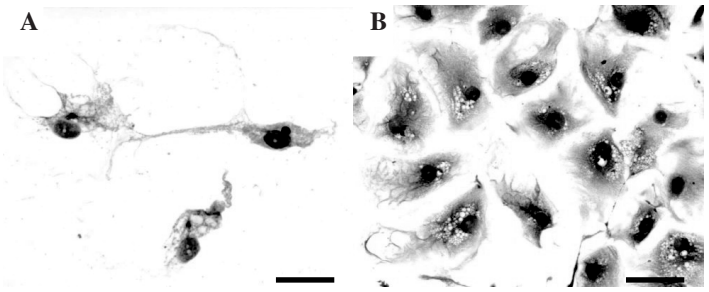
17. Sablitzky F, Moore A, Bromley M, Deed RW, Newton JS, Norton JD. Stage- and subcellular-specific expression of Id proteins in male germ and Sertoli cells implicates distinctive regulatory roles for Id proteins during meiosis, spermatogenesis, and Sertoli cell function. Cell Growth Differ 1998; 9:1015-1024.
18. Hamer G, Kal HB, Westphal CH, Ashley T, de Rooij DG. Ataxia telangiectasia mutated expression and activation in the testis. Biol Reprod 2004; 70:1206-1212.
19. Hamer G, Roepers-Gajadien HL, van Duyn-Goedhart A, Gademan IS, Kal HB, van Buul PP, Ashley T, de Rooij DG. Function of DNA-protein kinase catalytic subunit during the early meiotic prophase without Ku70 and Ku86. Biol Reprod 2003; 68:717-721.
20. Ahmed EA, van der Vaart A, Barten A, Kal HB, Chen J, Lou Z, Minter-Dykhouse K, Bartkova J, Bartek J, de Boer P, de Rooij DG. Differences in DNA double strand breaks repair in male germ cell types: lessons learned from a differential expression of Mdc1 and TP53. DNA Repair 2007; 6:1243-1254.
21. Anway MD, Folmer J, Wright WW, Zirkin BR. Isolation of sertoli cells from adult rat testes: an approach to ex vivo studies of Sertoli cell function. Biol Reprod 2003; 68:996-1002.
22. Gaemers IC, van Pelt AM, van der Saag PT, Hoogerbrugge JW, Themmen AP, de Rooij DG. Differential expression pattern of retinoid X receptors in adult murine testicular cells implies varying roles for these receptors in spermatogenesis. Biol Reprod 1998; 58:1351-1356.
23. Kanatsu-Shinohara M, Ogonuki N, Inoue K, Miki H, Ogura A, Toyokuni S, Shinohara T. Long-term proliferation in culture and germline transmission of mouse male germline stem cells. Biol Reprod 2003; 69:612-616.
24. Gobbel GT, Bellinzona M, Vogt AR, Gupta N, Fike JR, Chan PH. Response of postmitotic neurons to X-irradiation: implications for the role of DNA damage in neuronal apoptosis. J Neurosci 1998; 18:147-155.
25. Karran P, Ormerod MG. Is the ability to repair damage to DNA related to the proliferative capacity of a cell? The rejoining of X-ray-produced strand breaks. Biochim Biophys Acta 1973; 299:54-64.
26. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227:680-685.
27. Schlatt S, de Kretser DM, Loveland KL. Discriminative analysis of rat Sertoli and peritubular cells and their proliferation in vitro: evidence for follicle-stimulating hormone-mediated contact inhibition of Sertoli cell mitosis. Biol Reprod 1996; 55:227-235.
28. Hara E, Yamaguchi T, Nojima H, Ide T, Campisi J, Okayama H, Oda K. Id-related genes encoding helix-loop-helix proteins are required for G1 progression and are repressed in senescent human fibroblasts. J Biol Chem 1994; 269:2139-2145.
29. Lasorella A, Noseda M, Beyna M, Yokota Y, Iavarone A. Id2 is a retinoblastoma protein target and mediates signalling by Myc oncoproteins. Nature 2000; 407:592-598.
30. Nakayama K, Ishida N, Shirane M, Inomata A, Inoue T, Shishido N, Horii I, Loh DY, Nakayama K. Mice lacking p27(Kip1) display increased body size, multiple organ hyperplasia, retinal dysplasia, and pituitary tumors. Cell 1996; 85:707-720.
31. Holsberger DR, Buchold GM, Leal MC, Kiesewetter SE, O'Brien DA, Hess RA, Franca LR, Kiyokawa H, Cooke PS. Cell-cycle inhibitors $P27^{KIP1}$ and p21Cip1 regulate murine Sertoli cell proliferation. Biol Reprod 2005; 72:1429-1436.

32. Barone MV, Pepperkok R, Peverali FA, Philipson L. Id proteins control growth induction in mammalian cells. Proc Natl Acad Sci U S A 1994; 91:4985-4988.
33. Norton JD. ID helix-loop-helix proteins in cell growth, differentiation and tumorigenesis. J Cell Sci 2000; 113:3897-3905.
34. Yokota Y, Mansouri A, Mori S, Sugawara S, Adachi S, Nishikawa S, Gruss P. Development of peripheral lymphoid organs and natural killer cells depends on the helix-loop-helix inhibitor Id2. Nature 1999; 397:702-706.
35. Scobey MJ, Fix CA, Walker WH. The Id2 transcriptional repressor is induced by follicle-stimulating hormone and cAMP. J Biol Chem 2004; 279:16064-16070.
36. Steinberger A, Steinberger E. Replication pattern of Sertoli cells in maturing rat testis in vivo and in organ culture. Biol Reprod 1971; 4:84-87.
37. Wakayama T, Perry AC, Zuccotti M, Johnson KR, Yanagimachi R. Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. Nature 1998; 394:369-374.
38. Choi EK, Lee YH, Choi YS, Kwon HM, Choi MS, Ro JY, Park SK, Yu E. Heterogeneous expression of Ku70 in human tissues is associated with morphological and functional alterations of the nucleus. J Pathol 2002; 198:121-130.
39. Bouquet F, Muller C, Salles B. The loss of gammaH2AX signal is a marker of DNA double strand breaks repair only at low levels of DNA damage. Cell Cycle 2006; 5:1116-1122.
40. Audebert M, Salles B, Calsou P. Involvement of poly(ADP-ribose) polymerase-1 and XRCC1/DNA ligase III in an alternative route for DNA double-strand breaks rejoining. J Biol Chem 2004; 279:55117-55126.
41. Kratzer SS, Ulbright TM, Talerman A, Srigley JR, Roth LM,Wahle GR, Moussa M, Stephens JK, Millos A, Young RH. Large cell calcifying Sertoli cell tumour of the testis. Contrasting features of six malignant and six benign tumours and a review of the literature. Am J Surg Pathol 1997; 21:1271–1280.
42. Peters MA, de Rooij DG, Teerds KJ, van Der Gaag I, van Sluijs FJ. Spermatogenesis and testicular tumours in ageing dogs. J Reprod Fertil. 2000; 120:443-452
43. Brehm R, Rey R, Kliesch S, Steger K, Marks A, and Bergmann M. Mitotic activity of Sertoli cells in adult human testis: an immunohistochemical study to characterize Sertoli cells in testicular cords from patients showing testicular dysgenesis syndrome. Anat Embryol 2006; 211: 223-236.

SUPPLEMENTAL DATA LEGENDS



Suppl. Figure 1. *(A) Immunohistochemical staining for the Sertoli cell marker vimentin. Vimentin expression in mouse testis is restricted to the cytoplasm of Sertoli cells (arrows). (B) Mouse Sertoli cells of 6 days serum free culture positive for BrdU. (C) Adult Human Sertoli cells positive for CLU and BrdU. Bar represents 20µm.*



Suppl. Figure 2. *Immunohistochemical staining for the proliferation inducer ID2. The cultured mouse Sertoli cells are positive for ID2 after both 22h (A) and one week (B) of culture. Bar represents 20µm.*

Suppl. Table 1. *Summary of proteins expressed in Sertoli cells that regulate cell proliferation or are involved in DNA repair.*

Proteins expressed in Sertoli cells	**Repair pathway or function in proliferation**	**References**
TP53 - expressed after irradiation	Regulates cell proliferation and apoptosis after DNA damage	[15]
CDKN1B **-** 3 fold increase after X-irradiation	Cell cycle inhibitor, regulates cell proliferation and may be involved in the cellular response to DNA damage	[14, 25]
ID proteins - inhibitors of differentiation/DNA binding	These genes are often up-regulated in proliferating undifferentiated cells, and down-regulated upon induction of differentiation	[17]
H2A	Should become phosphorylated after DNA damage but not in Sertoli cells	S.1.
XRCC6/XRCC5	DSB repair by way of NHEJ	[19,38]
pAtm	DSB repair	[18]
TP53BP1 -foci are formed after irradiation	DSB repair	[20]
XRCC1	Base excision repair, single strand repair and alternative route of DSB repair by NHEJ	Fig.1
Phospho-XRCC1	Base excision repair, single strand repair and alternative route of DSB repair by NHEJ	Fig.1
PARP1	Base excision repair, single strand repair and alternative route of DSB repair by NHEJ	Fig.1

S.1. Bhatnagar, Y.M., Faulker, R.D., McCullar, M.K., 1985. Biochemical and immunological characterization of an H2A variant from the mouse testis. Biochim. Biophys. Acta. 827, 14-22.

Chapter 4

Parp1-XRCC1 and the repair of DNA double strand breaks in mouse round spermatids

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Submitted

Abstract

The repair of DNA double strand breaks (DSBs) in male germ cells is slower and differently regulated compared to that in somatic cells. Round spermatids show DSB repair and are radioresistant to apoptosis induction. Mutation induction studies using ionizing irradiation, indicated a high frequency of chromosome aberrations (CA) in the next generation. Since they are in a comparable G1 stage of the cell cycle, haploid spermatids are expected to repair DSBs by the non homologous end joining pathway (NHEJ). However, immunohistochemical evidence indicates that not all components of the classical NHEJ pathway are available since the presence of DNA–PKcs cannot be shown immunocytochemically. Therefore, we now have studied whether the alternative Parp1/XRCC1 dependent NHEJ pathway is active in these nuclei. We have found that round spermatids, as well as most other types of male germ cells express both Parp1 and XRCC1. To evaluate DSB repair in SCID mice, deficient for DNA-PKcs, and to study the involvement of the Parp1/XRCC1 dependant NHEJ pathway in round spermatids, the loss of γ-H2AX foci after irradiation was determined in nucleus spreads of round spermatids of SCID mice and in nucleus spreads and histological sections of Parp-inhibited mice and their respective controls. Results show that around half of the breaks in randomly selected round spermatids are repaired at 8 hr after irradiation. The repair of 16% of the induced DSBs requires DNA-PKcs and 21% Parp1. The numbers of foci in Parp-inhibited testes tended to be higher in spermatids of all epithelial stages reaching significance in stages I-III. These results indicate an active Parp1/XRCC1 dependent NHEJ pathway in round spermatids and a decreased repair capacity in later round spermatid stages.

1. Introduction

In contrast to the dynamic field of DNA repair in nuclei of somatic cells [1], insight into DNA repair mechanisms in the mammalian, mainly mouse, germline is only slowly developing (review [2]). The most risky DNA lesion, a DNA double strand break (DSB), is instrumental in the initiation of homologous recombination (HR) [3,4] and the mechanism of recombination is akin to somatic homologous recombination repair (HR). Apart from HR, DSBs are repaired by non homologous end-joining (NHEJ) [5-7]. The NHEJ pathway relies on a set of proteins that recognize, bind and repair the DSBs. These proteins include DNA-PKcs which is recruited by the Ku70 and Ku80 proteins to the site of damage and subsequently, both end-positioned Ku and DNA-PKcs mediate the recruitment of the XRCC4/DNA ligase IV complex which is responsible for the ligation step [8].

In response to DSBs, cells phosphorylate H2AX at serine 139, inducing the formation of nuclear foci of phosphorylated H2AX (γ-H2AX) at the sites of damage. These foci represent the DSBs in a 1:1 manner. Hence, γ-H2AX can be used as a marker (reviews [9,10]). Studies on

somatic cells [11,12] and on germ cells [13-15] have shown that the kinetics of γ-H2AX loss is related to DSB repair activity, especially at low doses of radiation [16]. Audebert et al [17] proposed an alternative route for *in vitro* end-joining activity, independent of the DNA-PKcs/XRCC4-ligase IV complex. This pathway requires a recently detected synaptic activity of Parp1 and the ligation activity of the XRCC1-DNA ligase III complex, proteins otherwise involved in base excision repair (BER). BER is a major pathway for removal of DNA lesions arising from endogenous processes as well as from exposure to exogenous chemicals and ionizing radiation [18] and is very active in spermatogenic cells [19]. When involved in DSBs repair, the Parp1/XRCC1 pathway may function as a backup for the DNA-PK dependent pathway in the absence of Ku and/or DNA-PKcs [20]. XRCC1 is reported to specifically associate with Parp1 and to regulate its activity following DNA damage [21]. Parp1 is expressed in the testis and *Parp1* knockout mice are viable and fertile but sensitive to ionizing radiation [22,23]. *XRCC1* gene knockout in mice results in embryonic lethality, demonstrating it is essential for development [24]. PCR results have shown that *XRCC1* is highly expressed in the mouse testis [25] (confirmed by Symatlas, https://biogps.gnf.org), but its role in spermatogenesis and in DNA repair in germ cells is unknown.

In male germ cells, the repair of DSBs differs from that described for somatic cell lines due to differences in the expression or, dependent on the cell type, even the absence of some DNA repair proteins, as usually evidenced by immunological in situ detection [13,26-28]. In early spermatocytes, during meiotic recombination, Ku70 is not expressed and NHEJ is suppressed [27,28]. In the haploid round spermatids, DSB repair takes place as shown by a diminution of the numbers of γ-H2AX foci with time after irradiation [13]. In these cells, repair most likely relies on NHEJ because of the inability of HR repair as only one copy of the DNA is available. Furthermore, immunohistochemical evidence indicates that DNA–PKcs is not expressed in round spermatids [27]. So while HR is not indicated in spermatids, classical NHEJ may not be functional either. Indications for a sensitivity of spermatids for the generation of chromosomal imbalances by ionizing irradiation induced DSBs, have been reported by Matsuda et al. [29], Russell et al. [30] and Tusell et al. [31]. Nevertheless round spermatids, do show repair of DNA DSBs [13]. However, while DNA DSB repair in irradiated somatic cells by DNA-PK dependent NHEJ is a very fast process, 80% of the breaks being rejoined in less than 2 hr and virtually 100% within 6 hr [32], round spermatids repair only 30% of breaks during the first 4 hr after irradiation [13]. Apparently, in round spermatids fast repair is not available.

We now have studied whether DSB repair in round spermatids is still carried out by the classical NHEJ pathway based on expression levels of DNA-PKcs levels below the immunohistochemical detection level, using DNA-PKcs deficient SCID mice. In addition, we explored the possibility that round spermatids use the alternative Parp1/XRCC1 pathway for NHEJ, following γ-H2AX foci numbers in control and Parp1-inhibited mice after irradiation.

Parp1 and XRCC1 expression in germ cells was localized and our data indicate that these proteins indeed have a role in the repair of DSBs in round spermatids.

2. Materials and methods

2.1. Animals, irradiation and fixation

Groups of C.B17 SCID and their wild type control (C.B17) mice were used (12 mice per group, 7 to 8 wk of age; Charles River, Maastricht, The Netherlands). These mice were either sham-irradiated (4 mice per group) or received a dose of 1 Gy of gamma-rays (whole body irradiation, 6 MV, 91 MU, Elekta, Crawley, UK). Irradiated mice were sacrificed 1 or 8 hr after irradiation. Mice were killed by CO_2 asphyxiation.

Two groups of male FVB mice (12 per group, 8 to 10 wk of age, Charles River) were used. Mice were either sham-irradiated or received a dose of 1 Gy of gamma-rays (whole body irradiation, 6 MV, 91 MU, Elekta). Groups received either injections of the Parp1 inhibitor, 3,4-dihydro-5-[4-(1-piperidinyl)butoxy]-1(2H)-isoquinolinone, DPQ (D5314 Sigma, 10 mg/kg of body weight) dissolved in DMSO or DMSO alone, 1 hr before irradiation (IR) and 3.5 hr later (for the 8 hr time point). Mice were killed by CO_2 asphyxiation, 1 or 8 hr after irradiation.

One testis was fixed in 4% paraformaldehyde in PBS for 24 hr at 4 °C and from the other testis nucleus spreads of spermatogenic cells were prepared (see below). Tissues were washed in 70% EtOH prior to embedding in paraffin (Stemcowax; Adamas Instruments, Amerongen, The Netherlands). The animals were used and maintained according to regulations provided by the animal ethical committee of the Utrecht University that also approved the experiments.

2.2. Immunohistochemistry

Sections of testes (5 µm) of normal and Parp-inhibited mice (irradiated or sham-irradiated were mounted together on TESPA (3-aminoproyl-tri-ethoxysilane)-coated glass slides and dried overnight at 37 °C. Sections were dewaxed in xylene and hydrated in a graded series of alcohols. For Parp1, XRCC1 and γ-H2AX staining, sections were boiled twice for 10 min in 0.01 M sodium citrate using a microwave oven (H2500; Bio-Rad, Hercules, USA). Sections were incubated in 0.35% H_2O_2 in PBS for 10 min. Blocking was done in 5% BSA (Sigma, St. Louis, USA, A-7906)/5% goat serum (Vector laboratories, S-1000, Burlingame, CA, USA) in PBS. The primary antibodies were: pre-diluted mouse monoclonal anti-XRCC1 (Abcam ab54393, Cambridge, UK), rabbit polyclonal anti-Parp1 (Abcam, ab2168.500, Cambridge, UK) and antiphospho-H2AX [Ser139] (JBW-301, 05-636, Upstate Biotechnology, Lake Placid, USA). The slides were washed in PBS and then incubated with secondary antibody,

PowerVision Poly Hrp-anti mouse/rabbit/rat (ImmunoVision Technologies, Co. Brisbane, CA 94005, USA), ready to use, for 40 min at room temperature. Bound antibodies were visualized using 0.3 µg/µl 3,3'-diaminobenzidine (DAB, Sigma) in PBS, to which 0.03% H_2O_2 was added. Sections were counterstained with Mayer hematoxylin. For negative controls, primary antibodies were replaced by rabbit or mouse IgGs. Sections were dehydrated in a series of graded alcohols and xylene and mounted with Pertex (Cellpath Ltd., Hemel Hempstead, U.K.).

2.3. *Surface-spread preparations*

Nucleus spreads were made as previously described by Peters et al [33] and van der Heijden et al [34]. Briefly, a suspension of spermatogenic cells in MEM was obtained and then incubated with a hypotonic buffer (17 mM sodium citrate, 50 mM sucrose, 30 mM Tris HCl, pH 8.2). After centrifugation, the pellet was carefully resuspended in a 100 mM sucrose solution and applied over a PFA-coated glass slide (1% PFA, 0.15% Triton-X-100, pH 9.2–9.5). The slides were kept in a humidified atmosphere in a box to slow down drying out. After 1.5 hr the box was opened and the slides were washed in 0.08% photo-flo (Sigma, P7417 St. Louis, USA).

2.4. Immunofluorescence

For immunofluorescence, slides were washed in PBS and incubated for 10 min in PBS including 0.04% Triton X and then incubated with blocking solution (10% goat serum and 10% BSA in PBS). For recognizing pachytene and diplotene spermatocytes, slides were incubated with rabbit polyclonal antibody anti-phospho-XRCC1 (Bethyl Laboratories, ICH00116, Montgomery, USA) and antiphospho-H2AX [Ser139] (JBW-301, 05-636, Upstate Biotechnology). The secondary antibodies, goat anti-rabbit (Alexa fluor 488, A-11008), goat anti-mouse (Alexa fluor 488, A-21121 and 594, A-21125) and Texas Red-labeled goat anti-mouse, were obtained from Jackson ImmunoResearch (West Grove, USA). The slides were incubated with DAPI (for 10 min) and mounted in VECTAshield (Vector lab. H-1000) and viewed with an Olympus AX70 microscope (Olympus Optical Co. ltd., Tokyo, Japan) and images were recorded digitally.

2.5. The kinetics of γ-H2AX loss after irradiation

Numbers of γ-H2AX foci were counted in round spermatids in testis sections and in nucleus spreads prepared from irradiated wild type and Parp1-inhibited mice, both at 1hr and 8 hr after irradiation. Stage recognition in sections was carried out according to Ahmed and de Rooij [35].

2.6. Statistics

Statistical analysis between groups from four different experiments was done by one way analysis of variance (Dunnett's Multiple Comparison Test) using GraphPad software (GraphPad Software, Inc., CA 92037 USA). Figures are given with the standard error of the mean as the indicator of variance.

3. Results

3.1. γ-H2AX foci numbers in round spermatids of SCID mice

Round spermatids do not contain DNA-PKcs at a level that can be detected by immunohistochemistry [28]. In order to see whether low levels of DNA-PKcs in round spermatids might allow the observed repair of DSBs via the classical NHEJ pathway, in nucleus spread preparations we compared the repair capacity in round spermatids in SCID mice (Fig. 1A), which are deficient for DNA-PKcs, and in wild type mice. γ-H2AX foci were counted at 1 and 8 hr after irradiation. In both SCID and wild type mice, an average of 7 foci per nucleus was seen 1 hr after a dose 1 Gy (Fig. 1B). At 8 hr after irradiation, 46.2 ± 1.1% of foci were repaired in round spermatids of control mice. In DNA-PKcs deficient SCID mice, this percentage was significantly reduced to 30.1 ± 1.8% ($p < 0.01$, Table 1), indicating that some DNA-PKcs dependent NHEJ is taking place in round spermatids, accounting for about 16% of the DSBs. However, most of the repair of DSBs in these cells must follow another NHEJ pathway.

To see whether NHEJ in round spermatids can follow the Parp1/XRCC1 pathway we localized the expression of these proteins in the testis. In the control testis**,** a dense nuclear staining for XRCC1 was found in A spermatogonia (Fig. 2A) while in In and B spermatogonia this staining was weak. During early meiotic prophase, a few foci for XRCC1 were found in leptotene and zygotene spermatocytes (Fig. 2B). A dense nuclear staining was found in late spermatocytes and in round and elongated spermatids up till stage XI. XRCC1 was also expressed in Sertoli cells**.**

***Table 1**. Numbers of γ-H2AX foci at 1 and 8 hr after irradiation and relative percentage of breaks repaired in spread nuclei from SCID and wild type C.B17 mice, and in Parp1-inhibited and control FVB mice. Statistical analysis (Dunnett's Multiple Comparison Test), [a] P< 0.01 with C.B17, [b] P< 0.001 with control (FVB).*

Mice	C.B17	SCID	Control (FVB)	Parp1-inhibited
Foci numbers ± SEM **1 hr** **8 hr**	 6.99 ± 0.03 3.90 ± 0.16	 7.15 ± 0.11 5.02± 0.06	 6.01 ± 0.11 2.73 ± 0.06	 6.47 ± 0.16 4.33 ± 0.19
Repaired % after 8 hr	46.2 ± 1.1%	30.1± 1.8% [a]	54.4 ± 0.2%	33.3± 4.0% [b]
Analyzed cells per mouse (number of mice)	40 (3)	40 (3)	50 (3)	50 (3)

3.2. XRCC1 expression in control and irradiated testis

In the irradiated testis, the expression pattern of XRCC1 was similar except that in the unirradiated testis more foci were seen in early spermatocytes (Fig. 2C). In addition there were some occasional XRCC1 foci in Sertoli cells. The dense nuclear staining for XRCC1 in late spermatocytes was also found after irradiation (Fig. 2CD). In nucleus spreads, double staining for phospho-XRCC1 and γ-H2AX revealed that the dense staining of late prophase spermatocytes was due to XRCC1 localization in the axial elements of sex chromosomes (Fig 2EF) and axial/lateral SC elements, extending into the surrounding chromatin.

3.3. Parp1 expression in control and irradiated testis

An abundant nuclear staining for Parp1 was observed in A spermatogonia. In type In and type B spermatogonia and in early spermatocytes the staining was weak. A strong staining was seen again in late spermatocytes and in round spermatids. In elongated spermatids, Parp1 staining was observed till stage XI, but this staining was in the acrosomes of these cells (Fig 3A). Sertoli cells also showed a strong nuclear staining for Parp1. No differences in Parp1 staining were seen between irradiated (Fig 3B) and non-irradiated testes (Fig 3A).

3.4. Parp inhibition reduced DNA DSBs repair kinetics in round spermatids

Parp1 and XRCC1 being expressed in round spermatids, we subsequently established the possible involvement of Parp1 and XRCC1 in the repair of DNA DSBs in these cells. In spread preparations from irradiated normal and Parp1-inhibited mice, γ-H2AX foci were counted in the nuclei of the round spermatids. From 1 to 8 hr after irradiation in normal mice, there was a 54.4 ± 0.2% reduction in the number of γ-H2AX foci in round spermatids indicating active DNA DSB repair (foci counts in spermatids in stages I–VIII taken together). In Parp1-inhibited mice, the reduction in foci number in round spermatids was significantly lower (33.3 ± 4.0%; $p < 0.0001$) (Table 1, Fig. 4A).

3.5. DSBs in round spermatids of different stages

We next studied whether the capacity to repair DSBs in round spermatids depends on the developmental step these cells are in. Counts of γ-H2AX foci were carried out in testis sections (Figs. 4B). Random counting of foci in all stages in sections indicated that 51.4 ± 3.3% of foci were repaired after 8 hr in control mice, which was significantly higher compared to the repair in Parp1-inhibited mice (35.6 ± 1.4%, Fig. 4D). Foci were counted in round spermatids in stages I-III, IV-V, V-VII and VIII-IX (Fig 4E). The data indicate DSB repair to be most efficient in round spermatids in steps 1 to 3 of their development (by definition present in epithelial stages I-III). These cells repaired 65.1 ± 2.1% of the DSBs between 1 and 8 hr after irradiation. Numbers of γ-H2AX foci per cell in early spermatids (stages I-III) were lower in number but bigger in size compared to those in late spermatids (stages VI-VII) (data not shown). In Parp1-inhibited mice only 35.7 ± 2.5% of the breaks were repaired, which is significantly lower ($p < 0.001$) (Fig. 4F, Table 2). While in the other epithelial stages, numbers of foci in round spermatids of Parp1-inhibited testes tended to be higher in spermatids as well, the difference with controls did not reach sigificancy (Fig 4E).

***Table 2**. Numbers of γ-H2AX foci at 1 and 8 hr after irradiation and relative percentage of breaks repaired in testis sections from Parp1-inhibited and control, FVB mice. Statistical analysis (Dunnett's Multiple Comparison Test), [a] P< 0.01 with control.*

Mice	Control (FVB)	Parp1-inhibited
Foci numbers ± SEM **1 hr** **8 hr**	 5.35 ± 0.26 2.64 ± 0.05	 5.23 ± 0.09 3.32 ± 0.24
Repaired % after 8 hr	51.4 ± 3.3%	35.6± 1.4% [a]
Repaired % after 8 hr (stage I-III)	65.1 ± 2.1%	35.7 ± 2.5% [a]

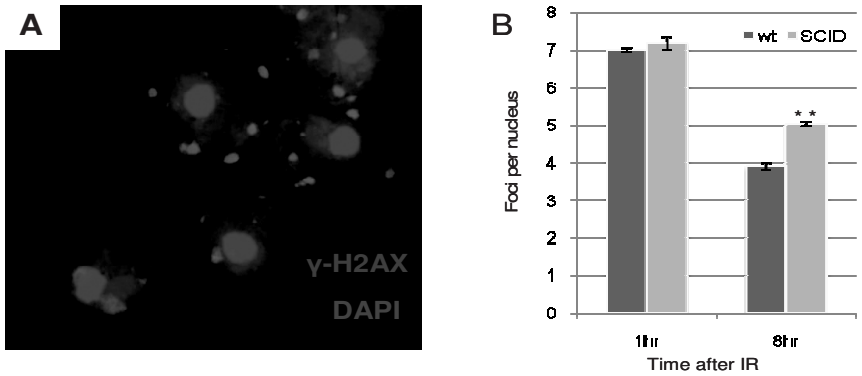

Fig. 1. *γ-H2AX foci in SCID round spermatids. (A) Round spermatids stained with anti-γ-H2AX and DAPI, 8hr after irradiation. (B). γ-H2AX foci numbers at 1 and 8 hr after irradiation in SCID and wild type C.B17 nuclei. Data are presented as mean ± SEM, n=3. ** P < 0.01.*

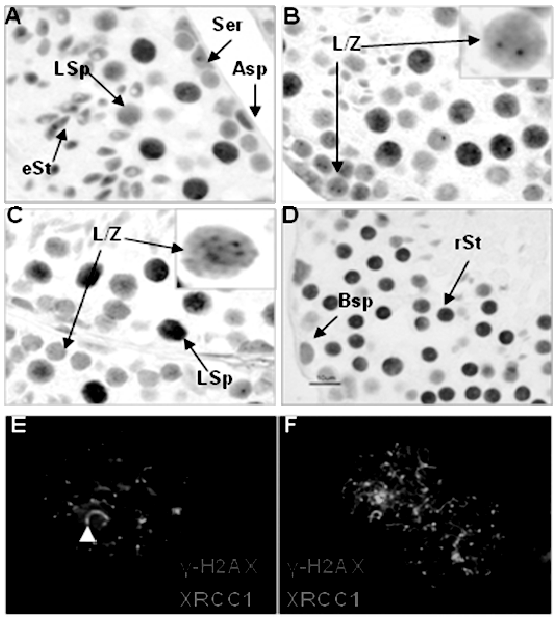

Fig. 2. *Localization of XRCC1 in control (A, B) and irradiated (C, D, E, F) testes of FVB mice and in surface spread preparations of late pachytene and diplotene spermatocytes. Asp – Type A spermatogonia; Bsp – B spermatogonia; L/Z – spermatocytes in leptotene/zygotene stages of meiotic prophase; LSp – late spermatocytes in late pachytene/ diplotene; rSt – round spermatids; eSt – elongating spermatids; Ser – Sertoli cells.*

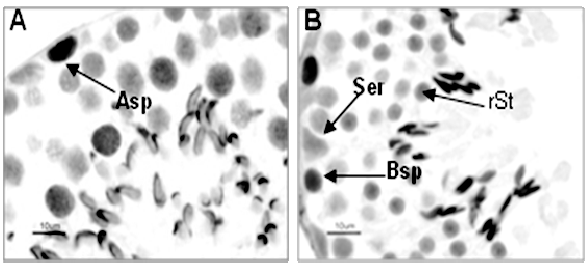

Fig. 3. *Localization of Parp1 in control (A) and irradiated (B) testis of FVB mice. Asp – Type A spermatogonia; Bsp – B spermatogonia; rSt – round spermatids; Ser – Sertoli cells.*

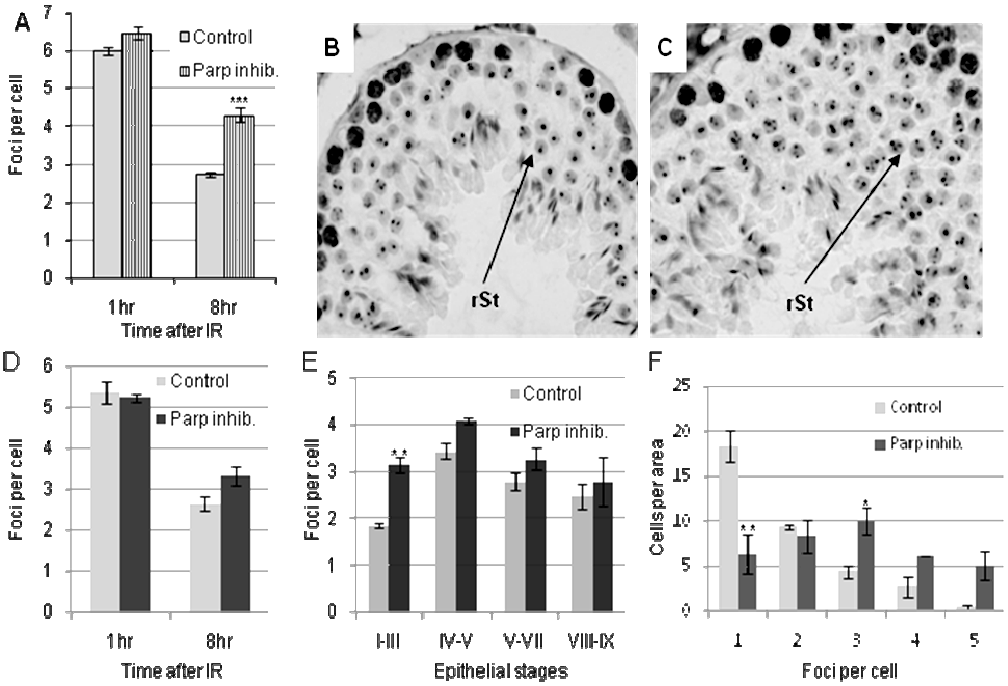

Fig. 4. *Parp1 inhibition reduces the rate of DSB repair in round spermatids. (A). Average numbers of γ-H2AX foci, 1 and 8 hr after irradiation in control and Parp1-inhibited mice, determined in nucleus spread preparations. (B and C) Demonstration of γ-H2AX foci in histological sections of control and Parp1-inhibited mice, respectively. (D) Foci numbers in round spermatids 1 and 8 hr after IR in control and Parp1-inhibited mice, determined in histological sections. (E) Foci numbers in grouped round spermatid stages 8 hr after irradiation. (F) Frequency distribution of foci numbers in round spermatid in epithelial stages I – III, 8hr after irradiation. Data are presented as mean ± SEM, n=3. * P < 0.05; ** P < 0.01; *** P < 0.001. rSt – round spermatids.*

4. Discussion

Here, we have studied the mechanisms involved in the previously detected repair of DSBs in mouse round spermatids [13]. Our experiments on SCID mice revealed that there is a small residual capacity to carry out classical NHEJ in these cells. Our results also indicate a significant contribution to DSB repair from an alternative NHEJ pathway, involving Parp1 and XRCC1 [17].

In order to find out how round spermatids repair DSBs, we first checked whether a possible expression level of DNA-PKcs in round spermatids, too low to detect by immunohistochemistry, still allows classical NHEJ. In wild type mice, counts of γ-H2AX foci in round spermatids in spread preparations showed that 46% of the breaks are repaired within 8 hr after irradiation. These data, and those of Ahmed et al. [13] indicate a slower rate of repair in round spermatids

than in somatic cell lines in which around 80% of the breaks (induced by irradiation) are rejoined in less than 2 hr (fast component) and rejoining is practically complete within 6 hr (slow component) [31,36]. These results provide another example of the notion that DSB repair in germ cells differs from that in somatic cells. In SCID mice 8 hr after irradiation, 30% of breaks had been repaired, which is significantly lower than in wild type mice. Apparently, low expression levels of DNA-PKcs exist in round spermatids [28] enough for about one third of the repair of the DSBs seen in wild type mice. DSB rejoining in DNA-PKcs deficient cells [32] is reported to be impaired with fewer breaks rejoined at any given time and only between 20 and 30% of DSBs are removed with fast kinetics (within 2 hr). Mutants deficient in Ku [37] and DNA ligase IV [38] showed the same residual end-joining repair capacity (20 to 30%). Taken together, the slow rate of repair (no fast end-joining) and the low number of DSBs repaired in SCID mice (around 35% relative to the control), indicate the involvement of other DSB repair pathways in round spermatids. Clearly, classical NHEJ is not the only DNA repair pathway in this spermatogenic cell type.

As in round spermatids we found a strong immunohistochemical staining for both Parp1 and XRCC1, we then tested for NHEJ via the recently described Parp1/XRCC1 pathway [17]. Parp1 was inhibited in vivo by DPQ, a very potent inhibitor of Parp1 compared to the classical inhibitor 3AB. While in normal mice, round spermatids repaired more than half of their DSBs within 8 hr after irradiation, administration of DPQ significantly diminished the reduction of foci numbers by about 40%. This indicates that the Parp1/XRCC1 repair pathway is indeed active in round spermatids and that DNA-PKcs-dependent and Parp1/XRCC1 dependent end-joining are of about equal importance in round spermatids.

Studying γ-H2AX foci numbers in round spermatid nuclei in testis sections allowed us to study repair capacity relative to the developmental stage of these cells. In young round spermatids in stages I-III of the epithelial cycle, the reduction in γ-H2AX foci (8 hr after irradiation) was larger than in later stages, indicating that the repair capacity is highest in early round spermatids. Earlier, Matsuda et al. [29] had shown that when sperm, irradiated at various spermatid developmental steps, was allowed to progress to the cauda epididymis and subsequently was used in mouse in vitro fertilization followed by chromosome analysis, zygote repair inhibitors caffeine (for Atm) and 3AB (for Parp1) could increase the yield of chromosome aberrations (CA). The yield of CA was highest for round spermatids around stage III, both with and without repair inhibitors. This conclusion was also reached at by Tusell et al. [31] using in vivo fertilization followed by zygote chromosome analysis. These data substantiate our findings that DSB repair is incomplete already at the early round spermatid stage but subsequently also suggest that slow NHEJ (as shown here) is leading to a high misrepair rate, creating CA and/or that zygote backing-up repair of these early DSBs is inaccurate, resulting into an extra increase of CA. We have also observed that foci of stages I-III were bigger in size (suggesting delayed repair, misrepair or no repair at all) than those of later stages 8 hr after irradiation, which may

explain the sensitivity of early spermatids found by Tusell et al. [31] and Matsuda et al. [29]. Round spermatids also show high yields of prenatal dominant lethals (caused by chromosome imbalance) and dominant visible and recessive point mutations, data derived from classical mouse mutagenesis studies [30]. Collectively, these data underscribe the mutation sensitivity of round spermatids, helped by the fact that round spermatids are resistant for apoptosis induction by irradiation [39]. Taking together, round spermatids repair around 50% of the breaks within 8 hr post irradiation using both Parp1/XRCC1 and DNAPK-dependent NHEJ. The repair is slow compared to other germ cells and is not completed during the life time of the spermatids during which apoptosis is not activated and the damage could be transmitted to the next generation.

With respect to the other types of germ cells, spermatogonia were found to express both Parp1 and XRCC1 before and after irradiation. These cells express most proteins required for NHEJ including the components of the DNA-PK complex: Ku70, Ku86, and DNA-PKcs [28], 53BP1 [13] and show γ-H2AX foci [40] In addition, in spermatogonia, contrary to round spermatids, HR is also available as Rad51 foci are formed in these cells after irradiation [13]. Despite the fact that spermatogonia can repair DSBs by HR, classical NHEJ involving DNA-PKcs is important for these cells as SCID mice are more sensitive for X-ray-induced cell killing of spermatogonial stem cells [41]. In view of this, DSB repair through the Parp1/XRCC1 pathway may be less important in spermatogonia.

During early meiotic prophase, in leptotene spermatocytes a few foci for XRCC1 were found before and after irradiation. These foci may arise after the endogenous induction of DSBs by SPO11 [3]. Mutant cells lacking XRCC1 were sensitive to IR and showed an elevated frequency of spontaneous chromosomal aberrations and deletions, and null mutant mice exhibited an embryonic lethal phenotype indicating the importance of XRCC1 for genetic stability and development [24]. XRCC1 was reported to physically interact with Parp1 through its BRCT1 domain (reviewed in [42]). In view of the expression of XRCC1 in early spermatocytes, XRCC1 was proposed to be involved in DNA strand-break repair associated with meiotic recombination [25,43].

In late spermatocytes, a dense nuclear staining for XRCC1 and Parp1 was found both before and after irradiation. Meiotic spreads from these cells showed that the staining for XRCC1 is over the whole nucleus, but accumulates on axial/lateral synaptic elements, especially of the sex chromosomes. In view of this localization, it is not clear whether the Parp1/XRCC1 pathway for NHEJ is available for late spermatocytes. The observed high expression of both Parp1 and XRCC1 in late spermatocytes and round spermatids in control and irradiated testes may also be related to BER, active in the removal of DNA lesions arising from endogenous processes and from irradiation. Indeed, a highly efficient BER has been found in these types of germ cells [19].

Parp1 has been suggested to play a role in chromatin remodeling in elongating spermatids as evidenced by the simultaneous presence of Parp1, the polyribosylation product PAR and γ-H2AX in testis cryosections of the rat [44]. In view of the immunocytochemical localization of PAR expression, the indicator of Parp activity, in round and elongating human spermatids [45], Meyer-Ficca et al. [46] hypothesized that the expression of γ-H2AX and the formation of ADP-ribose polymer in elongated spermatids during the chromatin remodeling steps, may facilitate DNA strand break management of sperm cell maturation [46]. However, the observed acrosomal localization of Parp1 in elongating spermatids does not support this hypothesis. Possibly, Parp1 levels in the nuclei below the immunohistochemical detection level are sufficient to carry out this function.

In conclusion, we have shown that the seemingly puzzling capacity of round spermatids to carry out DSB repair is based in part on classical NHEJ through a low level of DNA-PKcs expression in these cells and also on NHEJ via the Parp1/XRCC1 dependent pathway. Both pathways are about equally important in mouse round spermatids. The repair capacity in round spermatids diminishes with the ongoing development of these cells. These findings can be of significance in understanding male mutagenesis. To our knowledge this is the first demonstration of a functional Parp1/XRCC1 pathway for the repair DSB in an in vivo system.

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References

[1] E. Riballo, M. Kuhne, N. Rief, A. Doherty, G.C. Smith, M.J. Recio, C. Reis, K. Dahm, A. Fricke, A. Krempler, A.R. Parker, S.P. Jackson, A. Gennery, P.A. Jeggo, M. Lobrich, A pathway of double-strand break rejoining dependent upon ATM, Artemis, and proteins locating to gamma-H2AX foci, Mol. Cell. 16 (2004) 715–724.

[2] W.M. Baarends, R. van der Laan, J.A. Grootegoed, DNA repair mechanisms and gametogenesis, Reproduction 121 (2001) 31-39.

[3] F. Baudat, K. Manova, J.P. Yuen, M. Jasin, S. Keeney, Chromosome synapsis defects and sexually dimorphic meiotic progression in mice lacking Spo11, Mol. Cell. 5 (2000) 989-998.

[4] S.K. Mahadevaiah, J.M. Turner, F. Baudat, E.P. Rogakou, P. de Boer, J. Blanco-Rodriguez, M. Jasin, S.Keeney, W.M. Bonner, P.S. Burgoyne, Recombinational DNA double-strand breaks in mice precede synapsis, Nat. Genet. 27 (2001) 271-276.

[5] S.P. Jackson, Sensing and repairing DNA double-strand breaks, Carcinogenesis 23 (2002) 687-696.

[6] L. Krejci, L. Chen, S. van Komen, P. Sung, A. Tomkinson, Mending the break: two DNA double-strand break repair machines in eukaryotes, Prog. Nucleic Acid Res. Mol. Biol. 74 (2003) 159-201.

[7] K. Valerie, L.F. Povirk, Regulation and mechanisms of mammalian double-strand break repair, Oncogene 22 (2003) 5792-5812.

[8] P. Calsou, C. Delteil, P. Frit, J. Drouet, B. Salles, Coordinated assembly of Ku and p460 subunits of the DNA-dependent protein kinase on DNA ends is necessary for XRCC4-ligase IV recruitment, J. Mol. Biol. 326 (2003) 93-103.

[9] D.R. Pilch, O.A. Sedelnikova, C. Redon, A. Celeste, A. Nussenzweig, W.M. Bonner, Characteristics of gamma-H2AX foci at DNA double-strand breaks sites, Biochem. Cell Biol. 81 (2003) 123-129.

[10] O. Fernandez-Capetillo, A. Lee, M. Nussenzweig, A. Nussenzweig, H2AX: the histone guardian of the genome, DNA Repair 3 (2004) 959-967.

[11] I.B. Nazarov, A.N. Smirnova, R.I. Krutilina, M.P. Svetlova, L.V. Solovjeva, A.A. Nikiforov, S.L. Oei, I.A. Zalenskaya, P.M. Yau, E.M. Bradbury, N.V. Tomilin, Dephosphorylation of histone gamma-H2AX during repair of DNA double-strand breaks in mammalian cells and its inhibition by calyculin A, Radiat. Res. 160 (2003) 309-317.

[12] K. Rothkamm, I. Krüger, L.H. Thompson, M. Löbrich, Pathways of DNA double-strand break repair during the mammalian cell cycle, Mol. Cell. Biol. 16 (2003) 5706-5715.

[13] E.A. Ahmed, A. van der Vaart, A. Barten, H.B. Kal, J. Chen, Z. Lou, K. Minter-Dykhouse, J. Bartkova, J. Bartek, P. de Boer, D.G. de Rooij, Differences in DNA double strand breaks repair in male germ cell types: Lessons learned from a differential expression of Mdc1 and 53BP1, DNA Repair 6 (2007) 1243-1254.

[14] A. Chicheportiche, J. Bernardino-Sgherri, B. de Massy, B. Dutrillaux, Characterization of Spo11-dependent and independent phospho-H2AX foci during meiotic prophase I in the male mouse, J. Cell Sci. 120 (2007) 1733-1742.

[15] A. Derijck, G. van der Heijden, M. Giele, M. Philippens, P. de Boer, DNA double-strand break repair in parental chromatin of mouse zygotes, the first cell cycle as an origin of de novo mutation, Hum. Mol. Genet. 17 (2008) 1922-1937.

[16] F. Bouquet, C. Muller, B. Salles, The loss of gammaH2AX signal is a marker of DNA double strand breaks repair only at low levels of DNA damage, Cell Cycle 10 (2006) 1116-1122.

[17] M. Audebert, B. Salles, P. Calsou, Involvement of poly(ADP-ribose) polymerase-1 and XRCC1/DNA ligase III in an alternative route for DNA double-strand breaks rejoining, J. Biol. Chem. 279 (2004) 55117-55126.

[18] H.E. Krokan, H. Nilsen, F. Skorpen, M. Otterlei, G. Slupphaug, Base excision repair of DNA in mammalia cells, FEBS Lett. 476 (2000) 73–77.

[19] A.K. Olsen, H. Bjørtuft, R. Wiger, J. A. Holme, E.C. Seeberg, M. Bjørås, G. Brunborg, Highly efficient base excision repair (BER) in human and rat male germ cells, Nucleic Acids Res. 8 (2001) 1781-1790.

[20] M. Wang, W. Wu, W. Wu, B. Rosidi, L. Zhang, H. Wang, G. Iliakis, PARP-1 and Ku compete for repair of DNA double strand breaks by distinct NHEJ pathways, Nucleic Acids Res. 34 (2006) 6170-6182.

[21] M. Masson, C. Niedergang, V. Schreiber, S. Muller, J. Menissier-de Murcia, G. de Murcia, XRCC1 is specifically associated with poly(ADP-ribose) polymerase and negatively regulates its activity following DNA damage, Mol. Cell Biol. 6 (1998) 3563-3571.

[22] J.M. de Murcia, C. Niedergang, C. Trucco, M. Ricoul, B. Dutrillaux, M. Mark, F.J. Oliver, M. Masson, A. Dierich, M. LeMeur, C. Walztinger, P. Chambon, G. de Murcia, Requirement of poly(ADP-ribose) polymerase in recovery from DNA damage in mice and in cells, Proc. Natl. Acad. Sci. U S A 94 (1997) 7303-7307.

[23] Z.Q. Wang, B. Auer, L. Stingl H. Berghammer, D. Haidacher M. Schweiger E.W. Wagner, Mice lacking AD-PRT and poly_(ADP-ribosyl.ation) develop normally but are susceptible to skin disease, Genes Dev. 9 (1995) 509–520.

[24] R.S. Tebbs, M.L. Flannery, J.J. Meneses, A. Hartmann, J.D. Tucker, L.H. Thompson, J.E. Cleaver, R.A. Pedersen, Requirement for the Xrcc1 DNA base excision repair gene during early mouse development, Dev. Biol. 2 (1999) 513–529.

[25] C.A. Walter, D.A. Trolian, M.B. McFarland, K.A. Street, G.R. Gurram, J.R. McCarrey, Xrcc-1 expression during male meiosis in the mouse, Biol. Reprod. 55 (1996) 630-635.

[26] T.L. Beumer, H.L. Roepers-Gajadien, I.S. Gademan, P.P. van Buul, G. Gil-Gomez, D.H. Rutgers, D.G. de Rooij, The role of the tumor suppressor p53 in spermatogenesis, Cell Death Differ. 5 (1998) 669-677.

[27] W. Goedecke, M. Eijpe, H.H. Offenberg, M. van Aalderen, C. Heyting, Mre11 and Ku70 interact in somatic cells, but are differentially expressed in early meiosis, Nat. Genet. 23 (1999) 194-198.

[28] G. Hamer, H.L. Roepers-Gajadien, A. van Duyn-Goedhart, I.S. Gademan, H.B. Kal, P.P. van Buul, T. Ashley, D.G. de Rooij, Function of DNA-protein kinase catalytic subunit during the early meiotic prophase without Ku70 and Ku86, Biol. Reprod. 68 (2003a) 717-721.

[29] Y. Matsuda, N. Seki, T. Utsugi-Takeuchi, I. Tobari, X-ray- and mitomycin C (MMC)-induced chromosome aberrations in spermiogenic germ cells and the repair capacity of mouse eggs for the X-ray and MMC damage, Mutat. Res. 211 (1989) 65-75.

[30] W.L. Russell, J.W. Bangham, L.B. Russell, Differential response of mouse male germ-cell stages to radiation-induced specific-locus and dominant mutations, Genetics 148 (1998) 1567–1578.

[31] L. Tusell, L. Latre, I. Ponsa, R. Miro, J. Egozcue, A. Genesca, Capping of radiation-induced DNA breaks in mouse early embryos, J. Radiat. Res. 45 (2004) 415–422.

[32] G. Iliakis, H. Wang, A.R. Perrault, W. Boecker, B. Rosidi, F. Windhofer, W. Wu, J. Guan, G. Terzoudi, G. Pantelias, Mechanisms of DNA double strand break repair and chromosome aberration formation, Cytogenet. Genome Res. 104 (2004) 14-20.

[33] A.H. Peters, A.W. Plug, M.J. van Vugt, P. de Boer, A drying-down technique for the spreading of mammalian meiocytes from the male and female germline, Chromosome Res. 5 (1997) 66-68.

[34] G.W. van der Heijden, A.A. Derijck, E. Posfai, M. Giele, P. Pelczar, L. Ramos, D.G. Wansink, J. van der Vlag, A.H. Peters, P. de Boer, Chromosome-wide nucleosome replacement and H3.3

incorporation during mammalian meiotic sex chromosome inactivation, Nat. Genet. 39 (2007) 251-258.

[35] E.A. Ahmed, D.G. de Rooij, Staging of mouse seminiferous tubule cross-sections. In: Meiosis. Ed. S. Keeney, Methods in Molecular Biology, (2009) Humana Press, Totowa, USA. In press.

[36] S.J. DiBiase, Z-C. Zeng R. Chen, T. Hyslop, W.J. Curran G. Iliakis, DNA-dependent protein kinase stimulates an independently active, nonhomologous, end-joining apparatus, Cancer Res. 60 (2000) 1245–1253.

[37] H. Wang, Z-C. Zeng, T-A. Bui, E. Sonoda, M. Takata, S. Takeda, G. Iliakis, Efficient rejoining of radiation- induced DNA double-strand breaks in vertebrate cells deficient in genes of the RAD52 epistasis group, Oncogene 20 (2001a) 2212–2224.

[38] H. Wang Z. Zhao-Chong, A.R. Perrault, X. Cheng W. Qin, G. Iliakis, Genetic evidence for the involvement of DNA ligase IV in the DNA-PK-dependent pathway of non-homologous end joining in mammalian cells, Nucleic Acids Res. 29 (2001b) 1653–1660.

[39] E.F. Oakberg, R.L. Diminno, X-ray sensitivity of primary spermatocytes of the mouse, Int. J. Radiat. Biol. 2 (1960) 196-209.

[40] G. Hamer, H.L. Roepers-Gajadien, A. van Duyn-Goedhart, I.S. Gademan, H.B. Kal, P.P. van Buul, D.G. de Rooij, DNA double-strand breaks and gamma-H2AX signaling in the testis, Biol. Reprod. 68 (2003b) 628-634.

[41] P.P. van Buul, D.G. de Rooij, I.M. Zandman, M. Grigorova, A. van Duyn-Goedhart, X-ray-induced chromosomal aberrations and cell killing in somatic and germ cells of the scid mouse, Int. J. Radiat. Biol. 5 (1995) 549-455.

[42] W.C. Ladiges, Mouse models of XRCC1 DNA repair polymorphisms and cancer, Oncogene 25 (2006) 1612–1619.

[43] C.A. Hoy, J.C. Fuscoe, L.H. Thompson, Recombination and ligation of transfected DNA in CHO mutant EM9, which has high levels of sister chromatid exchange, Mol. Cell. Biol. 7 (1987) 2007-2011.

[44] R. Jha, A. Agarwal, R. Mahfouz, U. Paasch, S. Grunewald, E. Sabanegh, S.P. Yadav, R. Sharma, Determination of Poly (ADP-ribose) polymerase (PARP) homologues in human ejaculated sperm and its correlation with sperm maturation, (2008) Fertil Steril. In press.

[45] B.B. Maymon, M. Cohen-Armon, H. Yavetz , L. Yogev, B. Lifschitz-Mercer, S.E. Kleiman, A. Botchan, R. Hauser, G. Paz, Role of poly(ADP-ribosyl)ation during human spermatogenesis, Fertil. Steril. 86 (2006) 1402-1407.

[46] M.L. Meyer-Ficca, H. Scherthan, A. Bürkle, R.G. Meyer, Poly(ADP-ribosyl)ation during chromatin remodeling steps in rat spermiogenesis, Chromosoma 114 (2005) 67-74.

Chapter 5

Genetic probing of homologous recombination and non homologous end joining during meiotic prophase in irradiated mouse male germ cells

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Abstract

This study was designed to obtain a better insight into the relative contribution of homologous recombination (HR) and non homologous end joining (NHEJ) to the repair of radiation induced DNA double strand breaks (DSBs) during early pachytene stage in primary spermatocytes and, after crossing over, in late pachytene and early diplotene spermatocytes. We studied the kinetics of γ-H2AX chromatin foci removal (large - L and small - SL) after irradiation of mice potentially deficient for HR and mice deficient for NHEJ. Unirradiated spermatocytes of *Rad54/Rad54B* deficient mice show incomplete meiotic recombination repair. In these mice, 8 hr after irradiation, early pachytene spermatocytes showed a reduction of the numbers of γ-H2AX foci by 52% compared to 82% in the wild type, the difference being significant. However, after crossing over (in late pachytene and early diplotene), no effect of *Rad54/Rad54B* deficiency on the reduction of irradiation induced foci was observed. In NHEJ deficient scid mice repair kinetics in early spermatocytes were similar to those in wild type mice. However, in late pachytene and early diplotene scid spermatocytes and after 1 hr of irradiation, 1.7 times more foci were found than in wild type mice. This difference might be related to the absence of a DNA-PKcs dependent fast repair component in scid mice. As subsequent repair is normal, HR likely is taking over. Taken together, results from studying the repair kinetics in *Rad54/Rad54B* deficient mice and in scid mice, we could establish that DSB repair in early pachytene spermatocytes is exclusively carried out through HR. In late spermatocytes (late pachytenes and early diplotenes) both NHEJ and HR pathways are active, NHEJ probably being the most important.

Introduction

In response to DNA double strand breaks (DSBs), cells phosphorylate H2AX at serine 139, after which it is called γ-H2AX, leading to the formation of nuclear foci at the sites of damage [1]. These foci represent the DSBs in a 1:1 manner. Hence, γ-H2AX can be used as a marker for DNA DSBs (reviews [2, 3]). Studies on somatic cells and on mouse germ cells have shown that the kinetics of γ-H2AX loss are related to DSB repair activity, especially at low doses of radiation [4-6]. Compared to somatic cell systems, the number of DNA repair studies on male germ cells is still limited.

In early primary spermatocytes, at the leptotene stage of meiotic prophase, endogenous DSBs are introduced by the topoisomerase family member SPO11 [7]. Some 400 RAD51 foci are formed in mouse leptotene spermatocytes coinciding with a massive spread of γ-H2AX [8]. When synapsis is established during zygonema, γ-H2AX staining gradually decreases as it disappears from the synapsed regions and by pachynema, it is largely undetectable on autosomal chromatin. During pachynema and diplonema, γ-H2AX staining is very prominent

over sex body chromatin [9]. Recently, two types of foci have been described at the pachytene stage: small γ-H2AX foci, most extending just beyond the area of the synaptonemal complex (SC) specifically indicate sites of SPO11-induced DSBs involved in recombination. Larger γ-H2AX signals on chromatin loops emanating from the SC, target both unrepaired SPO11-DSBs and radiation induced, SPO11-independent DSBs [5]. Chicheportiche et al., [5] noted a reduction in numbers and size of foci with time after irradiation. These findings help to discriminate between the repair of endogenous DSBs by meiotic homologous recombination (HR), and the repair of radiation induced DSBs by HR and/or non homologous endjoining (NHEJ). The extent of HR repair during meiotic prophase has been determined by Schoenmakers et al., [10]. As inferred from the kinetics of RAD51 foci, 30 hr after 4 Gy of whole body irradiation, in which time spermatocytes progress from leptotene to zygotene, some 80% of DSBs are repaired. After in vitro irradiation of pachytene spermatocytes, DSB repair can likewise be followed by counting RAD51 foci [11]. Irradiation induced immunofluorescent foci (IRIF's) of γ-H2AX and MDC1 partially overlap with RAD51 foci in late pachytene and early diplotene spermatocytes, indicating a role for HR repair beyond the recombination window [4, 12]. When, after irradiation, IRIF loss in histological sections of mouse seminiferous tubules was followed in spermatocytes after stage IV, DSB repair appeared to be slow [4] compared to the repair kinetics in somatic nuclei [13, 14].

The role of NHEJ in the repair of radiation induced DSBs in early meiotic prophase may not be an important one, since KU proteins, which are essential in this repair pathway, are not expressed during leptotene and zygotene [15]. The KU signal returns at a low level over autosomal chromatin at pachytene with a distinct signal in the sex body. These results have been explained by a competition between HR and NHEJ at the initiation of recombination [15]. An indication for a role of DNA-PKcs at meiotic recombination was found by Hamer et al., [16] who observed an enhanced number of apoptotic pachytene spermatocytes at stage IV of the cycle of the seminiferous epithelium in DNA.PKcs hypomorphic scid mice. In epithelial stage IV a meiotic checkpoint has been suggested to exist [17]. In the haploid round spermatids, HR is not indicated. In these cells we established a role for both classical NHEJ involving DNA-PKcs / KU proteins and NHEJ through the recently discovered PARP1-XRCC1 pathway, in the repair of radiation induced DSBs (Ahmed et al., submitted). In somatic cells, a fast removal of DSBs occurs when all essential components of NHEJ are available and about 80% of the breaks can be rejoined within 2 hr [18, 19]. However, when one of the components of the NHEJ pathway is absent as is the case in DNA-PKcs or KU deficient cells, the rate of DSB repair is decreased: 80% were rejoined in 8 hr [19, 20].

To obtain a better insight into the relative contribution of HR and NHEJ to the repair of exogenously induced DSBs during early pachytene stage and after crossing over in late pachytene and early diplotene spermatocytes, we studied the kinetics of γ-H2AX foci removal in mutant mice deficient for one of these pathways. In C.B17 mice, the severe combined

immunodeficient (scid) mutation, coding for a severely hypomorphic DNA-PKcs protein, confers a 2- to 3-fold hypersensitivity to ionizing radiation and a deficiency in DNA DSBs repair by NHEJ [21]. Studies have shown that DSB rejoining in scid cells is impaired with fewer breaks rejoined at any given time. Only between 20 and 30% of DSBs are removed with fast kinetics (within 2 hr) and these cells require 4 to 6 hr to reach a level of repair, similar to that in wild type mice [19]. The *Rad54* and *Rad54B* genes are involved in HR and depletion of both RAD54 and RAD54B results in a defective HR, especially in embryonic and pluripotent cell lines [22, 23]. Both scid and *Rad54/Rad54B* double knockout males are fully fertile and no effect on litter size is apparent. Our data show involvement of both pathways in the repair of radiation induced DSBs during meiotic prophase and a scenario describing the interplay between HR and NHEJ is presented in the discussion.

Materials and methods

Animals and irradiation

Male C.B17 scid mice and their wild type C.B17 controls (7 to 8 wk of age; Charles River, Maastricht, The Netherlands) and *Rad54/Rad54B* deficient *mice* and their wild type control, a B6.129 synthetic (2 to 4 months of age) were used. The latter have been described by Wesoly et al., [23] and were bred to homozygosity, together with the B6.129 synthetic control as described by Derijck et al [6]. Both lines are kept in a closed colony, B6.129 being marked by homozygosity for non-agouti (a/a). Mice were either sham-irradiated (4 mice per group) or received a dose of 1 Gy of gamma-rays (whole body irradiation, 6 MV, 91 MU, Elektra, Crawley, United Kingdom). Irradiated mice were sacrificed 1 or 8 hr after irradiation. Mice were killed by CO_2 asphyxiation. Nucleus spreads of spermatogenic cells were prepared (see below). The animals were used and maintained according to regulations provided by the animal ethical committee of Utrecht University that also approved of the experiments.

Surface spread preparations

Nucleus spreads were made as previously described by Peters et al [24] and van der Heijden et al., [25]. Briefly, a suspension of spermatogenic cells in MEM-α was obtained, followed by incubation with a hypotonic buffer (17 mM sodium citrate, 50 mM sucrose, 30 mM Tris HCl, pH 8.2). After centrifugation, the pellet was carefully resuspended in a 100 mM sucrose solution and applied over a paraformaldehyde (PFA) covered glass slide (1% PFA, 0.15% Triton-X-100, pH 9.2–9.5). The slides were kept in a humidified atmosphere in a box to

slow down drying out. After 1.5 hr the box was opened and the slides were washed in 0.08% photo-flo (Sigma P7417, St. Louis, USA).

Fluorescent immunostaining

For immunofluorescence, nuclei were washed in PBS and incubated for 10 min in PBS including 0.04% Triton-X-100 and then incubated with blocking solution (10% goat serum and 10% BSA in PBS for 30 min.). After blocking, primary antibodies were applied, diluted in blocking solution, for 2 hr at room temperature. The slides were washed in PBS and incubated with the diluted secondary antibodies in blocking solution for 1 hr at room temperature. Thereafter the nuclei were stained with DAPI (for 10 min), mounted in VECTAshield (Vector lab. H-1000) and viewed with an Olympus microscope (Olympus Tokyo, Japan). Images were recorded digitally.

Antibodies

The primary antibodies were rabbit polyclonal anti-SCP3 (1:400, ab15092, Abcam Cambridge, UK) and mouse monoclonal anti-phospho-H2AX [Ser139] (1:400 Upstate Biotechnology JBW-301, 05-636 Lake Placid, USA). The secondary antibodies, goat anti-rabbit (Alexa fluor 488, A-11008. Invitrogen, Breda, Netherlands), goat anti-mouse (Alexa fluor 488, A-21121 and 594, A-21125, Invitrogen, Breda, Netherlands) and Texas Red-labeled goat anti-mouse (T6134), were obtained from Jackson ImmunoResearch (West Grove, USA) and applied at a 1:1000 dilution.

The kinetics of γ-H2AX loss after irradiation and foci scoring in spermatocytes

Foci were scored according to Chicheportiche et al. [5], with some minor variations. SPO11-dependent minute S-foci located in the fluorescent area of the autosomal synaptonemal complexes (SCs) were not included. We counted chromatin loop foci (irradiation dependent large “L” and small “SL” foci) that originated from the SC. SL foci only marginally cover the looped domains. L foci occasionally extend into both directions from the SC (Fig. 1 A,B). L and SL foci were counted separately (see Table 1) and regarded to be one event. L foci could also be positioned at the telomeric attachment plaques of the SC’s (Fig. 1 A,B) but the foci positioned in this way were not recorded separately.

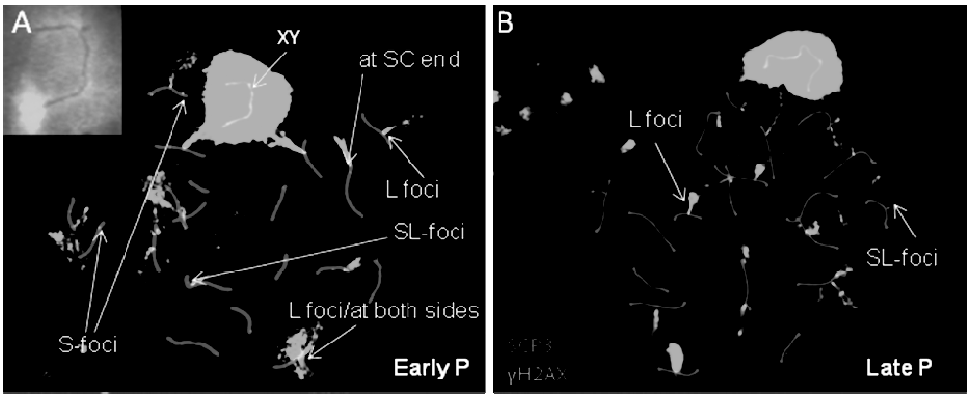

Fig. 1. *γ-H2AX foci in spermatocytes 1 hr after IR. Foci are attached to the synaptonemal complex (SC) and follow chromatin loops, L, 'large' foci, SL, 'small' foci and foci at SC's ends. Minute or small foci (S-foci) located directly at the SC's were not counted. DAPI in blue showing sex chromosomes (XY). P-pachytene.*

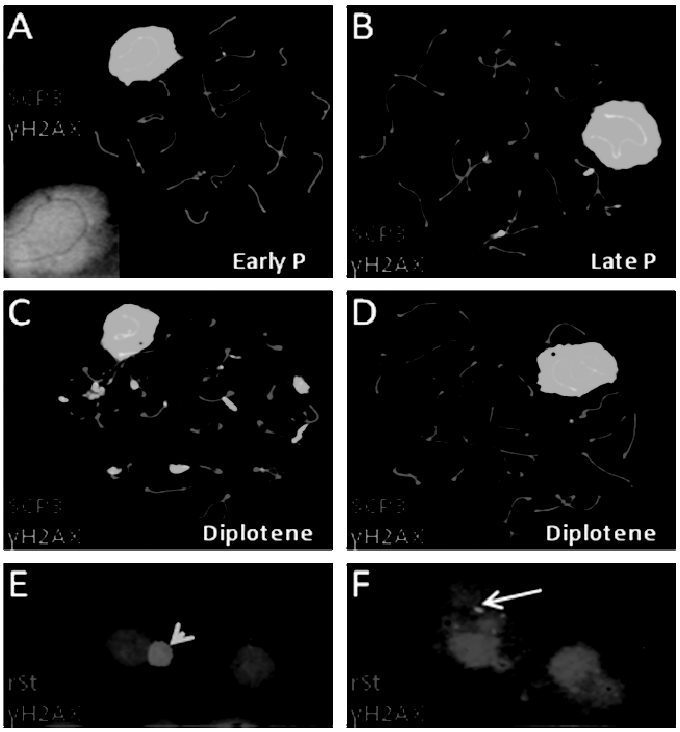

Fig. 2. *γ-H2AX foci in the un-irradiated Rad54/Rad54B deficient (A,B,C,E,F) are similar to IRIF's. Wild type control with no foci (D). rSt- round spermatids.*

Results

Incomplete meiotic recombination repair in spermatocytes lacking both Rad54 and Rad54B

In the unirradiated Rad54/Rad54B deficient mice, we have observed a number of γ-H2AX L foci attached to the SCs in early pachytene spermatocytes (Fig 2A). These L foci increased in number in late pachytene spermatocytes (3.70 ± 0.45 per cell, Fig 2B, Table 1) and in diplotene spermatocytes (4 to 10 L foci per cell, Fig 2C, see in discussion). In contrast, these L foci were rare in early and late spermatocytes in unirradiated control B6.129 mice (Fig 2D). Large and small foci were also found in round spermatids of unirradiated *Rad54/Rad54B* deficient mice (Fig 2E, F) but not in round spermatids from B6.129 mice (not shown). These results indicate incomplete recombinational repair in *Rad54/Rad54B* deficient mice as γ-H2AX foci persist after the completion of meiotic HR and even into the round spermatid stage.

Reduction of γ-H2AX foci removal during and after crossing over in Rad54/Rad54B deficient *mice*

In spread preparations of early pachytene spermatocytes from irradiated wild type mice, 82% of the radiation induced γ-H2AX (L+ SL) foci present after 1 hr had disappeared at 8 hr after irradiation. In contrast, in irradiated *Rad54/Rad54B* deficient mice only 52% of the foci had disappeared which is significantly less than in the irradiated wild type mice (82.2%) (Table 1). In the wild types, and to a greater extent in the mutant, the decrease in L foci was accompanied by an increase in SL foci. In late pachytene and early diplotene spermatocytes, the reduction in the numbers of both L and SL foci was similar in the double knockout (75.8%) and in the wild type (78%). Assuming that repair is associated with a reduction of L foci in number and/or in size (giving SL foci) [5], our results indicate that for the repair of exogenously induced breaks, HR is more important in early pachytene than in late pachytene and early diplotene spermatocytes**.**

Table 1. *Frequency of γ-H2AX foci (+/- SEM) in spermatocytes in Rad54/Rad54B deficient and wild type spermatocytes. Background foci were subtracted at both 1 hr and 8hr followed by assessment of the percentage of reduction (Foci numbers "L+SL" at 1 hr after IR minus "L+SL" at 8 hr divided by "L+SL" at 1 hr) x100. P – pachytene spermatocytes; Dip – diplotene spermatocytes; L – large; SL – small; SC – synaptonemal complex.* [a] *P< 0.01 compared with B6.129.*

Mice	**B6.129**		***Rad54*$^{-/-}$ *Rad54B*$^{-/-}$**	
	Early P	Late P/early Dip.	Early P	Late P/early Dip.
Background foci in unirradiated mice				
L foci	0.56±0.14	0.25±0.06	0.45±0.10	3.70±0.45
Small foci	0.85±0.40	0.15±0.05	1.88±0.14	1.25±0.25
Attached L foci (at SC's end/one/two sides)				
1 hr	10.70±1.30	14.49 ±0.37	12.23 ±0.07	14.75 ±0.73
8 hr	1.36±0.23	2.83 ±0.18	2.36 ±0.46	4.67 ±0.32
Attached L foci (two sides)				
1 hr	1.26 ±0.14	1.35 ±0.38	1.36 ±0.45	1.74 ±0.10
8 hr	0.05±0.04	0.04 ±0.01	0.03 ±0.02	0.40 ±0.09
Attached SL foci				
1 hr	1.07±0.49	0.37 ±0.15	0.65 ±0.41	0.70 ±0.29
8 hr	1.89±0.23	0.75 ±0.10	5.03 ±0.73[a]	2.82 ±0.78[a]
% of reduction (SL and L) after 8 hr (corrected of for background foci)	82.2%	78.0%	52.0%[a]	75.8%
cells analyzed				
Unirradiated (3 mice)	30	30	30	30
1 hr (3 mice)	45	52	54	54
8 hr (5 mice)	84	117	60	145

Table 2*. Reduction of γ-H2AX foci in round spermatids from Rad54/Rad54B deficient mice compared to wild types. Statistical analysis (Dunnett's Multiple Comparison Test),* [a] *P< 0.01 compared with B6.129, n = 4 mice per genotype, 40 nuclei scored per mouse.*

Mice	**B6.129**	***Rad54*$^{-/-}$ *Rad54B*$^{-/-}$**
Foci numbers ± SEM 1 hr	6.92 ± 0.34	7.41 ± 0.22
8 hr	3.84 ± 0.18	2.22 ± 0.47
Repaired % after 8 hr	44.16 ± 2.95%	67.87± 7.37%[a]
Number of large foci after 8 hr	1.99 ± 0.38	2.78 ± 0.09

Reduction of γ-H2AX foci in round spermatids from Rad54/Rad54B deficient *mice*

In spread preparations from irradiated wild type and *Rad54/Rad54B* deficient mice, γ-H2AX foci were counted in nuclei of round spermatids (Table 2). Between 1 and 8 hr after irradiation, in *Rad54/Rad54B* deficient mice, 67.9± 7.4% of DSBs were repaired compared to 44.2 ± 3.0 % in B6.129, the difference being significant (Table 2). These results indicate a more effective reduction in the number of breaks in round spermatids potentially defective for HR.

Table 3. *Numbers of γ-H2AX foci (+/- SEM) in scid and wild type spermatocytes. For calculation of the reduction rates, see Table 1. Statistical analysis (Dunnett's Multiple Comparison Test),* [a] *P< 0.01 compared with C.B17.*

Mice	**C.B17**		**scid**	
	Early P	Late P/early Dip.	Early P	Late P/early Dip.
Attached L foci (at SC's end/one/two sides)				
1 hr	9.94 ± 0.62	11.69 ± 1.00	13.28 ± 0.36 [a]	19.31 ± 0.88 [a]
8 hr	1.60 ± 0.30	3.15 ± 0.24	2.61 ± 0.34	6.60 ± 0.44 [a]
Attached L foci (two sides)				
1 hr	1.27 ± 0.16	1.50 ± 0.15	2.11 ± 0.24	2.15 ± 0.17
8 hr	0.24 ± 0.09	0.24 ± 0.09	0.26 ± 0.10	0.50 ± 0.07
Attached SL foci				
1 hr	0.85 ± 0.17	0.35± 0.03	0.43 ± 0.10	1.00 ± 0.27
8 hr	1.73 ± 0.55	0.97 ± 0.29	1.53 ± 0.26	1.06 ± 0.37
% of reduction (SL and L) after 8 hr	68.5 %	65.8 %	69.8 %	62.3 %
cells analyzed				
1 hr (3 mice)	45	52	54	54
8 hr (5 mice)	84	117	60	145

γ-H2AX foci numbers in spermatocytes in scid mice

γ-H2AX foci were counted in late spermatocytes in irradiated scid and C.B17 wild type mice (Table 3). A reduction of L foci was observed, in both genotypes, in early pachytene spermatocytes as well as in late pachytene and early diplotene spermatocytes. However, comparing the number of foci at 1 hr after irradiation, especially in late pachytene/early diplotene a scid effect was obvious. In scid mice the number of foci was 19.3 ± 0.9 which is significantly more than in wild type mice (11.7 ± 1.0). In early pachytene, no significant difference in the kinetics of the disappearance of the γ-H2AX foci between scid and wild type mice was observed, although foci numbers at 1 hr were higher in scid mice (Table 3). Despite

the higher number of foci in late pachytene in scid mice, the relative rates of repair as indicated by signal loss were not different between scid and C.B17 mice. These results indicate that within 1 hr after IR, fast repair is not available in late pachytenes and early diplotenes in scid mice indicating a role for NHEJ after crossing over in wild type C.B17.

Discussion

In the present study we used mice deficient for *Rad54 and Rad54B* and their appropriate wild type controls (both irradiated and un-irradiated) to evaluate the contribution of HR to repair during and after crossing over at meiotic prophase, using the *in vivo* disappearance of γ-H2AX foci as a marker for DSB repair. For comparison, the scid mouse model was used to probe for the role of NHEJ. Analyzing γ-H2AX signals in unirradiated *Rad54/Rad54B* deficient mice, we found γ-H2AX SL foci during meiotic recombination (in early pachytene spermatocytes) and L foci thereafter (in late pachytene spermatocytes) (Table 1). These L foci likely correspond to the Rad51 foci observed by Wesoly et al., [23]. We have found as much as 10 foci in some non-irradiated *Rad54/Rad54B* deficient mouse diplotene spermatocytes which is high enough to represent a repair defect, leading to cell loss at spermatogenesis. Similar γ-H2AX foci were found in spermatocytes of an azoospermic man with a meiotic maturation arrest exhibiting a defect in meiotic DSB repair [27] and also in mice carrying mutations that affect the repair of meiotic DSBs (*Dmc1, Msh5, Atm*) [26]. We also found some round spermatids in unirradiated double KO mice showing large and small focus formation for γ-H2AX. Taken together, the pronounced increase in the numbers of γ-H2AX and RAD51 foci in late spermatocytes, when meiotic recombination should have been completed, the similar decrease in genetic recombination in the double knockout over two chromosomal intervals (as measured by the single-sperm typing technique [23] and the persistence of some γ-H2AX foci into the round spermatid stage, indicate an effect of RAD54 on meiotic recombination.

One hour after 1 Gy of whole body irradiation of *Rad54/Rad54B* deficient mice and their wild type controls, 10 to 12 L foci were seen in early pachytene spermatocytes. Our results indicate that the extent of γ-H2AX foci (L+ SL) loss in *Rad54/Rad54B* deficient spermatocytes that are engaged in crossing over is significantly reduced from 82% in wild type to 52% in *Rad54/Rad54B* deficient mice (Table 1). The occurrence of HR repair during leptotote and zygotene stages of meiotic prophase was inferred from positive immunofluorescent staining for RAD51 after 4Gy of whole body irradiation [10]. Comparing the loss of foci at early pachytene in double knockout mice versus wild type indicates that HR repair of exogenously induced DSBs is hampered to about the same extent as repair of endogenously induced SPO11 breaks, as reflected by decreased HR [23]. From this, together with data from Schoenmakers et al., [10], it follows that the HR machinery also takes care of exogenously induced breaks. In agreement with this supposition, there was no difference in the repair kinetics in early pachytene

spermatocytes from scid and wild type mice. Hence, there seems to be no role for DNA.PK dependent NHEJ at early pachytene. This supports the “repair pathway conflict hypothesis” [15, 16]. From the absence of KU80 during leptotene and zygotene, these authors proposed that no competition for DSBs between HR and NHEJ is allowed during the initiation of meiotic recombination.

In late pachytene and early diplotene spermatocytes, the role of HR seems diminished as the difference in foci loss between *Rad54/Rad54B* deficient and wild type mice becomes lost (Table 1). The existence of HR during late meiotic prophase was already apparent from the presence of RAD51 foci at this stage in *Rad54/Rad54B* deficient spermatocytes [23]. At the same time, an indication for NHEJ activity was obtained in that the numbers of L foci were higher in late pachytene and early diplotene scid spermatocytes, 1 hr after irradiation, compared to wild type spermatocytes, indicating less repair in scid mice (Table 3). This effect is reminiscent of the rise in γ-H2AX foci in sperm that was observed shortly after gamete fusion of a 3 Gy irradiated wild type sperm cell with a scid oocyte [6]. Then, postulating a quick component [18, 19], late meiotic prophase cells repair radiation-induced DNA DSBs mainly by NHEJ and, as speculated for somatic cells [20], the contribution of HR repair could come later after the initial rejoining. If this holds true for late meiotic prophase, the absence of a scid effect in the relative loss of γ-H2AX signal (between 1 and 8 hr, see Table 3) can be explained by the contribution of HR. This might involve upregulation of HR when NHEJ is compromised.

An interplay between HR and NHEJ, compensating for each others deficits, was first demonstrated for somatic nuclei by Allen et al., [28] and Fukushima et al., [29]. Recently the same principle was shown to apply for mouse zygote DSB repair [6]. At late pachytene and early diplotene, HR could well be stimulated by the reduction in DNA.PK dependent NHEJ, masking the scid effect. This may explain the small scid effect that was found by cytogenetic analysis of chromosome and chromatid fragments at diakinesis and metaphase I. Twenty four hr after irradiation at diplotene in scid mice, a 1.4 increase in both chromatid- and chromosome type aberrations was found, compared to wild types [30]. Counting γ-H2AX foci in round spermatids of *Rad54/Rad54B* deficient and control mice (for scid and wild type see Ahmed et al., submitted) a significant difference in the clearance rate was observed. In the mutants 1.67-fold more foci were lost (Table 2). One explanation could be that due to a less efficient HR, NHEJ becomes up-regulated. So, the deficit in HR is compensated for by upregulation of NHEJ, even though HR is not effective anymore at this stage. In round spermatids, it cannot be decided if upregulation concerns DNA-PK dependent NHEJ or the PARP1-XRCC1 dependent variant (Ahmed et al., submitted).

The general trend of our data, being that at 1 hr after radiation more foci were found at late pachytene and early diplotene compared to early pachytene (which was found for 3 out of 4 genotypes, Tables 1,3) is also noticeable by irradiation cytogenetics [31, 32]. A trend towards higher radiation sensitivity for diplotene was apparent when scoring diakinesis and metaphase I

spermatoctes for chromosome and chromatid fragments. It is tempting to speculate that when later in meiotic prophase, the role of NHEJ is growing; the rate of non-repair is rising. An interplay between HR and NHEJ has been reported in Drosophila in which the survival of both *lig4*- and *rad54*-deficient males was reduced to 25% after DSBs induction in comparison to the wild type. However this percentage increased to 70% when either HR or NHEJ was available [33].

In conclusion, by using *Rad54/Rad54B* deficient mice and *DNA-PKcs* deficient scid mice, we have found clear indications for the involvement of both HR and NHEJ in the repair of radiation induced breaks during meiotic prophase. Also, results were best explained by assuming an interplay between these repair pathways. When HR is to some extend affected, NHEJ can take over and vice versa.

References

1. Rogakou EP, Boon C, Redon C, Bonner WM. Megabase chromatin domains involved in DNA double-strand breaks in vivo. J Cell Biol 1999; 146: 905-916.
2. Fernandez-Capetillo O, Lee A, Nussenzweig M, Nussenzweig A. H2AX: the histone guardian of the genome. DNA Repair (Amst) 2004; 3: 959-967.
3. Pilch DR, Sedelnikova OA, Redon C, Celeste A, Nussenzweig A, Bonner WM. Characteristics of gamma-H2AX foci at DNA double-strand breaks sites. Biochem Cell Biol 2003; 81: 123-129.
4. Ahmed EA, van der Vaart A, Barten A, Kal HB, Chen J, Lou Z, Minter-Dykhouse K, Bartkova J, Bartek J, de Boer P, de Rooij DG. Differences in DNA double strand breaks repair in male germ cell types: lessons learned from a differential expression of Mdc1 and 53BP1. DNA Repair (Amst) 2007; 6: 1243-1254.
5. Chicheportiche A, Bernardino-Sgherri J, de Massy B, Dutrillaux B. Characterization of Spo11-dependent and independent phospho-H2AX foci during meiotic prophase I in the male mouse. J Cell Sci 2007; 120: 1733-1742.
6. Derijck A, van der Heijden G, Giele M, Philippens M, de Boer P. DNA double-strand break repair in parental chromatin of mouse zygotes, the first cell cycle as an origin of de novo mutation. Hum Mol Genet 2008; 17: 1922-1937.
7. Baudat F, Manova K, Yuen JP, Jasin M, Keeney S. Chromosome synapsis defects and sexually dimorphic meiotic progression in mice lacking Spo11. Mol Cell 2000; 6: 989-998.
8. de Vries FA, de Boer E, van den Bosch M, Baarends WM, Ooms M, Yuan L, Liu JG, van Zeeland AA, Heyting C, Pastink A. Mouse Sycp1 functions in synaptonemal complex assembly, meiotic recombination, and XY body formation. Genes Dev 2005; 19: 1376-1389.
9. Mahadevaiah SK, Turner JM, Baudat F, Rogakou EP, de Boer P, Blanco-Rodriguez J, Jasin M, Keeney S, Bonner WM, Burgoyne PS. Recombinational DNA double-strand breaks in mice precede synapsis. Nat Genet 2001; 27: 271-276.
10. Schoenmakers S, Wassenaar E, van Cappellen WA, Derijck AA, de Boer P, Laven JS, Grootegoed JA, Baarends WM. Increased frequency of asynapsis and associated meiotic silencing of heterologous chromatin in the presence of irradiation-induced extra DNA double strand breaks. Dev Biol 2008; 317: 270-281.
11. Matulis S, Handel MA. Spermatocyte responses in vitro to induced DNA damage. Mol Reprod Dev 2006; 73: 1061-1072.
12. Bekker-Jensen S, Lukas C, Kitagawa R, Melander F, Kastan MB, Bartek J, Lukas J. Spatial organization of the mammalian genome surveillance machinery in response to DNA strand breaks. J Cell Biol 2006; 173: 195-206.
13. Nazarov IB, Smirnova AN, Krutilina RI, Svetlova MP, Solovjeva LV, Nikiforov AA, Oei SL, Zalenskaya IA, Yau PM, Bradbury EM, Tomilin NV. Dephosphorylation of histone gamma-H2AX during repair of DNA double-strand breaks in mammalian cells and its inhibition by calyculin A. Radiat Res 2003; 160: 309-317.
14. Rothkamm K, Kruger I, Thompson LH, Lobrich M. Pathways of DNA double-strand break repair during the mammalian cell cycle. Mol Cell Biol 2003; 23: 5706-5715.
15. Goedecke W, Eijpe M, Offenberg HH, van Aalderen M, Heyting C. Mre11 and Ku70 interact in somatic cells, but are differentially expressed in early meiosis. Nat Genet 1999; 23: 194-198.
16. Hamer G, Roepers-Gajadien HL, van Duyn-Goedhart A, Gademan IS, Kal HB, van Buul PP, Ashley T, de Rooij DG. Function of DNA-protein kinase catalytic subunit during the early meiotic prophase without Ku70 and Ku86. Biol Reprod 2003; 68: 717-721.
17. de Rooij DG, de Boer P. Specific arrests of spermatogenesis in genetically modified and mutant mice. Cytogenet Genome Res 2003; 103: 267-276.
18. DiBiase SJ, Zeng ZC, Chen R, Hyslop T, Curran WJ, Jr., Iliakis G. DNA-dependent protein kinase stimulates an independently active, nonhomologous, end-joining apparatus. Cancer Res 2000; 60: 1245-1253.
19. Iliakis G, Wang H, Perrault AR, Boecker W, Rosidi B, Windhofer F, Wu W, Guan J, Terzoudi G, Pantelias G. Mechanisms of DNA double strand break repair and chromosome aberration formation. Cytogenet Genome Res 2004; 104: 14-20.
20. Wang H, Zeng ZC, Perrault AR, Cheng X, Qin W, Iliakis G. Genetic evidence for the involvement of DNA ligase IV in the DNA-PK-dependent pathway of non-homologous end joining in mammalian cells. Nucleic Acids Res 2001; 29: 1653-1660.
21. Biedermann KA, Sun JR, Giaccia AJ, Tosto LM, Brown JM. scid mutation in mice confers hypersensitivity to ionizing radiation and a deficiency in DNA double-strand break repair. Proc Natl Acad Sci U S A 1991; 88: 1394-1397.

22. Essers J, Hendriks RW, Swagemakers SM, Troelstra C, de Wit J, Bootsma D, Hoeijmakers JH, Kanaar R. Disruption of mouse RAD54 reduces ionizing radiation resistance and homologous recombination. Cell 1997; 89: 195-204.
23. Wesoly J, Agarwal S, Sigurdsson S, Bussen W, Van Komen S, Qin J, van Steeg H, van Benthem J, Wassenaar E, Baarends WM, Ghazvini M, Tafel AA, Heath H, Galjart N, Essers J, Grootegoed JA, Arnheim N, Bezzubova O, Buerstedde JM, Sung P, Kanaar R. Differential contributions of mammalian Rad54 paralogs to recombination, DNA damage repair, and meiosis. Mol Cell Biol 2006; 26: 976-989.
24. Peters AH, Plug AW, van Vugt MJ, de Boer P. A drying-down technique for the spreading of mammalian meiocytes from the male and female germline. Chromosome Res 1997; 5: 66-68.
25. Derijck AA, van der Heijden GW, Ramos L, Giele M, Kremer JA, de Boer P. Motile human normozoospermic and oligozoospermic semen samples show a difference in double-strand DNA break incidence. Hum Reprod 2007; 22: 2368-2376.
26. Barchi M, Mahadevaiah S, Di Giacomo M, Baudat F, de Rooij DG, Burgoyne PS, Jasin M, Keeney S. Surveillance of different recombination defects in mouse spermatocytes yields distinct responses despite elimination at an identical developmental stage. Mol Cell Biol 2005; 25: 7203-7215.
27. Sciurano RB, Rahn MI, Pigozzi MI, Olmedo SB, Solari AJ. An azoospermic man with a double-strand DNA break-processing deficiency in the spermatocyte nuclei: case report. Hum Reprod. 2006, 21:1194-1203.
28. Allen C, Kurimasa A, Brenneman MA, Chen DJ, Nickoloff JA. DNA-dependent protein kinase suppresses double-strand break-induced and spontaneous homologous recombination. Proc Natl Acad Sci USA 2002; 99: 3758-3763.
29. Fukushima T, Takata M, Morrison C, Araki R, Fujimori A, Abe M, Tatsumi K, Jasin M, Dhar PK, Sonoda E, Chiba T, Takeda S. Genetic analysis of the DNA-dependent protein kinase reveals an inhibitory role of Ku in late S-G2 phase DNA double-strand break repair. J Biol Chem 2001; 276: 44413-44418.
30. van Buul PP, Abramsson-Zetterberg L, Zandman IM, van Duyn-Goedhart A. Further characterization of the radiosensitivity of the scid mouse. Int J Radiat Biol 1998; 74: 35-41.
31. Matsuda Y, Ohara H, Tobari I. Studies on radiation-induced chromosome aberrations in mouse spermatocytes. II. Dose-response relationships of chromosome aberrations induced at zygotene stage in mouse primary spermatocytes following fast neutron- and 60Co gamma-irradiations. Mutat Res 1987; 176: 251-257.
32. Walker HC. Comparative sensitivities of meiotic prophase stages in male mice to chromosome damage by acute X-and chronic gamma-irradiation. Mutat Res 1977; 44: 427-432.
33. Romeijn RJ, Gorski MM, van Schie MA, Noordermeer JN, Mullenders LH, Ferro W, Pastink A. Lig4 and rad54 are required for repair of DNA double-strand breaks induced by P-element excision in Drosophila. Genetics. 2005; 169:795-806.

Chapter 6

SUMMARIZING DISCUSSION

SUMMARIZING DISCUSSION

In this research project, we have first studied a number of proteins involved in the repair of DNA double strand breaks (DSBs) in the mouse, covering both the various types of germ cells and supporting cells (chapter 2). From the insight thus obtained, a further three projects arose. Signals of active DSBs in quiescent Sertoli cells have been followed up in chapter 3. Studies into the mechanism of DSB repair, the kinetics of which were shown in chapter 2, were carried out in chapter 4 for round spermatids and in chapter 5 for early and late pachytene spermatocytes. The main observations will be discussed below.

In the last decennium, insight has been developed as to how to follow DNA repair at the microscopical level. DNA DSBs stand out as being most suitable for microscopy after immunodetection of histone changes and repair proteins. In this thesis these methods have been applied to spermatogenic cells and their supportive Sertoli cells. As the differentiating spermatogenic cells do not flourish in culture, genotoxic insult has been applied in vivo.

Immunodetection of the chromatin neighbouring a DSB, using antibodies that recognize the addition of a phosphate group at serine 139 of histone H2AX, enables one to recognize DSBs after their induction. The generation of γ-H2AX foci has been the subject of many studies, including the proteins that assemble at such a focus and their putative roles. After irradiation and immunodetection, these foci are collectively called irradiation induced immunofluorescent foci (IRIF's) [1]. Studies on somatic cells and on mouse germ cells have shown that the kinetics of the loss of γ-H2AX foci are related to DSB repair activity [2-5]. The disappearance of foci with time, due to the dephosphorylation of γ-H2AX, is generally interpreted to represent the completion of repair. However, γ-H2AX foci that do not disappear but increase in size after irradiation could be related to a slow or incomplete repair as are small foci that accumulate (Chapters 2, 4 and 5).

1. Differential expression of DNA double strand breaks repair proteins in male germ cells

The special position of germ cells in DSB repair is born out by the fact that SPO11 induced DSBs are used to achieve homologous chromosome synapsis and homologous recombination. Interestingly, although MRE11 (a sensor protein for DNA DSBs) and KU70 (a key protein in NHEJ) interact in somatic cells and MRE11 is highly abundant in early spermatocytes, KU70 is not expressed in these cells, indicating no role for KU proteins during meiotic recombination and differential regulation of DNA repair proteins in meiotic cells [7,8]. Also, DNA–PKcs another key protein involved in NEHJ, is not expressed in round spermatids [8] while NHEJ should be

the main DSB repair pathway in these haploid cells. On the other hand, the ATM-CHK2 module and γ-H2AX are physiologically active in early human spermatocytes in which DNA DSBs occur during meiotic recombination [9], demonstrating that the upstream signaling from DSB lesions is functional in at least some stages of spermatogenesis. Clearly, DSB repair in male germ cells does not necessarily follow the principles established for somatic cells, making it important to find out more about the possibilities of male germ cells to repair the DSBs and the differential expression of repair proteins in different germ cell types.

1.1. Differential expression of MDC1 and γ-H2AX

Data obtained from somatic cells indicate that MDC1 recognizes phosphorylated H2AX via its tandem BRCT domain directly after DSB induction (endogenously or exogenously by IR or other damaging agents). Then MDC1 acts as a mediator/adaptor protein providing a "landing-platform" for other DNA repair proteins at the damaged site, establishing a full H2AX phosphorylation response [10, 11]. Our data from studying the expression and the co-localization of MDC1 and γ-H2AX in germ cells indicate that MDC1 and γ-H2AX follow somatic cell rules during meiotic recombination (Ch2). In leptotene stage of meiotic prophase, after the induction of meiotic DSBs by the SPO11 protein, MDC1 forms foci that show overlap (see Fig 3, Ch 2 upper panels) with γ-H2AX foci but MDC1 foci are smaller and lower in number. MDC1 also co-localizes with γ-H2AX in the tadpole-shaped structure containing the sex chromosomes during late zygotene and in the sex body in pachytene spermatocytes.

Radiation induces MDC1 foci in mid and late spermatocytes and in round spermatids that overlap completely with γ-H2AX foci (Ch2), indicating their involvement in the repair of DSBs in these cells. *Mdc1*$^{-/-}$ *mice* show an arrest in the spermatogenic process at the level of pachytene spermatocytes in stage IV, indicating a role for MDC1 in processing recombination related DSBs also. This arrest at stage IV appears similar to that described for H2AX$^{-/-}$ mice [13], both showing the same phenotype [4, 14]. Interestingly, MDC1 is not expressed in spermatogonia and preleptotene spermatocytes although γ-H2AX foci are induced in the nuclei of these cells (Ch 2). These findings clearly indicate a differential expression of MDC1 and γ-H2AX in different germ cell types.

1.2. Differential expression of MDC1 and 53BP1

53BP1 is expressed in spermatogonia and preleptotene spermatocytes before and after irradiation and in the absence of MDC1 forms foci in these nuclei that co-localize with γ-H2AX foci [4]. Surprisingly, recently it was established that in HeLa cells a direct interaction between 53BP1 and MDC1 is required for the recruitment and focus formation of 53BP1 and its maintenance at the site of damage [15]. As this interaction is not required in the germline (Ch 2), this provides another example of differential expression of important "DSB –repair proteins" in germ cells.

Studies in somatic cells have shown a role for 53BP1 in NHEJ but not during HR [16]. In spermatocytes, during meiotic recombination, 53BP1 is not expressed. Apparently, 53BP1 is not required for meiotic recombination. The absence of 53BP1 during meiotic prophase up to stage IV/V and also the absence of the Ku proteins in this period, indicate the absence of NHEJ during meiotic recombination, explaining why *53BP1$^{-/-}$ male mice* are fertile [4,16, 17]. The localization pattern of 53BP1 provides another example of the differential expression of DNA DSB repair proteins in the male germline.

2. Male germ cells repair DSBs slowly

A fast removal of DSBs occurs when all essential components of NHEJ are available and if this is the case in somatic cells, about 80% of the breaks can be rejoined within 2 hr [18, 19]. Repair by HR is slower and also, when one of the components of the NHEJ pathway is absent as is the case in DNA-PKcs or KU deficient cells, the rate of DSB repair is decreased [19, 20].

Within 5 min after ionizing radiation, focus formation of MDC1 and γ-H2AX occurs in mid to late pachytene and diplotene spermatocytes and in round spermatids, as established in histological sections (Ch 2). The kinetics of MDC1 and γ-H2AX focus formation in these germ cells seem comparable to that in somatic cell lines in which MDC1 foci are found within a minute after irradiation [11]. Foci of γ-H2AX and 53BP1 have also been shown within a few minutes after irradiation in spermatogonia and preleptotene spermatocytes (Ch2). When we studied the kinetics of the disappearance of these foci at various intervals after irradiation in spermatogonia and preleptotene spermatocytes, a 40% reduction for both γ-H2AX and 53BP1 foci was found at 16 hr after irradiation. This indicates much slower and incomplete repair in spermatogonia and preleptotene spermatocytes, compared with dividing somatic cells, possibly due to the absence of MDC1 (Ch2). The efficiency of the DSB repair as determined by the disappearance of the foci in round spermatids was comparable to that in late spermatocytes, leading to an about 70% reduction in 16 hr. These results indicate that DSB repair in male germ cells is slow (Ch 2). An exception to this rule could be found in late pachytene spermatocytes. In irradiated scid mice, at 1 hr after irradiation we observed a 1.7 fold higher number of γ-H2AX foci numbers than in irradiated wild type mice, indicating that within an hour in the wild type mice, repair already had considerably diminished the number of DSBs (Ch5). This difference may implicate the presence of fast repair by NHEJ late pachytene spermatocytes in wild type mice while this is missing in scid mice.

3. DNA DSBs repair pathways available for spermatogenic cell types

MDC1 is not expressed in spermatogonia (Ch 2), which may cause insufficient NHEJ and the absence of fast repair in these cells, leaving only slow repair. Focus formation by 53BP1

and γ-H2AX can be related to NHEJ repair in spermatogonia (Ch 2) but RAD51 foci are equally induced implicating that HR functions as well in spermatogonia. A role of scid in the repair of radiation damage in mouse spermatogonial stem cells has been demonstrated by van Buul et al. (1995) [21]. The scid mutation causes increased cell killing of spermatogonial stem cells. Clearly, NHEJ is playing a role in stem spermatogonia also and the slow loss of γ-H2AX foci after irradiation (Ch 2) and the sensitivity of dividing spermatogonia for apoptosis [22] may be related to some checkpoint mechanism.

The presence of HR repair during meiotic prophase can be inferred from the immunofluorescential detection of RAD51 after a 4 Gy dose of irradiation of mice which induces 25% extra DSBs at leptotene [23] and after in vitro irradiation of spermatocytes [24]. Since KU proteins are not expressed during meiotic recombination, repair of radiation induced DSBs likely does not occur by way of NHEJ at this time [7, 8]. Studying DSB repair kinetics in spreads of early pachytenes derived from irradiated scid mice and their wild type controls indicated similar repair kinetics (Ch 5). These data confirm the suppression of NHEJ in early spermatocytes. In early pachytene spermatocytes from irradiated *Rad54*$^{-/-}$ *Rad54B*$^{-/-}$ *double knock out mice*, which potentially have a less effective HR pathway, 52% of the DSBs were repaired between 1 and 8 hr after irradiation, which was significantly lower than in the irradiated wild type mice (82%) confirming the low efficiency of DSB repair in these mutant mice. Moreover, in the mutant an excess of small γ-H2AX foci was found after 8 hr, which can be interpreted as an indication for slow repair. As both localization data [7,8] and functional data (Ch 5) point at the absence of NHEJ during zygotene, early pachytene, the most likely explanation for the repair still present in *Rad54*$^{-/-}$*Rad54B*$^{-/-}$ *mice* at early pachytene is the residual repair capacity of HR in meiocytes of this mutant, as is evidenced by the male and female fertile status that requires homologous recombination. So RAD54 and RAD54B are not essential for HR but deficiency for these genes slows down the repair process.

Meiotic recombination ends by the mid to late pachytene stage (Ch 5) and after that both HR and NHEJ may contribute to repair of the irradiation-induced DSBs. In late pachytene to early diplotene spermatocytes, scid mice and their wild type controls have the same repair kinetics between 1 and 8 hr after irradiation. However, a scid effect was obvious when we studied γ-H2AX foci numbers in late pachytenes 1 hr after irradiation. We found a 1.7 fold higher number of DSBs in scid mice compared to in wild types. As introduced in the previous section this might be related to the fast repair component of NHEJ in wild type mice which is missing in scid mice and *DNA-PK-deficient* somatic cells in vitro [19]. This finding is compatible with the speculation that NHEJ is the main pathway for rejoining of radiation-induced DNA DSBs and that the contribution of HR repair follows after initial rejoining through the NHEJ fast component [20,25]. Could this be the case in late pachytene spermatocytes? If the presence of MDC1 signifies a preference for HR as proposed by Xie et al., (2007) [26] for human HR/SCR cells, the kinetics of MDC1 foci as reported in Ch 2 would favour HR. However, the difference in

repair kinetics 1 or 8 hr after irradiation in *Rad54*$^{-/-}$ *Rad54B*$^{-/-}$ mice and in wild type B6.129 mice was not significant (Ch 5). This result in combination with the "scid effect" discussed above, challenges the proposed link between MDC1 and HR. In round spermatids, when HR is no longer expected on theoretical grounds and Rad51 is not found (Ch 2), MDC1 IRIFs are conspicuous, again challenging the exclusive reporter function of MDC1 for HR in the testis.

It would appear that RAD54 is dispensible for HR in spermatocytes, in view of the fully fertile status of male *Rad54 knockout mice*. The data of Ch 5 are compatible with a system in which HR and NHEJ act as reciprocal backups, despite the slow acquisition of 53BP1 in late pachytene (Ch 2). Nevertheless, NHEJ may be the main repair pathway in late pachytene and early diplotene spermatocytes.

Since they are in G1 stage of the cell cycle, haploid spermatids are expected to repair DSBs by way of NHEJ. However, immunohistochemical evidence indicates that not all components of the classical NHEJ pathway are available since the presence of DNA–PKcs could not be shown immunocytochemically [8]. DSB repair kinetics of γ-H2AX foci in nucleus spreads of scid round spermatids and in nucleus spreads and histological sections of PARP1-inhibited mice and their respective controls indicate that round spermatids repair around 50% of the breaks within 8 hr post irradiation using both PARP1/XRCC1 and DNAPK-dependent NHEJ (Ch 4). As in scid mice 1 hr after irradiation of the numbers γ-H2AX foci in spermatids are similar as in wild type mice (Ch 4), it can be concluded that no fast component of NHEJ is active [19] in these nuclei, that would fit with the slow acquisition of 53BP1 at γ-H2AX foci (Ch 2). When the kinetics of the loss of γ-H2AX foci after irradiation are compared between spermatocytes (Ch 5) and spermatids (Ch 4), 8 hr after irradiation, repair is more extensive in spermatocytes. When chromosome aberrations are scored at metaphase of the zygote, as early as the round spermatid stage, the male germline is sensitive for the transmission of DNA lesions [27]. Hence, repair is not completed during the life time of the spermatids and part of the DNA damage is transmitted to the next generation. This might be a reason for the sensitivity of round spermatids to the generation of chromosomal imbalance and mutation by ionizing radiation [28].

4. Somatic cells in the testis and the repair of DNA DSBs

Our immunohistochemical data show no detectable MDC1, 53BP1, RAD51 and γ-H2AX in interstitial somatic cells, peritubular cells and myoid cells before and after irradiation. Quiescent cells will generally not be bothered by the presence of DSBs, unless some vital gene is involved in the break and indeed these types of quiescent somatic cells in the testis do not express DNA repair proteins. This is in sharp contrast with the proliferating somatic cells usually used to perform DNA repair studies. However, there was one unexpected exception, Sertoli cells did express 53BP1 and after irradiation 53BP1 foci were rapidly formed (Ch 2).

4.1. Sertoli cells

Our data from the in vitro culture of Sertoli cells indicate that postpubertal Sertoli cells cannot be regarded as terminally differentiated cells that have lost the capacity to proliferate. In vivo, the testis barrier and the formation of tight junction after maturation may prevent Sertoli cells from reentering the cell cycle. Also, there seems to exist a balance between cell cycle inhibitors and inducers that in the normal in vivo situation keeps the cells quiescent. Probably the residual proliferation capacity of Sertoli cells in vivo is the underlying reason for the rare Sertoli cell tumors seen in humans [29] and more often in the dog [30]. Also in nodules in testes of infertile human patients mitoticaly active Sertoli cells have been found [31]. After 20 days of culture in DMEM/F12 medium containing 5% FCS, about 36% of mouse Sertoli cells, identified by immunohistochemical staining for the marker vimentin, incorporated BrdU. The renewed proliferation was associated with a 70% decrease in the expression of the cell cycle inhibitor CDKN1B ($P27^{kip1}$) and a 2-fold increase in the level of the proliferation inducer ID2. Probably in vivo, the balance between cell cycle inhibitors and inducers is such that the cells remain quiescent, while in culture the balance is disturbed and Sertoli cells start to proliferate again. The renewed proliferative activity of Sertoli cells in culture could be further confirmed by double staining for BrdU and the Sertoli cell marker clusterin (CLU), showing about 25% of the CLU positive cells to be also positive for BrdU after 13 days of culture. Radiobiologically, Sertoli cells also do not behave as the other terminally differentiated quiescent somatic testicular cells. Interestingly, the numbers of 53BP1 foci in Sertoli cells showed a reduction of 60% at 16 hr after irradiation suggesting the presence of a DSBs repair system in Sertoli cells in vivo (Ch 2). Sertoli cells in vivo were also found to express PARP1, XRCC1 and other DNA repair proteins. A comet assay on irradiated Sertoli cells revealed a 70% reduction in tail length and moment at 20 hr after irradiation indicating the ability of Sertoli cells to repair the DSBs. Our finding that Sertoli cells do repair DSBs is compatible with the notion that these cells still have the capacity to proliferate, making it necessary to remove DSBs. However, it will be interesting to study the regulatory mechanisms governing Sertoli cell proliferation *in vitro*, in further detail. This aspect could have significance for seasonal breeders, may be providing a clue as to the pathway(s) involved.

5. Concluding remarks

Our conclusions can be summarized as follows:

- In chapter 2, the results of our studies on the expression of important DNA repair proteins indicate that these proteins (MDC1, 53BP1 and γ-H2AX) are differentially expressed in testicular cell types. The absence of MDC1 in spermatogonia could be the reason for the incomplete and slow repair seen in these cells. The repair kinetic data indicate that most germ cell types repair DNA DSBs slowly compared to somatic cells.

- In chapter 3, we describe that, in contrast to other testicular somatic cell types, Sertoli cells, express several DNA repair proteins and that these cells do repair DSBs, as shown by the in vivo disappearance of 53BP1 foci after irradiation (Ch 2) and the results of the comet assay. This, together with our finding that adult Sertoli cells resume proliferation in culture, indicates that adult Sertoli cells can no longer be regarded as a classical example of terminally differentiated cells.
- In chapter 4, we answered the question how round spermatids can repair DNA DSBs while these haploid cells cannot carry out HR and also do not, or at a very low level, express DNA-Pkcs, necessary for classical NHEJ. We showed that in round spermatids DSB repair is carried out by NHEJ via the newly described Parp1/XRCC1 dependent pathway. In addition, in these cells a low level of DSB repair occurs via the classical NHEJ pathway.
- In Chapter 5, from the repair kinetic data obtained in normal, *Rad54/Rad54B* deficient mice and SCID mice, we could establish that DSB repair in early spermatocytes is exclusively carried out through HR. However, in late spermatocytes both NHEJ and HR pathways are active, NHEJ probably being the most important.

5. References

1. Bekker-Jensen S, Lukas C, Kitagawa R, Melander F, Kastan MB, Bartek J, Lukas J. Spatial organization of the mammalian genome surveillance machinery in response to DNA strand breaks. J Cell Biol 2006; 173: 195-206.
2. Nazarov IB, Smirnova AN, Krutilina RI, Svetlova MP, Solovjeva LV, Nikiforov AA, Oei SL, Zalenskaya IA, Yau PM, Bradbury EM, Tomilin NV. Dephosphorylation of histone gamma-H2AX during repair of DNA double-strand breaks in mammalian cells and its inhibition by calyculin A. Radiat Res 2003; 160: 309-317.
3. Rothkamm K, Kruger I, Thompson LH, Lobrich M. Pathways of DNA double-strand break repair during the mammalian cell cycle. Mol Cell Biol 2003; 23: 5706-5715.
4. Ahmed EA, van der Vaart A, Barten A, Kal HB, Chen J, Lou Z, Minter-Dykhouse K, Bartkova J, Bartek J, de Boer P, de Rooij DG. Differences in DNA double strand breaks repair in male germ cell types: lessons learned from a differential expression of Mdc1 and 53BP1. DNA Repair 2007; 6: 1243-1254.
5. Chicheportiche A, Bernardino-Sgherri J, de Massy B, Dutrillaux B. Characterization of Spo11-dependent and independent phospho-H2AX foci during meiotic prophase I in the male mouse. J Cell Sci 2007; 120: 1733-1742.
6. de Rooij DG, Russell LD. All you wanted to know about spermatogonia but were afraid to ask. J Androl 2000; 21: 776-798.
7. Goedecke W, Eijpe M, Offenberg HH, van Aalderen M, Heyting C. Mre11 and Ku70 interact in somatic cells, but are differentially expressed in early meiosis. Nat Genet 1999; 23: 194-198.
8. Hamer G, Roepers-Gajadien HL, van Duyn-Goedhart A, Gademan IS, Kal HB, van Buul PP, Ashley T, de Rooij DG. Function of DNA-protein kinase catalytic subunit during the early meiotic prophase without Ku70 and Ku86. Biol Reprod 2003; 68: 717-721.
9. Bartkova J, Bakkenist CJ, Rajpert-De Meyts E, Skakkebaek NE, Sehested M, Lukas J, Kastan MB, Bartek J. ATM activation in normal human tissues and testicular cancer. Cell Cycle 2005; 4: 838-845.
10. Goldberg M, Stucki M, Falck J, D'Amours D, Rahman D, Pappin D, Bartek J, Jackson SP. MDC1 is required for the intra-S-phase DNA damage checkpoint. Nature 2003; 421: 952-956.
11. Stewart GS, Wang B, Bignell CR, Taylor AM, Elledge SJ. MDC1 is a mediator of the mammalian DNA damage checkpoint. Nature 2003; 421: 961-966.
12. Mahadevaiah SK, Turner JM, Baudat F, Rogakou EP, de Boer P, Blanco-Rodriguez J, Jasin M, Keeney S, Bonner WM, Burgoyne PS. Recombinational DNA double-strand breaks in mice precede synapsis. Nat Genet 2001; 27: 271-276.
13. Celeste A, Petersen S, Romanienko PJ, Fernandez-Capetillo O, Chen HT, Sedelnikova OA, Reina-San-Martin B, Coppola V, Meffre E, Difilippantonio MJ, Redon C, Pilch DR, Olaru A, Eckhaus M, Camerini-Otero RD, Tessarollo L, Livak F, Manova K, Bonner WM, Nussenzweig MC, Nussenzweig A. Genomic instability in mice lacking histone H2AX. Science 2002; 296: 922-927.
14. Lou Z, Minter-Dykhouse K, Franco S, Gostissa M, Rivera MA, Celeste A, Manis JP, van Deursen J, Nussenzweig A, Paull TT, Alt FW, Chen J. MDC1 maintains genomic stability by participating in the amplification of ATM-dependent DNA damage signals. Mol Cell 2006; 21: 187-200.
15. Eliezer Y, Argaman L, Rhie A, Doherty AJ, Goldberg M. The direct interaction between 53BP1 and MDC1 is required for the recruitment of 53BP1 to sites of damage. J Biol Chem 2009; 284: 426-435.
16. Nakamura K, Sakai W, Kawamoto T, Bree RT, Lowndes NF, Takeda S, Taniguchi Y. Genetic dissection of vertebrate 53BP1: a major role in non-homologous end joining of DNA double strand breaks. DNA Repair 2006; 5: 741-749.
17. Ward IM, Minn K, van Deursen J, Chen J. p53 Binding protein 53BP1 is required for DNA damage responses and tumor suppression in mice. Mol Cell Biol 2003; 23: 2556-2563.
18. DiBiase SJ, Zeng ZC, Chen R, Hyslop T, Curran WJ, Jr., Iliakis G. DNA-dependent protein kinase stimulates an independently active, nonhomologous, end-joining apparatus. Cancer Res 2000; 60: 1245-1253.
19. Iliakis G, Wang H, Perrault AR, Boecker W, Rosidi B, Windhofer F, Wu W, Guan J, Terzoudi G, Pantelias G. Mechanisms of DNA double strand break repair and chromosome aberration formation. Cytogenet Genome Res 2004; 104: 14-20.
20. Wang H, Zeng ZC, Bui TA, Sonoda E, Takata M, Takeda S, Iliakis G. Efficient rejoining of radiation-induced DNA double-strand breaks in vertebrate cells deficient in genes of the RAD52 epistasis group. Oncogene 2001; 20: 2212-2224.
21. Van Buul PP, De Rooij DG, Zandman IM, Grigorova M, Van Duyn-Goedhart A. X-ray-induced chromosomal aberrations and cell killing in somatic and germ cells of the scid mouse. Int J Radiat Biol 1995; 67: 549-555.
22. Beumer TL, Roepers-Gajadien HL, Gademan IS, van Buul PP, Gil-Gomez G, Rutgers DH, de Rooij DG. The role of the tumor suppressor p53 in spermatogenesis. Cell Death Differ 1998; 5: 669-677.

23. Schoenmakers S, Wassenaar E, van Cappellen WA, Derijck AA, de Boer P, Laven JS, Grootegoed JA, Baarends WM. Increased frequency of asynapsis and associated meiotic silencing of heterologous chromatin in the presence of irradiation-induced extra DNA double strand breaks. Dev Biol 2008; 317: 270-281.
24. Matulis S, Handel MA. Spermatocyte responses in vitro to induced DNA damage. Mol Reprod Dev 2006; 73: 1061-1072.
25. Xu X, Stern DF. NFBD1/MDC1 regulates ionizing radiation-induced focus formation by DNA checkpoint signaling and repair factors. FASEB J 2003; 17: 1842-1848.
26. Xie A, Hartlerode A, Stucki M, Odate S, Puget N, Kwok A, Nagaraju G, Yan C, Alt FW, Chen J, Jackson SP, Scully R. Distinct roles of chromatin-associated proteins MDC1 and 53BP1 in mammalian double-strand break repair. Mol Cell 2007; 28: 1045-1057.
27. Matsuda Y, Seki N, Utsugi-Takeuchi T, Tobari I. X-ray- and mitomycin C (MMC)-induced chromosome aberrations in spermiogenic germ cells and the repair capacity of mouse eggs for the X-ray and MMC damage. Mutat Res 1989; 211: 65-75.
28. Russell WL, Bangham JW, Russell LB. Differential response of mouse male germ-cell stages to radiation-induced specific-locus and dominant mutations. Genetics 1998; 148: 1567-1578.
29. Kratzer SS, Ulbright TM, Talerman A, Srigley JR, Roth LM, Wahle GR, Moussa M, Stephens JK, Millos A, Young RH. Large cell calcifying Sertoli cell tumor of the testis: contrasting features of six malignant and six benign tumors and a review of the literature. Am J Surg Pathol 1997; 21: 1271-1280.
30. Peters MA, de Rooij DG, Teerds KJ, van Der Gaag I, van Sluijs FJ. Spermatogenesis and testicular tumours in ageing dogs. J Reprod Fertil 2000; 120: 443-452.
31. Brehm R, Rey R, Kliesch S, Steger K, Marks A, Bergmann M. Mitotic activity of Sertoli cells in adult human testis: an immunohistochemical study to characterize Sertoli cells in testicular cords from patients showing testicular dysgenesis syndrome. Anat Embryol 2006; 211: 223-236.

Samenvatting

Samenvatting

DNA dubbelstrengs breuken (DSBn) kunnen ontstaan door ioniserende straling of tijdens de meiotische recombinatie. De twee belangrijkste mechanismen waarmee DSBn worden gerepareerd zijn homologe recombinatie (HR) en “non homologous end-joining” (NHEJ). De celsoorten waarlangs het proces van de zaadcelvorming, ofwel spermatogenese, verloopt kunnen worden verdeeld in drie hoofdgroepen: spermatogoniën, spermatocyten en spermatiden.

De spermatogoniën zijn de delende cellen in het spermatogenetische epitheel en omvatten de spermatogoniale stamcellen en opeenvolgende generaties van delende spermatogoniën. Spermatogoniën brengen verschillende DNA reparatie eiwitten tot expressie zoals y-H2AX (een marker voor DSBn) en p53 binding protein1 (53BP1). MDC1 (mediator of DNA damage checkpoint), een eiwit dat een belangrijke rol speelt tijdens HR en NHEJ, komt niet tot expressie in spermatogoniën. Hierin verschillen spermatogoniën van delende niet spermatogenetische, ofwel somatische, cellen en dit zou mogelijk verklaren waarom onze resultaten aantonen dat DSB herstel in spermatogoniën langzaam en incompleet is. Uit de laatste deling van de spermatogoniën ontstaan de zogenaamde spermatocyten die de meiose uitvoeren. Tijdens de meiotische recombinatie, in vroege spermatocyten (leptoteen, zygoteen en vroege pachytene stadia van de meiotische profase) onstaan DSBn. Onze resultaten laten zien dat de foutgevoelige reparatie door NHEJ niet plaatsvindt in vroege spermatocyten, ook niet tijdens de reparatie van DSBn welke zijn verzoorzaakt door straling. Ten eerste, brengen deze cellen geen 53BP1 tot expressie, een belangrijk eiwit in NHEJ. Ten tweede, laten we zien dat de reparatie van recombinatie gerelateerde DSBn en stralings-geinduceerde DSBn in scid muizen (deficiënt voor NHEJ) niet verschilt van wild type muizen. Dus, het herstel van DSBs in vroege spermatocyten gebeurt uitsluitend via HR.

In late spermatocyten (mid-pachyteen tot en met diploteen stadium) komen alle belangrijke eiwitten (voor NHEJ) tot expressie en gebruikmakend van *Rad54/Rad54B* deficiënte muizen, welke mogelijk deficiënt zijn voor HR, kunnen we laten zien dat zowel NHEJ als HR mechanismes actief zijn in late spermatocyten en dat NHEJ waarschijnlijk het meest belangrijk is in deze cellen. De meiotische delingen resulteren in ronde spermatiden die elongeren en differentiëren tot spermatozoa. In spermatiden kan HR niet plaatsvinden omdat deze cellen haploid zijn. Wat betreft NHEJ hebben eerdere onderzoeken laten zien dat DNA-PKcs, een eiwit onmisbaar voor de klassieke NHEJ, niet of nauwelijks tot expressie komt in spermatiden Niettemin, laat het verdwijnen van y-H2AX foci, die de plaats van DSBs markeren, zien dat deze cellen toch in staat zijn tot herstel van DSBn. Teneinde deze puzzel op te lossen hebben wij gebruik gemaakt van scid muizen, deficiënt voor klassieke NHEJ, en PARP1 deficiente muizen, deficiënt voor een onlangs beschreven alternatieve route voor NHEJ. Hiermee konden wij aantonen dat het herstel in spermatiden voor de helft plaatsvindt d.m.v. een rest

herstelactiviteit via klassieke NHEJ en voor de andere helft via de alternatieve route via PARP1 en XRCC1. Desondanks, is DSB herstel in spermatiden traag en onvolledig.

De spermatogenetische cellen in het epitheel worden ondersteund door een somatische celsoort, de cellen van Sertoli. Door middel van de zogenaamde “comet assay” en het aantonen van het verdwijnen van 53BP1 foci in vivo, hebbben we aangetoond dat ook Sertoli cellen DSBn repareren. Deze cellen brengen ook verscheidende DNA reparatie eiwitten tot expressie in tegenstelling tot de andere somatische cellen in de testis. Dit, samen met onze bevinding dat Sertoli cellen de celdeling hervatten in kweek, impliceert dat volwassen Sertoli cellen niet langer kunnen worden beschouwd als een klassiek voorbeeld van terminaal gedifferentieerde cellen die niet meer kunnen delen, niet in staat zijn tot reparatie van DNA DSBn, en alleen maar een bepaalde functie uitvoeren.

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List of publications

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Ahmed, E.A., de Boer, P., Philippens, M., Kal H.B., and de Rooij, D.G. Parp1-XRCC1 and the repair of DNA double strand breaks in mouse round spermatids. Submitted.

Ahmed E.A., D.G. de Rooij (2009). Staging of mouse seminiferous tubule cross-sections. In: Meiosis. Ed. S. Keeney. *Methods in Molecular Biology*, Humana Press, Totowa, USA. In press.

E.A. Ahmed, A.D. Barten-van Rijbroek, H.B. Kal, H. Sadri-Ardekani, S.C. Mizrak, A.M.M. van Pelt, D.G. de Rooij (2009). In vitro proliferative activity and DNA repair indicate that adult mouse and human Sertoli cells are not terminally differentiated quiescent cells. *Biology of Reproduction,* In Press.

Ahmed, E.A., Van der Vaart, A., Barten, A., Kal, H.B., Chen, J., Lou, Z., Minter-Dykhouse, K., Bartkova, J., Bartek, J., De Boer, P. and De Rooij, D.G. (2007) Differences in DNA double stand breaks repair in male germ cell types: Lessons learned from a differential expression of Mdc1 and 53BP1. *DNA Repair* 6: 1243-1254.

Curriculum Vitae

Emad Abdel Aziz Ahmed was born in El-Minia, Egypt on the 20th of August, 1973. After high school, in 1992, he studied Biology at the Faculty of Science, Minia University, Egypt, for four years. In June 1996, he graduated from the department of Zoology, Faculty of Science, Minia University, with very good-honor.

In January 1997, he started a position of Demonstrator at the department of Zoology, Faculty of Science, Assiut University, Egypt, to do his postgraduate studies and to participate in the teaching. Emad completed the courses (Physiology, Biochemistry, Toxicology, Histochemistry, Immunology and Biostatistics) required for his Master degree within one academic year, all with very good grades. In April 2001, he obtained his Master degree that was entitled "Physiological effect of tannic acid as antioxidant on long-term aluminium exposure in rats" under supervision of prof.dr. Khadiga A. Hassan and prof.dr. Hossam M. Omar. In May 2001, he continued the work in the same department as assistant lecturer.

In October 2003, he got a ten-months scholarship provided by the Norwegian Research Council, and worked as a research student at the department of Experimental Pathology, Faculty of Medical Biology, Tromso University, Norway.

In 2004, he was granted a 4-year scholarship from the Egyptian government to do his PhD in The Netherlands. In April 2005, he started his PhD at the University of Utrecht, Department of Biology, Division of Endocrinology and Metabolism under supervision of Prof.dr. Dirk G. de Rooij and Dr. Peter de Boer. Finally after obtaining his PhD, he will be working as a lecturer postdoc at the department of Zoology, Faculty of Science, Assiut University, Egypt.

Arabic summary

الملخص العربي لرسالة الدكتوراه المقدمة إلى جامعة أوتريخت بهولندا تحت عنوان " مسارات إصلاح تكسر الحامض النووي المزدوج الشريط (DNA) فى الخلايا التناسلية لذكور الفئران".

مقدمه من عماد عبد العزيز أحمد عبد جابر المولود فى 20 أغسطس 1973 بمحافظة المنيا، جمهورية مصر العربية، بعثة خارجية ، مجال دراسة عام (فسيولوجي) - دقيق (بيولوجية جزيئية).

- يمكن تلخيص نتائج الدراسة في النقاط التالية:

أوضحت الدراسة أن العديد من البروتينات التي تعمل معا بصورة رئيسية لإصلاح تكسرالحامض النووي في الخلايا الجسدية تتمايز في التعبير البروتيني في الخلايا التناسلية لذكور الفئران وأن بروتين رئيسي (MDC1) من هذه البروتينات غير موجود في الخلايا المولدة للنطاف وذلك ما قد يكون سببا في عدم اكتمال وبطء الإصلاح بهذه الخلايا وقد يفسر الحساسية الشديدة لهذه الخلايا كما وجد أن الفئران التي أزيل منها الجين المسؤول عن هذا البروتين قد توقف بها الانقسام الميوزي داخل الخصية عند المرحلة IV، عند هذه المرحلة لا تنمو الخلايا المنوية الثانوية و إنما تذهب في موت فسيولوجي منظيم.

أظهرت الدراسة قيام الخلايا التناسلية بإصلاح تكسر الحامض النووي المزدوج الشريط ببطء مقارنة بالخلايا الجسدية.

خلافا لغيرها من الخلايا الجسدية البالغة داخل الخصية وجد أن لخلايا سرتولى القدرة على إصلاح الـ AND داخل " in vivo" وخارج الجسم "in vitro" كما أنها تستعيد القدرة على الانقسام في خارج الجسم وهو ما يضع علامة استفهام امام اعتبارها مثالا تقليديا للخلايا جانبية التمايز " التي لا تنقسم".

بوجه عام هناك مسارين رئيسيين لإصلاح تكسر الحامض النووي المزدوج الشريط . الأول دقيق ويحتاج نسخة أخري من المادة الوراثية ويسمى HR والاخر غير دقيق ويؤدي غالبا إلى أخطاء في الإصلاح ويسمى NHEJ. حديثا اقترحت الدراسات مسارا آخر يعمل بديلا للمسار الثاني و يعتمد على بروتين PARP1 وبروتين XRCC1 وعندما قمنا بمعاملة الفئران بمثبط لنشاط البروتين PARP1 أشارت النتائج إلى أن كلا من المسارين الثاني والبديل نشط في خلايا الأذناب المدورة ويقومان وببطء بإصلاح نصف عدد كسورالحامض النووي بعد 8 ساعات من تعريض الفئران للأشعة السينية.

باستخدام فئران أزيلت منها إحدى الجينات المسؤولة عن الإصلاح بواسطة HR أو NHEJ نستطيع أن نؤسس أن إصلاح تكسر الـ AND خلال عملية العبور بين الكروموسومات الذاتية (يحدث طبيعيا أثناء الانقسام الميوزي) وكذلك التكسر الناتج عن التعرض للأشعة السينية كلاهما يتم إصلاحه حصريا بواسطة المسار الأول الدقيق بينما كلا من المسارين متاح للخلايا المنوية الثانوية بعد عملية العبور وقد يكون NHEJ الأكثر نشاطا.