DNA DOUBLE-STRAND BREAKS & APOPTOSIS IN THE TESTIS

A SUMMARIZING DISCUSSION

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In comparison to most other tissues the DNA damage responses in the testis are probably most divers. For instance, the mitotically dividing spermatogonia are radiosensitive and easily undergo apoptosis in response to DNA damage (1), whereas, in comparison, the meiotic and haploid germ cells appear very resistant for the direct effects of ionizing radiation (IR) (2). However, independent from any treatment with DNA damaging agents, many cells in the testis also “spontaneously” undergo apoptosis (3). Even DNA damage is a naturally occurring event in the testis as DNA double-strand breaks (DSBs) are endogenously induced during the first meiotic prophase (4). These breaks are necessary for the meiotic cell division and are repaired by meiotic recombination (5).

During male germ cell development, mitotically dividing spermatogonia differentiate into meiotic spermatocytes, into elongating haploid cells and eventually into spermatozoa. In other words: the DNA of the germ cells first has to undergo several mitotic divisions, followed by the two subsequent meiotic cell divisions, including meiotic recombination, after which it has to be condensed in a tight package upon delivery to fertilize an oocyte. These changes imply major changes in chromatin structure and as a consequence also in the molecular mechanisms by which genome integrity is maintained. Therefore, DNA damage responses in testis are not only very divers but also tightly regulated.

This thesis is mainly focused on the spermatogonial DNA damage response and meiotic recombinational repair during the first meiotic prophase. During both stages of male germ cell development different DNA damage response pathways are active. Studying expression and regulation of proteins involved in these pathways during spermatogenesis can give insight in how cells might regulate the proper DNA damage response at given moment during their development.

SPERMATOGONIAL APOPTOSIS & INTERCELLULAR BRIDGES

Spermatogonia do not only undergo apoptosis in response to DNA damage but also, when the ratio spermatogonia/ Sertoli cells is too high, as a way of germ cell density regulation (3). Although both leading to spermatogonial apoptosis, these two processes use different molecular pathways. During DNA-damage induced spermatogonial apoptosis the tumor suppressor p53 plays a central role, this protein being induced shortly after irradiation (6-8). In contrast, spermatogonial degeneration during germ cell density regulation depends on the Bcl-2 family member Bax (9, 10), which has been shown not to be involved in the spermatogonial response to DNA-damage (9).

As they divide, spermatogonia stay cytoplasmically connected by intercellular bridges leading to large spermatogonial “clones” (11, 12). However, in response to irradiation and subsequent DNA damage, single spermatogonia undergo apoptosis without affecting their neighbouring interconnected cells (13). This means that apoptotic signals induced in one damaged cell nucleus are not necessarily transmitted through the cytoplasmic bridges. In contrast, during germ cell density regulation, spermatogonia do not undergo apoptosis individually but as whole clones of interconnected cells (14). Apparently, in spermatogonia, the apoptotic effect of spermatogonial p53 signaling differs from the effect of the pathway involving Bax. Unfortunately, it is very difficult to perform immunohistochemistry using whole mount seminiferous tubules, making it hard to investigate proteins in the p53 or Bcl2-family signaling cascades in relation to intercellular connections. In order to study these aspects, a spermatogonial culture system in which intercellular relations are clearly visible would be very beneficial and probably required.
c-Abl & p73

Although very important, p53 is not always essential for spermatogonial apoptosis (15) and also p53 deficient spermatogonia are able to undergo apoptosis in response to irradiation (6). One alternative is provided by the p53 homologue p73. p73 exists in various isoforms that can be pro- and anti-apoptotic (16-20), however, we found that in the testis the full length pro-apoptotic p73α is present (21).

p73 is activated by the non-receptor tyrosine kinase c-Abl (22-25), which gets activated in response to IR (26, 27). In most cell types activated p73 then functions as a transcription factor (16-20). Also in spermatogonia c-Abl phosphorylates p73 in response to irradiation (21). However, in spermatogonia both c-Abl and p73 reside in cytoplasm, excluding direct functions as transcriptional activators.

Since c-Abl is not present in the spermatogonial nucleus, it is not very likely that, in these cells, DNA damage directly activates c-Abl. More likely reactive oxygen species, induced by IR, directly activate cytoplasmic c-Abl (28, 29). c-Abl has multiple cytoplasmic functions, including regulation of mitogenesis (30), and even directly at the mitochondria triggering the release of the apoptosis inducing cytochrome c (29, 31, 32). In the testis c-Abl also phosphorylates the pro-apoptotic p73α, giving rise to the next question: how can cytoplasmic p73 induce apoptosis?

Sub-cellular localization of p73, and also p53, are under strict regulation of both nuclear export as well as import sequences (33-37). However, most authors assume that cytoplasmic p73 is not functional. This assumption is not in line with the fact that, although phosphorylated by c-Abl, spermatogonial p73 does not translocate to the nucleus in response to IR. More likely p73 can also be active in the cytoplasm, probably acting directly via mitochondria, a route that also has been described for p53 (38, 39). The role for and regulation of c-Abl and p73 will thus differ between various tissues (40) and depends on sub-cellular localization of these proteins and the presence of specific p73 isoforms.

γ-H2AX

The most severe cellular damage induced by irradiation is the generation of DNA double-strand breaks (DSBs) (41-51), which in spermatogonia will induce p53 (6). In response to especially DSBs histone H2AX gets phosphorylated at serine 139 (52), and within minutes γ-H2AX (phosphorylated H2AX) foci appear specifically at the damaged sites containing DSBs (53, 54). Each individual focus represents a single DSB and there is a linear effect between the dose of IR and the number of γ-H2AX foci, making quantification of foci a reliable method for measuring induction and repair of DSBs (55, 56). The exact mechanisms by which γ-H2AX foci are induced remain to be elucidated, however, they are certainly essential for the sequestration of several DNA-repair factors and DNA damage-signaling proteins to IR induced foci (IRIF) (54, 57-59). Also in the testis γ-H2AX foci are induced by IR, which in spermatogonia interact with p53 (60).

Additionally, in the testis, DSBs do not only occur in response to irradiation, they are also endogenously induced during the first meiotic prophase (4, 61). These meiotic DSBs have been described to occur predominantly during the leptotene stage and thus before synopsis of the homologous chromosomes (4). However, radiation-independent γ-H2AX is already present in all intermediate and B spermatogonia, remaining up until zygotene spermatocytes and the sex bodies of pachytene spermatocytes (60). This expression pattern in the testis seems to contradict the assumed restriction of γ-H2AX localization to DSBs, at least during spermatogenesis. Otherwise DSBs would remain unrepaired during two mitotic divisions and
the pre-meiotic S phase. More likely, H2AX phosphorylation has a testis specific role in the remodeling of germ cell chromatin as during spermatogenesis chromatin is constantly being reconstructed (62).

Especially intriguing is the presence of γ-H2AX in the sex bodies that contain the X and Y-chromosomes. This presence clearly has a function since in the H2AX−/− testis the sex chromosomes fail to pair, and are fragmented or associated with autosomal chromosomes (63). Moreover, H2AX−/− spermatocytes do not initiate meiotic sex chromosome inactivation and fail to recruit several sex body specific proteins (64). As a consequence, spermatogenesis in the H2AX−/− mice is arrested at what, as far as can be concluded from the published figures, seems to be pachytene stage IV-V (63, 64).

Although γ-H2AX has been widely used as marker for DSBs, it clearly also has other functions during spermatogenesis. Therefore it would be very interesting to compare the presence of γ-H2AX (4, 60) with the staining pattern of another marker for DSBs in the testis. A good candidate might be the p53 binding protein 1 (53BP1). In somatic cells this protein co-localizes with γ-H2AX and also accumulates in foci at DSBs in response to IR (65-73). It would be of interest to see when and where γ-H2AX and 53BP1 overlap during spermatogenesis and to investigate whether γ-H2AX in the testis exists without co-localizing with 53BP1.

**REGULATION OF DNA-PK & THE CHOICE BETWEEN HRR & NHEJ**

DNA damage can be repaired in a variety of ways of which the very accurate homologous recombinational repair (HRR) and the more error prone non-homologous end joining (NHEJ) are most important for the repair of DSBs (41-51) (Figure 6, Chapter 1). During every step of development or cell differentiation a “choice” between these two pathways has to be made (41). For instance, there appear to be differences in the response to IR at different developmental stages, HRR being important for DSB-repair during early mouse development and NHEJ in the adult mouse (74).

A major player in the repair of DSBs is the DNA-dependent protein kinase (DNA-PK). This protein complex consists of a heteromeric DNA binding component (Ku70 and Ku86) and a DNA-PK catalytic subunit (DNA-PKcs) (75, 76). The Ku heterodimer binds to single- and especially double-stranded DNA breaks (DSBs) and subsequently recruits DNA-PKcs that plays a key role in non-homologous end joining of the damaged DNA (77-81).

In spermatogonia, DNA-PK detects and assists in repair of DSBs by NHEJ, for instance caused by IR (82). In pachytene spermatocytes, however, DSBs have to be repaired by meiotic recombination, a specialized form of HRR. Proper meiotic recombination is essential for the entire meiotic process (5) and, moreover, NHEJ would be too error prone to repair the many DSBs induced during meiosis. The complete DNA-PK holo-enzyme has been shown to suppress homologous recombination (83), so presence of all the DNA-PK components in early spermatocytes would seriously interfere with meiotic recombination. Additionally, interruption of the DNA-PK has been found to stimulate homologous recombination (84, 85). During male meiosis, this interruption is achieved by the very specific down regulation of the Ku proteins in early spermatocytes until pachytene stage IV of the seminiferous epithelium (Figure 3, Chapter 4) (82, 86). Regulated expression of the Ku heterodimer during meiosis could be the major mechanism that stimulates meiotic recombination and suppresses NHEJ.

DNA-PK activity also appears to be regulated through an auto-regulatory feedback mechanism that involves *in trans* auto-phosphorylation of DNA-PKcs (87, 88). The exact characteristics of this auto-regulatory feedback loop remain to be elucidated. However, it does not seem very likely that this mechanism is involved in suppressing NHEJ during meiotic
recombination since it requires the Ku proteins, which are not present throughout the pachytene stage.

Even without the Ku heterodimer, DNA-PKcs still has a function in early spermatocytes. Until now, Ku independent DNA binding and activation of DNA-PKcs only has been described in vitro (89). However, the significantly higher number of apoptotic early spermatocytes in the DNA-PKcs deficient scid testes suggests a Ku-independent function for DNA-PKcs in these early meiotic cells (82). The majority of these apoptotic spermatocytes is present at stage IV of the seminiferous epithelium. At this epithelial stage spermatogenesis is known to abrogate at the spermatocyte level in several knockout mice deficient for DNA damage response proteins (90), which suggests that a stage IV meiotic checkpoint exists, selecting spermatocytes with damaged DNA to undergo apoptosis.

REGULATION OF ATM DURING SPERMATOGENESIS

Like DNA-PK, also ATM appears to differentially regulated during spermatogenesis. In spermatogonia ATM dimers get auto-cross-phosphorylated in response to IR (91) and initiate the spermatogonial DNA damage response, including the appearance of γ-H2AX foci at the sites of DSBs (52, 60, 92, 93) and induction of p53 (94-97), which will lead to spermatogonial apoptosis or cell cycle arrest (6-8).

However, although ATM clearly is essential for meiotic recombination (98, 99), it remains non-phosphorylated during the meiotic prophase. This in contrast to spermatogonia and other mitotic cells (91) in which ATM gets activated by phosphorylation. Hence, like DNA-PK, also ATM is differently regulated during the meiotic prophase and does not need to be phosphorylated at this stage in order to be functional.

An exception during meiosis, however, is the constitutively presence of phosphorylated ATM in the sex bodies. In these structures phosphorylated ATM might very well be responsible for phosphorylation of γ-H2AX (52, 60, 92, 93) which is, as mentioned earlier, required for meiotic X-chromosome inactivation (64, 100, 101) and keeping the X and Y chromosomes intact during the first meiotic prophase (63). The reason why DNA damage repair proteins are present in the sex bodies in a constitutively activated state remains to be resolved. One explanation could be that the X and Y chromosomes are in a constant state of damage. However, it seems more likely that, in the sex bodies, these proteins also can have a more preventive function rather than a reparative. The fact that some of these proteins are described to be required for meiotic X-inactivation (64, 100, 101) could also lead to another explanation. Perhaps, when transcription is being repressed during DNA damage or repair by these specific proteins, their presence at the sex chromosomes could lead to meiotic X-inactivation, even without the actual presence of DNA damage.

QUESTIONS & ANSWERS

In reality DNA repair during spermatogenesis will appear to be much more complex, involving many other proteins, like BRCA1 and 2 (101-106), the MRE11 complex (107) and the more recently discovered Artemis (108, 109). Whereas BRCA1 and 2 (110-119) and the MRE11 (107) complex function during HRR, Artemis is involved in NHEJ (108). In addition, also many proteins of the mismatch-repair system (41-51) are involved in DNA repair during spermatogenesis and especially meiotic recombination (120-141). Although its function is not entirely understood, mismatch-repair protein MHL1 is even a commonly used marker for
meiotic crossovers (129). Expression and regulation of all these and other proteins determine the “choice” between different DNA repair pathways at different steps during spermatogenesis. Current research, using cell culture and gene over-expressing systems, shows many possibilities for a great variety of DNA damage response pathways. Also many cell cycle and apoptosis regulating proteins, like Chk2 (142, 143), the E2F family of transcription factors (143-145) and MDC1 (146-148), are continuously being discovered or further investigated. Additionally, in many cases, cross-talk between different pathways has been described and the borders between all these different routes may not be as strict as often assumed. Of many of these pathways it has not even been studied yet whether they are relevant in vivo in the testis, or other tissues, or involved in various cancers. Spermatogenesis provides a good model to study these processes because male germ cells are constantly developing and very precisely regulate their response to DNA damage. Therefore, also the general knowledge about DNA damage responses can benefit from testis research.
REFERENCES


