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Review

The in vivo role of α-mannosidase IIx and its role in processing of *N*-glycans in spermatogenesis

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Abstract. The surfaces of mammalian cells are covered by a variety of carbohydrates linked to proteins and lipids. *N*-glycans are commonly found carbohydrates in plasma membrane proteins. The structure and biosynthetic pathway of *N*-glycans have been analyzed extensively. However, functional analysis of cell surface *N*-glycans is just under way with recent studies of targeted disruption of genes involved in *N*-glycan synthesis. This review briefly introduces the potential role of processing α-mannosidases in *N*-glycan biosynthesis and recent findings derived from the α-mannosidase IIx (MX) gene knockout mouse, which shows male infertility. Thus, the MX gene knockout experiment unveiled a novel function of specific *N*-glycan, which is *N*-acetylglucosamine-terminated and fucosylated triantennary structure, in the adhesion between germ cells and Sertoli cells. Analysis of the MX gene knockout mouse is a good example of a multidisciplinary approach leading to a novel discovery in the emerging field of glycobiology.

Key words. Spermatogenesis; *N*-glycan; mannosidase; lectin; gene targeting; mutation; cell adhesion.

Introduction

Reproduction allows organisms to maintain their own species by passing genetic information from one generation to the next. Sexual reproduction may have an advantage over asexual reproduction, as this method enables genetic information to be passed through male and female gametes, thus providing offspring with increasing variation and tolerance against environmental pressure [1]. Spermatogenesis generates an actively mobile male germ cell to fertilize a female oocyte. Morphologically, the processes of spermatogenesis are similar to each other among different mammalian species [2].

In mammals, spermatogenesis takes place in seminiferous tubules in the testis. The spermatogenic stem cells or spermatogonia adhere to the inner wall of the seminiferous tubule. At a certain point, a spermatogonium enters meiosis and differentiates into sperm. During meiosis and differentiation processes, germ cells adhere to Sertoli cells, long epithelial cells stemming from the basement membrane of the inner face of the seminiferous tubule [3]. Thus Sertoli cells and germ cells interact with each other through direct cell-to-cell contact and also indirectly through a paracrine mechanism by secreting factors. During differentiation, germ cells move from the basal position to the apical region of Sertoli cells. Matured germ cells or spermatozoa reach the apical end of the Sertoli cell and then move from the testis to the epididymis, where spermatozoa undergo further maturation [4]. Adhesion between germ cells and Sertoli cells has been studied in many laboratories, leading to suggestions of various cell adhesion molecules [2, 3, 5–9]. However, the molecular mechanisms underlying this adhesive interaction remained largely speculative until now.

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Many studies suggested the importance of carbohydrates in spermatogenesis [10–13]. Histochemical analyses of testis using various lectins, each of which recognizes a specific carbohydrate structure, show remarkable staining patterns associated with developmental stages of spermatogenic cells [14–17]. Thus in each mammalian species, alteration of carbohydrate structures is associated with differentiation of spermatogenic cells, which has been supported by structural analysis of glycoprotein carbohydrates [18, 19]. Biochemical analyses of testis carbohydrates show the presence of unusual glycoconjugates in this organ. For example, seminolipid is a sulfated glycolipid uniquely found in testis and brain [20, 21]. Two types of mutant mice were generated, lacking either the galactosyltransferase or sulfotransferase involved in the biosynthesis of seminolipid. Both mutant mice showed male infertility due to severe spermatogenesis failure associated with elevated apoptosis [22, 23]. In addition, mutant mice lacking one of sialyltransferases involved in the synthesis of gangliosides or sialylated glycolipids showed male infertility. Analysis of this mutant revealed that gangliosides are essential for nonspermatogenic Leydig cells to effectively transport testosterone [24].

Biosynthesis of *N*-glycan carbohydrates in mammalian cells

Majority of proteins synthesized by the sorting pathway in mammalian cells is glycosylated. Two major glycosylations are known, *N*-glycosylation and *O*-glycosylation. *O*-glycans are synthesized by each glycosyltransferase step by step, and participate in a variety of functions, including the inflammatory response and lymphocyte homing [25, 26]. *N*-glycans are synthesized through a complex mechanism that includes processing high mannose oligosaccharides (fig. 1). Thus, biosynthesis of *N*-glycans includes 'en-block' transfer of oligosaccharides made of glucose (Glc), mannose (Man) and *N*-acetylglucosamine (GlcNAc) in the endoplasmic reticulum (ER) from a dolichol-lipid donor to an asparagine residue of a protein acceptor [27]. A high mannose type *N*-glycan thus formed is processed by glucosidases and mannosidases to $Man_5GlcNAc_2$, which is modified further to the so-called hybrid type and then to complex type oligosaccharides (fig. 1).

The importance of *N*-glycans in mammals in vivo was demonstrated by the *N*-acetylglucosaminyltransferase-I (GnT-I) gene knockout mouse [28, 29]. Mouse embryos lacking GnT-I die at E9.5~10.5, showing a failure in neural tube formation, vascular system malformation and lack of bronchial epithelium. Since GnT-I null mice produce high-mannose-type but not complex-type *N*-glycans, this result demonstrated that high-mannose-type *N*-glycans alone cannot support mouse embryonic development beyond E9.5 and 10.5. Mutant mouse embryos lacking *N*-acetylglucosaminyltransferase-II (GnT-II) are able to synthesize hybrid-type but not complex-type *N*-glycans, and survived through all stages of embryonic development [30]. However, GnT-II null mice showed postnatal lethality and exhibited aberrant phenotypes, includ-

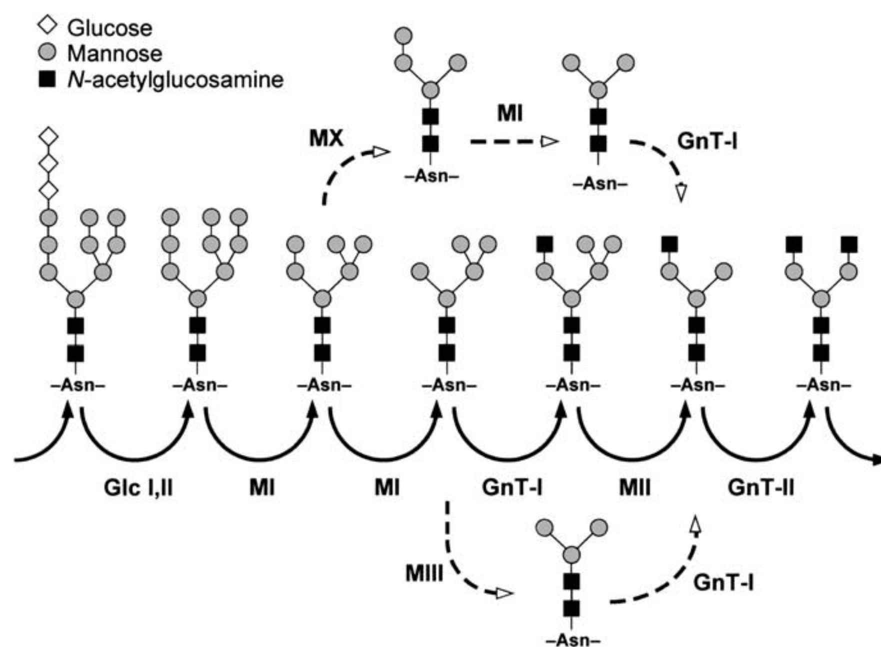

Figure 1. Steps of *N*-glycan processing. The synthesis of *N*-glycans initiates from the so-called high-mannose-type oligosaccharide, which is then trimmed by the processing glucosidases and mannosidases. This figure illustrates the reactions surrounding MII, including MX [34, 35] and putative enzyme MIII [31].

ing growth retardation, gastrointestinal dysfunction, impaired bone development, anemia and leukocyte deficiencies, and locomotor dysfunction.

Although the MII step occurs between GnT-I and -II (see fig. 1), the MII gene (*Man2a1*) knockout mouse shows mild phenotypes in homozygotes attributable to dyserythropoiesis, a condition similar to the human genetic disease congenital dyserythropoietic anemia type II (CDAII) [31]. Structural analysis of *N*-glycans from MII null mouse tissues indicates the presence of small amounts of complex-type *N*-glycans, the products downstream of MII step. This observation indicated the existence of an alternate pathway for detouring defective MII. In MII null liver tissue, an α-mannosidase activity hydrolyzing $Man_5GlcNAc_2$ oligosaccharide to produce $Man_3GlcNAc_2$ in a cobalt ion-dependent manner was found (fig. 1). This enzyme, termed MIII, is thought to be responsible for that alternate pathway [31]. Insect MIII has been cloned [32], but mammalian MIII has not yet been cloned. It remains unknown whether a putative mammalian homologue of MIII is responsible for an alternate pathway.

Previously we identified a human gene encoding an α-mannosidase highly homologous to MII, and named it α-mannosidase IIx (MX) [33]. Overexpression experiments of MX suggest that MX hydrolyzes $Man_6GlcNAc_2$ to $Man_4GlcNAc_2$ (fig. 1) [34]. However, the substrate specificity of MX has not been defined unambiguously by in vitro experiments due to its weak enzymatic activity [34]. Thus MX may be active only in the Golgi membrane in cells. Nonetheless, MX is a candidate α-mannosidase for the alternative pathway described above (fig. 1).

Phenotypes of MX null mutant mice

To determine the role of MX in vivo, we disrupted *Man2a2*, the gene encoding MX, in embryonic stem (ES) cells by targeted mutation [35]. A homologous recombinant ES clone was used to generate chimeric mice, which were crossed to 129/SVJ mice, and heterozygotes were crossed to produce homozygous mutant offspring. Genotyping of pups born from $MX^{+/-}$ male and female crosses revealed unusual proportions; $MX^{+/+}$: $MX^{+/-}$: $MX^{-/-}$, a 1.0 : 1.13 : 0.37, but not 1.0 : 2.0 : 1.0 ratio. Thus the proportion of $MX^{+/-}$ pups was significantly lower than the expected numbers by Mendelian rule, while some $MX^{-/-}$ pups were also born. $MX^{-/-}$ pups survived to adulthood with no apparent illness or behavioral anomaly. $MX^{-/-}$ females were fertile. However, $MX^{-/-}$ males were nearly infertile.

When $MX^{+/-}$ females were crossed with $MX^{+/+}$ males, equal numbers of $MX^{+/+}$ and $MX^{+/-}$ pups were born. However, when $MX^{+/+}$ females were crossed with $MX^{+/-}$ males, more $MX^{+/+}$ than $MX^{+/-}$ pups were produced. Such results suggested a deficiency in spermatogenic cells produced by $MX^{+/-}$ males.

As noted above, the surviving $MX^{-/-}$ mice showed no gross abnormalities. However, their testes were smaller than those of $MX^{+/+}$ or $MX^{+/-}$ littermates. Electron microscopy shows prominent intercellular spaces surround $MX^{-/-}$ spermatocytes (fig. 2, right panel), while spermatocytes of $MX^{+/+}$ mice are closely packed within the seminiferous tubule (fig. 2, left panel). This suggests a failure of germ cell adhesion to Sertoli cells within the seminiferous tubules. In addition, the caudal ductus epididymis from the $MX^{+/+}$ mouse was packed densely with mature spermatozoa, while that from the $MX^{-/-}$ mouse showed significantly reduced numbers of spermatozoa. Collectively, it appears that in $MX^{-/-}$ mouse testis, spermatogenesis is impaired due to the premature release of developing germ cells from the seminiferous epithelium to the epididymis.

Role of *N*-glycans in spermatogenesis

Quantitative structural analyses of *N*-glycans from $MX^{+/+}$ and $MX^{-/-}$ mouse testis were carried out using a two-dimensional mapping technique [36, 37]. These analyses showed that $MX^{+/+}$ testes contain significant amounts of GlcNAc-terminated complex type *N*-glycans, a result consistent with strong staining with *Grifornia simplicifolia* agglutinin II (GSAII), which recognizes GlcNAc-terminated carbohydrates [14, 35]. In $MX^{-/-}$ testes, the levels of GlcNAc-terminated oligosaccharides are reduced [35]. These findings suggest that a distinct set of glyco-

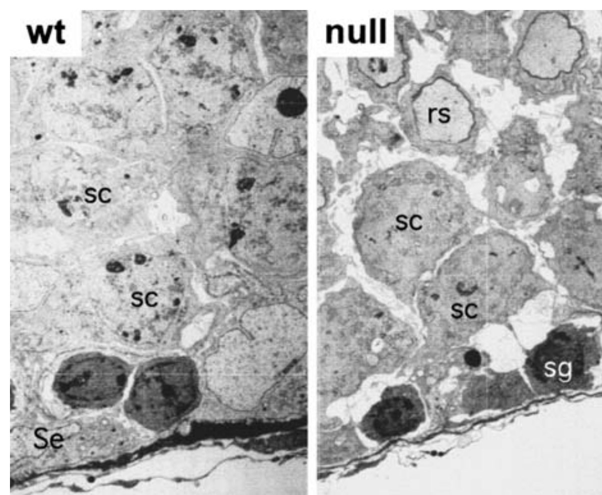

Figure 2. Electron micrographs of wild-type and MX null mutant mouse testes. Note that MX null spermatogenic cells are sparsely distributed inside the seminiferous tubules. sg, spermatogonia; sc, spermatocytes; rs, round spermatids; Se, Sertoli cells (modified from [35]).

proteins is synthesized through an MX-dependent pathway in spermatogenic cells. The mechanism underlying such differential processing is totally unknown.

An in vitro assay for adhesion of spermatogenic cells to Sertoli cells [6, 38] was carried out to determine the role of *N*-glycans on germ cell-Sertoli cell adhesion. Thus each purified and structurally defined mouse testis *N*-glycan oligosaccharide was tested for potential inhibition of germ cell adhesion to Sertoli cells. This analysis indicated that one of the unique *N*-glycans, GlcNAc-terminated triantennary and fucosylated *N*-glycan, is the critical structure required for the germ cell and Sertoli cell interaction (fig. 3). This conclusion is consistent with the biochemical analysis of $MX^{-/-}$ testis *N*-glycans, which showed a significant reduction of this key *N*-glycan structure. Collectively, MX null spermatogenic cells fail to adhere to Sertoli cells in seminiferous tubules, and they are prematurely released from the testis to the epididymis. Thus the failure of spermatogenesis in MX null mutant mice is characterized as hypospermatogenesis [35].

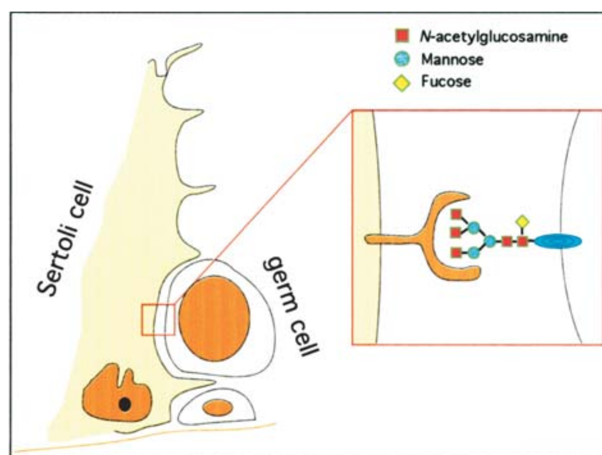

Figure 3. Schematic diagram of germ cell and Sertoli cell interaction through specific *N*-glycan. Structural analysis of *N*-glycans, and in vitro adhesion assays identifying critical *N*-glycan structure required for germ cell survival in spermatogenesis (modified from [46]).

Related work and future perspectives

The importance of α-mannosidases in spermatogenesis and fertilization has been suggested for some time, as farm animals grazing on locoweed develop male infertility [39], and the major 'loco-toxin' swainsonine is an α-mannosidase inhibitor [40]. Immunohistochemistry of *N*-glycan processing α-mannosidases in rat testis suggest that each processing α-mannosidase is expressed in a cell-type-specific manner [11]. Although *N*-glycan structures expressed on the surface of spermatogenic cells may vary significantly among mammalian species [14–16, 41, 42], the findings obtained by targeted disruption of the MX gene in the mouse have provided us with a model for analyzing the in vivo role of *N*-glycans in spermatogenesis in mammals.

Future studies should address identifying and characterizing the glycoprotein(s) carrying *N*-glycans that are synthesized by MX-dependent pathway [35]. Second, identification of the Sertoli cell receptor recognizing germ cell *N*-glycans is necessary, in order to analyze this cellular interaction at the molecular level. Finally, it should be determined whether this adhesive interaction through *N*-glycans affects cellular differentiation and oncogenic transformation of spermatogenic cells. Modifications of *N*-glycans are closely related to cancer malignancy and metastasis [43–45], indicating the importance of *N*-glycans in normal cellular functions in many cell types. As the specific *N*-glycan plays an important role in normal development of germ cells (fig. 3), alteration of *N*-glycans in spermatogenic cells potentially causes oncogenic transformation and malignancy of testicular germ cell tumors.

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