The role of Dicer1 in the male reproductive tract

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Dicer1 is an RNase III enzyme necessary for microRNA (miRNA) biogenesis, as it cleaves pre-miRNAs into mature miRNAs. miRNAs are important regulators of gene expression. In recent years, several miRNA-independent roles of Dicer1 have been identified. They include the production of endogenous small interfering RNAs, detoxifying retrotransposon-derived transcripts, and binding to new targets; messenger RNAs and long noncoding RNAs. Further, in this review, the functional significance of Dicer1 in the male reproductive tract is discussed. Conditional Dicer1 knock-out mouse models have demonstrated a requisite role for Dicer in male fertility. Deletion of Dicer1 from somatic or germ cells in the testis cause spermatogenic problems rendering male mice infertile. The lack of Dicer1 in the proximal epididymis causes dedifferentiation of the epithelium, with unbalanced sex steroid receptor expression, defects in epithelial lipid homeostasis, and subsequent male infertility. In addition, Dicer1 ablation from the prostate leads to increased apoptosis of the differentiated luminal cells, followed by epithelial hypotrophy of the ventral prostate. However, further studies are needed to clarify which functions of Dicer1 are responsible for the observed phenotypes in the male reproductive tract.

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DICER1 FUNCTIONS

MicroRNA biogenesis
Dicer1 was identified in 2001 as a central RNase III enzyme processing pre-miRNAs to mature microRNAs (miRNAs).1 Since then the canonical miRNA biogenesis pathway has been revealed. miRNAs are transcribed by RNA Polymerase II as long primary miRNA (pri-miRNA) transcripts. After transcription the nuclear double-stranded RNA-binding protein DiGeorge syndrome critical region 8 (DGCR8) directly interacts with pri-miRNAs and directs the cleavage by another RNase III enzyme, Drosha, which releases the individual pre-miRNAs by cleaving the strands at the base of the stem loops in the nucleus.2–4 Approximately 70-nt-long pre-miRNAs are exported to the cytoplasm by the nuclear export factor Exportin 5.5 In the cytoplasm, Dicer1 cleaves the loop structure, producing ≈ 22 nt-long mature miRNA.2–6 After cleavage, one miRNA strand and Argonaute (AGO) proteins are assembled into the RNA-induced silencing complex (RISC).7 miRNA targets the RISC complex to the 3’-untranslated region (UTR) of the target mRNA. Perfect complementarity of miRNA to the target site promotes cleavage of mRNA, whereas miRNAs having mismatch bulges generally result in translational repression.8

miRNA-independent RNA processing
Since the identification of the RNA interference pathway, a vast number of miRNA has been identified and to date close to 2000 mature miRNA sequences can be found in the miRBase databases for the mouse and human (http://www.mirbase.org). Many miRNAs are expressed in a tissue-, cell-specific or developmental stage-specific manner, thereby significantly contributing to cell-type-specific profiles of protein expression and different cellular functions. Although Dicer1 is required for miRNA production, studies within the last decade have clearly shown that this is not the only function of Dicer1, and the complexity of Dicer1 functions has started to be unraveled. The direct comparison of Dicer1-, Dgcr8- and Drosha-knock-out phenotypes have allowed identification of miRNA-dependent cellular functions. Those comparisons also demonstrated miRNA-independent roles for Dicer1 and Drosha, and to date it is known that they have diverse functions. In addition to pre-miRNAs, Dicer1 is able to process other dsRNAs to produce endogenous small interfering RNAs (endo-siRNA) and viral siRNAs, while a truncated Dicer1 containing only one RNAse III domain has a DNAse capability and a role in DNA degradation and subsequent apoptosis.9 From the above-mentioned functions, only the role in producing endo-siRNAs has been demonstrated in higher eukaryotes. Endo-siRNAs are important regulators of gene expression, for example in mammalian oocytes.10–12 The RNA cleavage function of Dicer1 was recently shown to be important for detoxifying retrotransposon-derived transcripts such as Alu elements. Down-regulation of Dicer1 in the human retinal pigmented epithelium induces accumulation of Alu RNA, and this cytotoxic RNA causes epithelial degeneration resulting in an untreatable advanced form of age-related macular degeneration, Geographic atrophy.13 A conditional mouse model has confirmed an miRNA-independent role of Dicer1 in this process.15

Nuclear Dicer1
In lower organisms, Dicer1 has been shown to have a role in nuclear functions, such as heterochromatin formation and chromosome segregation.14 Growing evidence suggests that Dicer1 is also present in the mammalian nucleus and may have an miRNA-independent
role there. Two studies have localized Dicer1 in the nucleus. First, Dicer1 was observed in association with ribosomal DNA chromatin; second, by the use of fluorescent cross-correlation spectroscopy, EGFP-tagged Dicer1 was found in the nucleus of stably transfected cell lines. Moreover, Doyle et al. demonstrated a nuclear localization signal and nuclear export signal in the human Dicer1 protein, indicating an ability of Dicer1 to shuttle between the cytoplasm and nucleus. The significance of this finding is still unclear, as the full-length Dicer1 is unable to enter the nucleus and gains this ability only after deletion of the helicase domain. Indirect support for nuclear localization and functions have arisen from Dicer1 knock-out studies, revealing for example, reduced silencing of centromeric repeats in mouse ES cells, defects in sister chromatid segregation during mitosis in a human-chicken hybrid cell line, and X-chromosomal inactivation, although this finding remains controversial. However, it is still unclear whether the above-mentioned phenotypes are caused by direct loss of Dicer1 or lack of miRNAs in the cytoplasm.

**Messenger RNA and long noncoding RNA as Dicer1 targets**

A recent study by Rybak-Wolf et al. demonstrated new binding targets for Dicer1. In addition to miRNAs and structural, promoter and mitochondrial RNA, Dicer1 was shown to bind to mRNAs and long noncoding (Inc) RNAs. Interestingly, it was discovered that in a majority of cases, Dicer1 binding to mRNAs and IncRNAs did not result in cleavage of the targets. Those binding sites were thus referred to as “passive” sites. Binding to passive sites seems to stabilize the targets and sequester Dicer1 and thus reduce miRNA levels. Furthermore, comparison of passive Dicer1 targets and genes required for RNA granule formation showed that a significant proportion of passive target transcripts was those found in RNA granules. In contrast, active targets were not associated with RNA granules. This, along with previous data indicating Dicer1 localization in germ granules in *C. elegans* and the chromatin body in mice, suggests a possibility that Dicer1 is involved in RNA localization in RNA granules. Further studies are needed to shed light onto this putative role of Dicer1.

Given the importance of Dicer1 in many cellular functions it is not surprising that the full knock-out of the gene is embryonically lethal. Mouse embryos lacking Dicer1 die at embryonic day (E) 7.5, presumably due to the stem cell loss observed in knock-out embryos. To be able to study the role of Dicer1 later during development and in adult tissues, conditional mouse models with a floxed RNAse III domain have been crossed with tissue- or cell-specific Cre-recombinase mouse lines. Those studies have demonstrated the role of Dicer1, of varying magnitude, from morphogenesis of several tissues to dysregulation of gene expression in adult tissues.

The importance of Dicer1 in male reproductive tissues has been indicated by tissue-, segment- and cell type-specific and age-dependent miRNA expression. Next we review the relevance of Dicer1 in the male reproductive organs, revealed by studying the conditional Dicer1 knock-out mouse models (Table 1).

### TISSUE-SPECIFIC DELETION OF DICER1 IN THE MALE REPRODUCTIVE TRACT

#### The testis

The necessity of Dicer1 in spermatogenesis has been shown using several conditional knock-out mouse models. Deletion of Dicer1 from testicular somatic cell progenitors in the adreno-gonadal primordium at E10 by Steroidogenic factor 1 (Sf1)-Cre did not affect fetal testicular development. However, shortly before birth, Sertoli and Leydig cells underwent rapidly progressing apoptosis leading to the degeneration of the most testicular cords and somatic cells at postnatal (P) day 5. Furthermore, Dicer1 deletion in primordial germ cells around E10 using Tissue-nonspecific alkaline phosphatase (*Tnap*)-Cre cause a proliferation defect in primordial germ cells and postnatal defects of spermatogenesis.

Sertoli cell-specific Dicer1 deletion by anti-Müllerian hormone (*Amh*, previously also known as Müllerian inhibiting substance, Mis)-Cre at E14.5 leads to concurrent Sertoli cell proliferation and apoptosis after birth. Further, both the testicular transcriptome and proteome change markedly. The impaired Sertoli cell maturation and function result in subsequent loss of germ cells, testicular degeneration and infertility in *Amh*-CreDicer1 mice. The phenotypic severity of germ cell-specific Dicer1 models varies with the onset and cell type of Dicer1 recombination. Dicer1 ablation from spermatogonia at E18 using DEAD (Asp-Glu-Ala-Asp) box polypeptide 4 (*Ddx4*)-driven Cre, causes several cumulative defects leading to male infertility. The first meiotic division is affected; in Prophase I the transition from leptotene to zygotene/pachytene is delayed, and apoptosis of pachytene spermatocytes is significantly increased. Moreover, the few round spermatids that escape apoptosis do not develop normally, as spermiogenesis is also impaired in *Ddx4*-Cre;Dicer1 mice. Spermatids are present with multiple defects; fragmented acrosomes, abnormal shapes of the head and mitochondria and chromatin condensation. Interestingly, the expression of transposable elements of the short interspersed nuclear element (SINE) family is up-regulated in *Ddx4*-Cre;Dicer1 spermatocytes as found in mouse oocytes. In these cells, it was suggested that transposon

| Table 1: Conditional Dicer1 mouse models in the male reproductive tract |
|--------------------------|----------|----------------|-----------------|
| Mouse model              | Cre mediated Dicer1 recombination |
| Age                      | Cell type                  | Main defects                          | References |
| Sf1-Cre;Dicer1<sup>fl/fl</sup> | E10                  | Primordial somatic cell progenitors    | Sertoli and Leydig cell apoptosis | 32 |
| Tnap-Cre;Dicer1<sup>fl/fl</sup> | E10                  | PGCs                                  | PGC proliferation                | 33,34 |
| Amh-Cre;Dicer1<sup>fl/fl</sup> | E14.5          | Sertoli cells                          | Delay in Sertoli cell maturation, apoptosis | 35,37 |
| Ddx4-Cre;Dicer1<sup>fl/fl</sup> | E18          | Spermatogonia                          | Severe defects in meiosis and spermiogenesis | 38 |
| Stra8-Cre;Dicer1<sup>fl/fl</sup> | E14.5          | Meiosis, spermiogenesis                | Spermiogenesis; abnormal head, tail and chromatin condensation | 39,40 |
| Ngn3-Cre;Dicer1<sup>fl/fl</sup> | E14.5          | Spermatogonia                          | Spermiogenesis; abnormal head | 41 |
| Prm1-Cre;Dicer1<sup>fl/fl</sup> | E18          | Round spermatids                       | Spermiogenesis; abnormal head | 42 |
| HoxB7-Cre;Dicer1<sup>fl/fl</sup> | E11.5         | Wolffian duct                          | No apparent defects              | 47 |
| Defb41-Cre;Dicer1<sup>fl/fl</sup> | E12          | Epididymal principal cells             | Epithelial dedifferentiation, abnormal lipid homeostasis, sperm maturation defects | 49 |
| ARR-PB-Cre;Dicer1<sup>fl/fl</sup> | E14–21       | Prostatic luminal and basal cells      | Apoptosis, epithelial hypotrophy | 28 |

PGCs: primordial germ cells.
repeat-derived small RNAs regulate mRNAs in a Dicer1-dependent manner. On the other hand, in the human retinal pigmented epithelium, accumulation of Alu-transposable elements causes epithelial degeneration and cell death. Whether the accumulation of SINE elements in spermatocytes causes the observed increase in apoptosis remains to be explored.

Later deletion of Dicer1 in spermatogonia around P4-10, stimulated by retinoic acid gene 8 (Strahl- or Neurogenin3 (Ngn3)-driven Cre, results in less severe phenotypes. In the Ngn3-Cre;Dicer1 fl/fl mice, meiosis progresses normally; however, an increased number of apoptotic spermatocytes is detected. Spermiogenesis, instead, is heavily affected. Spermatozoan have an abnormal head shape and chromatin condensation, as well as a disrupted tail structure. In contrast to that in Defb41-Cre; Dcr1 fl/fl mice, expression of transposable elements is not up-regulated. Instead, induced transcription of centromeric major repeats is found. Effects in spermiogenesis are similar in Strahl-Cre;Dicer1 fl/fl mice, but in contrast to Ngn3-Cre;Dicer1 fl/fl, the Strahl-Cre-driven Dicer1 deletion seem to display a partial delay in meiosis. This could be due to slightly earlier deletion of Dicer1 from spermatogonia in the Strahl-Cre mouse model than in the Ngn3-Cre model. In addition, over-expression of genes located on sex chromosomes, including most known targets of meiotic sex chromosome inactivation (MSCI), was found in one of the published Strahl-Cre;Dicer1 fl/fl mouse models. On the contrary, in the other published Strahl-Cre;Dicer1 fl/fl paper, the authors failed to show any effect on MSCI. Thus, it seems likely that the observed over-expression actually reflects the higher number of pre-MSCI spermatocytes present in the whole testicular samples.

Even milder phenotypic changes are seen in Protamine 1 (Prtm1)-Cre; Dicer1 fl/fl tests, where deletion at P18 in round spermatids result in male subfertility, due to the increased apoptosis of round spermatids, acrosomal defects, improper histon compaction and head shape abnormalities. Furthermore, it has been suggested that Dicer1 and miRNAs are required for proper control of translation via actin-associated protein-mediated regulation in differentiating germ cells.

The testis is the only male reproductive organ from which comparative analyses of Dicer1, Drosha and Dcr8 have been performed, allowing estimation of whether the phenotypes obtained are due to changed miRNA or endo-siRNA levels. In both of the models, Ddx4-Cre;Dgcr8 fl/fl and Strahl-Cre;Dgcr8 fl/fl, the phenotype was reported to be similar to Dicer1 deletions, with delay in meiosis, increased apoptosis and impairment of spermiogenesis. However, all the defects were found to be milder in animals lacking Dgcr8 than in the Dicer1 knock-out, indicating that also endo-siRNAs have a role, although a milder one, in spermatogenesis.

Moreover, this result suggests that the meiotic defect is not due to a direct lack of Dicer but rather the lack of miRNA/endo-siRNA biogenesis. In contrast, spermatogenesis was reported to be more severely affected in Strahl-Cre;Dgcr8 fl/fl males than in Strahl-Cre;Dicer1 fl/fl males. This discrepancy can most likely be explained by the small RNA-independent functions of Drosha, i.e., mRNA cleavage and RNA cleavage-independent regulation of gene expression. Interestingly, in the ovary, Dicer1, but not Dcr8, is necessary for spindle formation and chromosomal alignment in meiosis I, suggesting that endo-siRNAs rather than miRNAs are essential for oocyte maturation. This intriguing difference in the control of meiotic processes during spermatogenesis versus oogenesis requires further studies.

The epididymis

Dicer1 does not seem to be required for the proper embryonal development of the epididymis. Homeobox B7 (HoxB7)-Cre mediated deletion of Dicer1 from the Wolffian duct at E11.5 does not cause any phenotypic changes in the epididymis. This might be due to low recombination levels in the epididymis when HoxB7-Cre is used (unpublished information, P Sipilä). However, another research group has generated HoxB7-Cre;Dicer1 fl/fl mice, where one Dicer1 allele is deleted and thus mice have only one conditional allele to be recombined by Cre recombinase. These animals had a stronger kidney phenotype than was reported by Pastorelli et al., which indeed suggests higher levels of Dicer1 deletion. Nevertheless, at the gross anatomical level the epididymes seemed to be normal in HoxB7-Cre;Dicer1 fl/fl mice at E18.5 (personal communication from Prof. B. Hinton).

The final differentiation of the epididymis takes place during puberty and in contrast to embryonal development, requires Dicer1. In the mice from which Dicer1 is deleted prepubertally from the proximal epididymis using a Defensin beta 41 (Defb41)-iCre knock-in mouse line, the differentiation of the initial segment (IS) and caput begin normally. At the age of 33 days, the IS is distinguishable from the caput by its high, columnar-shaped epithelial cells. However, by the age of 45 days the epithelium had regressed to resemble an undifferentiated epithelium. In adults, the dedifferentiation of the IS is clearly seen by the lack of intense vascularization typical of the IS and lack of endogenous β-galactosidase activity. The Defb41-iCre;Dicer1 fl/fl epididymes were also significantly smaller than the control epididymes. Surprisingly, despite the smaller size, the Defb41-iCre;Dicer1 fl/fl IS and caput epididymes presented with a significant increase in the number of proliferative cells; especially in the caput, which showed 6 times more proliferative cells than the control caput. Moreover, the number of apoptotic cells was also slightly increased in the Defb41-iCre;Dicer1 fl/fl epididymis, demonstrating a concurrent cellular proliferation and apoptosis.

The phenotype of the adult mice epithelium varies from a highly disorganized cell layer and tubules with small diameter, to an IS and caput that resembled the thin epithelial layer of corpus with an increased tubular diameter. A common feature of all the Defb41-iCre; Dicer1 fl/fl IS was a significant reduction in the height of the epithelium. In the Defb41-iCre;Dicer1 fl/fl epididymes, all the epithelial cell types were present, however, the layer of smooth muscle cells surrounding the duct was increased from one cell layer to three cell layers indicating a cell-cell crosstalk between epithelial and muscle cells. Even though all the epithelial cell types were present, the segment-specific gene expression pattern was disturbed. The expression of several IS-specific genes was down-regulated showing a lack of segment identity in the Defb41-iCre;Dicer1 fl/fl epididymes.

Epididymal development, tissue homeostasis and functions are highly dependent on androgens. Analyses of sex steroid receptors in Defb41-iCre;Dicer1 fl/fl epididymes showed down-regulation of the androgen receptor (Ar) and estrogen receptor 2 (Esr2), whereas the estrogen receptor (Esr1) was expressed in all epithelial cells of the IS, in contrast to narrow cell-specific expression in the controls. Esr1 is known to promote cell proliferation, thus high Esr1 levels in the Defb41-iCre;Dicer1 fl/fl IS could explain the observed increase in cell proliferation in that epididymal segment. Androgen and estrogen receptors are known targets of miRNAs, and they in turn regulate miRNA expression, creating a complex regulatory network for fine-tuning gene expression. It seems that in many cases the optimal balance between androgens and estrogens is more important for the cellular functions than the exact levels of individual hormones. Thus, the imbalance in sex steroid receptors due to lack of mature miRNAs in the Defb41-iCre;Dicer1 fl/fl epididymes could be at least a partial reason for the observed epithelial phenotype.
The changes in the epididymal epithelium of Defb41-iCre;Dicer1fl/fl males causes infertility,59 thus resembling the Ros1 knock-out and the GPX5-Tag2 transgenic mouse models, which also lack a fully developed IS.60,61 However, the sperm phenotype of these previous mouse models was mainly caused by a failure of sperm cells to regulate the cell volume, which led to hairpin formation of the sperm flagellum with no reduction in motility or sperm membrane fluidity.52 The Defb41-iCre;Dicer1fl/fl mice on the other hand, present with predominantly immotile spermatozoa, which display an increased breakage of the neck and acrosome region. These are indicators of sperm membrane instability that could be caused by an altered lipid constitution of the membrane. Indeed, it has been demonstrated that lipid homeostasis in the Defb41-iCre;Dicer1fl/fl epididymal epithelium is altered with a significant reduction in the gene expression of the elongation of very long chain fatty acids-like 2 (Elov2), an enzyme needed for production of long-chain polyunsaturated fatty acids (PUFAs), and the up-regulation of several genes in cholesterol synthesis and transportation pathways.59 This is reflected in the lipid constitution of the Defb41-iCre; Dicer1fl/fl sperm membrane, where the levels of long-chain PUFAs are decreased and cholesterol increased, resulting in an increased cholesterol:PUFA ratio – likely to be the cause of the immobility and breakage of the Defb41-iCre;Dicer1fl/fl sperm cells.59 These results, along with the growing number of miRNAs shown to target genes involved, for example in cholesterol synthesis and transport,61,64 suggest that miRNAs are important regulators of lipid homeostasis. Indeed, many miRNAs show promise as therapeutic targets for treating dyslipidaemias and cardiovascular diseases.

**The prostate**

The role of Dicer1 in early prostatic development has not been studied. A composite probasin (Pbsn, also termed PB) promoter (ARR, PB, containing two distinct AR-binding sites linked to the endogenous PB promoter sequence from – 244 to + 28 bp) drives Cre expression in luminal and basal cells of the prostatic epithelium. The expression starts at the age of 2–3 weeks, when prostate cell lineage commitment and glandular branching morphogenesis have largely been established. Deletion of Dicer1 with ARR, PB-Cre results in smaller prostates at the age of 2 months. By the age of 4 months, ARR, PB-Cre;Dicer1fl/fl mice have developed epithelial hypotrophy in the ventral prostates with concurrent increase in both apoptotic and proliferating epithelial cells.62 For the two epithelial cell types, apoptosis is more pronounced in the differentiated luminal cells, whereas proliferation is observed in both luminal and basal cells. Furthermore, the lack of Dicer1 affect the sphere-forming capacity of prostatic stem cells, indicating a defect in the proliferation of the cells lacking Dicer1. It has been suggested that differentiated luminal cells are more dependent on miRNA-mediated regulation than basal or stem cells and that the proliferation seen in the epithelial cells could be due to an intrinsic tissue repair response of the cells where Cre-mediated recombination of Dicer1 had not occurred.63 Interestingly, concurrent increase in cell proliferation and apoptosis has also been demonstrated in testicular Sertoli cells and epididymal epithelial cells lacking Dicer1. In those models, the increased proliferation was thought to be linked to delayed maturation or dedifferentiation, respectively, of the cell type modified.60,62

Deregulation or mutations of Dicer1 are observed in a wide variety of cancers, and the outcome depends on dosage effects and tissue- and cell-type-specific contexts.65 In human prostatic cancers, up-regulation of Dicer1 has been reported.66,67 In the in vivo mouse prostate cancer model and in prostatic cancer cell lines in vitro, the lack of Dicer1 is found to reduce the tumor or cell growth significantly. However, hemizygous or knock-down of Dicer1 causes enhanced invasiveness in the mouse prostatic cancer model and in some of the cancer cell lines studied.68

**The seminal vesicle and vas deferens**

The role of Dicer1 in the development and function of the seminal vesicles and vas deferens has not been studied extensively. However, deletion of Dicer1 using HoxB7-Cre demonstrated that Dicer1 is not absolutely necessary for the development of those organs.50 Nevertheless, in both studies also showing Dicer1 deletion in the seminal vesicle (Pastorelli et al.69 and Zhang et al.71) the results indicated that there were phenotypic changes in the seminal vesicles as well. In case of HoxB7-Cre deletion, the authors reported hypoplastic seminal vesicles with low penetrance.50 Moreover, ARR, PB-Cre; Dicer1fl/fl mice were reported to have lower total urogenital organ weight (prostate, seminal vesicles, bladder and urethra) than controls. As the smaller prostatic weight alone could not account for the weight difference of the total organs, the seminal vesicles may have been smaller, too. This assumption is further supported by the published Figure, which depicts a seemingly smaller seminal vesicle.71 In both of the above-mentioned studies, Dicer1 was also deleted from the vas deferens. Nevertheless, no phenotypic changes of the vas deferens were reported. Further studies are needed to clarify the role of Dicer1 in these two organs.

**CONCLUSIONS AND FUTURE PERSPECTIVES**

Conditional deletions of the mouse Dicer1 have revealed its necessity for normal development and function of the male reproductive tract. However, our understanding on the role of different Dicer1 functions in the male reproductive tract is still superficial. More specific models are needed in order to allocate observed phenotypes in our mouse models to miRNA-dependent and miRNA-independent roles of Dicer1. Controversial results of declining sperm quality among young men in industrialized countries have demonstrated the demand to understand better normal sperm production and maturation. Thus, we are in urgent need of basic research on pathways regulating those vital processes. Identification of novel molecules and pathways necessary for male fertility could provide tools for the development of diagnostic biomarkers for male idiopathic infertility, and on the other hand, novel male contraceptive targets.

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**COMPETING FINANCIAL INTERESTS**

The authors disclose no financial conflicts of interests.

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