Univerzita Karlova
1. lékařská fakulta

Autoreferát disertační práce/PhD Thesis Summary

UNIVERZITA KARLOVA
1. lékařská fakulta

Příprava a charakterizace myší s vyřazeným genem pro glutamátkarboxypeptidasu II

Generation and Characterization of Glutamate Carboxypeptidase II (GCPII)-Deficient Mice

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Disertační práce bude nejméně pět pracovních dnů před konáním obhajoby zveřejněna k nahlížení veřejnosti v tištěné podobě na Oddělení pro vědeckou činnost a zahraniční styky Děkanátu 1. lékařské fakulty.

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ABSTRACT

Glutamate carboxypeptidase II (GCPII) is a transmembrane glycoprotein, which consists of short intracellular and transmembrane domains, and a large extracellular domain possessing carboxypeptidase activity. In the human body, GCPII fulfils a neuromodulatory function in the brain and facilitates folate absorption in the small intestine. In addition to the brain and small intestine, high level of GCPII is also present in the prostate and kidney. However, GCPII function in these tissues has not been determined yet. To study the role of GCPII in detail, several research groups attempted to inactivate GCPII encoding gene *Folh1* in mice. Surprisingly, the experiments led to rather conflicting results ranging from embryonic lethality to generation of viable GCPII-deficient mice without any obvious phenotype. This dissertation project aimed to dissect the discrepancy using alternative strategy for gene modification.

For this purpose, we designed TALENs that specifically targeted exon 11 of *Folh1* gene and manipulated mouse zygotes of C57BL/6NCrl genetic background. We analysed all genetically modified mice of F0 generation for presence of TALEN-mediated mutations and established 5 different GCPII-mutant mouse colonies from founder mice that altogether carried 2 frame-shift mutations and 3 small in-frame deletions. We thoroughly characterized all 5 colonies and found out that GCPII is not expressed in any of mice homozygous for *Folh1*-mutant variant. We were thus able to generate viable GCPII-deficient mice that breed normally and do not show any overt phenotype.

Produced GCPII-deficient mice were utilized for investigation of potential role of GCPII in the urogenital system. It was revealed that aged GCPII-deficient mice possess increased propensity to enlarged seminal vesicles, though the origin of this dilation is yet to be determined. In contrast, kidneys from aged GCPII-deficient mice did not display any pathological abnormalities and targeted metabolomics of mouse urine showed only 3 out of 193 measured metabolites discriminating GCPII-deficient and wild type mice.

In addition to dissecting the discrepancy found in the literature by showing that GCPII-deficient mice are viable, this dissertation project may set the direction for revealing the GCPII function in reproduction. Strikingly, supposed unimportance of GCPII for kidney function may open the door for GCPII-targeted anti-cancer treatment.

KEY WORDS: GCPII, PSMA, Folh1, GCPII-deficient mice, knockout mice, TALEN, enlarged seminal vesicles, urogenital system, kidney, urine metabolomics, NAAG, folate

ABSTRAKT

Glutamátkarboxypeptidasa II (GCPII) je transmembránový glykoprotein skládající se z krátké intracelulární a transmembránové domény a velké extracelulární domény, která vykazuje karboxypeptidasovou aktivitu. GCPII se podílí na neuromodulaci v mozku a absorpci folátů v tenkém střevě. Kromě těchto tkání se GCPII též vyskytuje v prostatě a ledvinách. Zde je ale její role neznámá. Za účelem zjištění funkce GCPII se několik vědeckých skupin pokusilo inaktivovat gen *Folh1* (kódující GCPII) v myši. Tyto experimenty však vedly k rozporuplným výsledkům. Zatímco dvě studie ukázaly, že inaktivace genu *Folh1* v myši vede k embryonální letalitě, jiné dvě studie publikovaly, že i po zmíněné inaktivaci jsou myši stále viabilní a navíc nevykazují žádný výrazný fenotyp. Cílem tohoto projektu bylo pokusit se připravit a charakterizovat myši s inaktivovaným *Folh1* genem pomocí alternativní metody genetické modifikace.

Za tímto účelem byly vytvořeny TALENy, které specificky cílily 11. exon genu *Folh1* v myších zygotách genetického pozadí C57BL/6NCrl. Narozená mláďata generace F0 byla analyzována pro přítomnost mutací v rámci genu *Folh1* a myši, které dohromady nesly 5 různých delecí, byly vybrány pro vytvoření mutantních linií. Linie, které nesly mutantní varianty genu *Folh1* na obou alelách, neobsahovaly detekovatelnou hladinu GCPII. Bylo potvrzeno, že myši s vyřazeným genem pro GCPII jsou viabilní, nemají reprodukční problémy a obecně nevykazují žádný výrazný fenotyp.

Modifikované myši byly použity pro zkoumání funkce GCPII v urogenitálním systému. Bylo zjištěno, že starší myši s vyřazeným genem pro GCPII mají zvýšený sklon k obstrukci semenných váčků. Zdroj tohoto fenotypu se ale nepodařilo objasnit. Ledviny starších modifikovaných myší nevykazovaly patologické změny a metabolomická analýza moči poukázala pouze na 3 ze 193 možných metabolitů, jejichž hladiny se u modifikovaných myší a myší divokého typu významně lišily.

Kromě prokázání, že myši s vyřazeným genem pro GCPII jsou viabilní, by tento disertační projekt mohl napomoci k odhalení potenciální role GCPII v reprodukci. Pravděpodobný omezený vliv GCPII na funkci ledvin by poté mohl být užitečný pro vývoj protirakovinových léků cílených na GCPII.

KLÍČOVÁ SLOVA: GCPII, PSMA, Folh1, myši s vyřazeným genem pro glutamátkarboxypeptidasu II, geneticky modifikované myši, TALEN, zvětšené semenné váčky, urogenitální systém, ledviny, metabolomická analýza moči, NAAG, folát

1. INTRODUCTION

1.1. Human and mouse GCPII protein

GCPII is type II transmembrane glycoprotein consisting of a short intracellular domain, a single membrane-spanning domain and a large extracellular domain.[1,2] Human GCPII (hGCPII) contains 750 amino acids[3] and shares 86% sequence identity and 91% sequence similarity with mouse GPCII (mGCPII) that contains 752 amino acids.[2,4]

Both proteins contain 10 potential N-linked glycosylation sites from which at least some are glycosylated. Indeed, while the molecular weight of GCPII was predicted from the amino acid sequence to be around 84 kDa, the molecular weight of GCPII determined experimentally is around 100 kDa.[2,5] In addition, high sequence identity of both proteins ensures that some monoclonal antibodies produced as tools for hGCPII detection could be also used in mGCPII research[6]. Especially, the epitope of the antibody GCP2-04 is highly conserved (see PhD Thesis, Figure 1, p. 14).[6,7]

Tertiary structure of extracellular portion of GCPII was resolved by protein crystallography for hGCPII (see Figure 1)[8,9] but not yet for mGCPII. Nevertheless, due to the high sequence similarity, it can be expected that mGCPII 3D structure closely resembles the 3D structure of hGCPII.

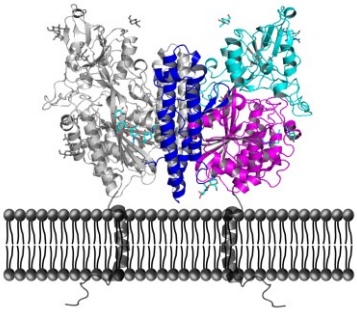

Figure 1: 3D structure of human GCPII. Crystal structure of extracellular part of human GCPII homodimer in cartoon representation (PDB identification code 2PVW) tethered to plasmatic membrane. One monomer is displayed in light grey; in the other monomer, individual domains are shown by distinct colours: the protease-like domain in pink, the apical domain in cyan, and the C-terminal domain (i.e. dimerization domain) in blue. Illustrative display of transmembrane and intracellular part of GCPII is coloured dark grey.

The extracellular part of human GCPII (amino acids 44-750) consists of three domains – the protease-like domain (amino acids 59-116 and 352-590), the apical domain (amino acids 117-351) and the C-terminal or the dimerization domain (amino acids 591-750) (see Figure 1).[8-10] Through the dimerization domain, the extracellular part of GCPII folds to a homodimer, which displays a pronounced overall fold similarity to the transferrin receptor.[9,11]

1.2. GCPII enzymology

As the name suggests, GCPII (EC 3.4.17.21) is a peptidase that hydrolyses its substrates at C-terminal glutamate. According to MEROPS database, GCPII belongs to a metallopeptidase subfamily M28B[12] since it requires 2 co-catalytic zinc cations for its activity.[13] The active site is conserved and harbours catalytic Glu424 (in hGCPII, Glu426 in mGCPII) and 2 zinc atoms.[9,13] The catalytic Glu424 is a general base in the catalytic mechanism[13]; it has been shown that a specific mutation E424A leads to complete loss of GCPII enzymatic activity.[14] The zinc atoms are coordinated by five residues – His377, Asp387, Glu425, Asp453 and His553 – which specific mutations result in severely reduced enzymatic activity.[13]

Due to the conservation of the active site, hGCPII and mGCPII possess similar substrate profiles. Indeed, both orthologues have been shown to process most of the N-acetylated dipeptides in a dipeptide library Ac-X-Glu-OH, where X is any proteinogenic amino acid.[4,15,16] With regard to the physiologically relevant substrates, both hGCPII and mGCPII hydrolyse *N*-acetyl-L-aspartyl-L-glutamate (NAAG) and poly-gamma-glutamylated folates ($FolGlu_n$).[2,17,18] While cleavage of NAAG yields *N*-acetyl-L-aspartate and L-glutamate, successive hydrolysis of $FolGlu_n$ eventually results in release of folate and several free L-glutamates (see Figure 2).[2,17,19]

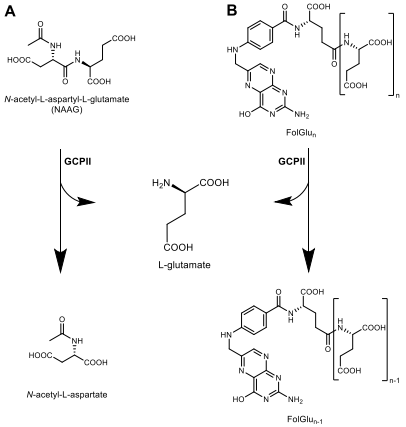

Figure 2: GCPII-catalysing hydrolysis of naturally occurring substrates. A: Catalysis of N-acetyl-L-aspartyl-L-glutamate (NAAG) cleavage. **B:** Catalysis of poly-gamma-glutamylated folates ($FolGlu_n$) hydrolysis.

1.3. Expression profile of human and mouse GCPII on the protein level

Over the years of GCPII research, wide variety of antibodies has been developed in order to examine expression profile of GCPII protein. In contrast, not many antibodies intended for mGCPII research are available. Due to the high sequence identity of both orthologues, some of the antibodies against hGCPII could be used for detection of mGCPII.[6] Nevertheless, while the data collected for hGCPII expression profile has been mostly generated by immunohistochemistry, Western blot analysis

and ELISA, mGCPII expression profile has been mainly generated using NAAG-hydrolysing assay.

A consensus has been reached on GCPII expression within human body in the prostate (acinar epithelium), nervous system (astrocytes and Schwann cells), small intestine and kidney (proximal tubules).[10,20-27] Several studies also showed that GCPII expression profile within the human body is much broader but the level of GCPII in other than the mentioned tissues is much lower. The distribution of hGCPII and mGCPII proteins within the body slightly differs. The mouse tissue with the highest amount of expressed GCPII is the kidney followed by the brain.[2,4] In contrast, almost no GCPII was detected in the mouse small intestine and prostate.[2,4]

1.4. GCPII functions

In spite of the fact that GCPII is expressed in numerous healthy tissues, its biological role has been only established in the brain and small intestine. The GCPII functions in both tissues correspond with its two enzymatic activities and are thus distinct. While in the brain, GCPII fulfils a neuromodulatory role, in the small intestine, it is involved in folate metabolism. In addition, GCPII has been suggested to play several distinct (though also contradictory) roles in healthy prostate and different carcinomas but not enough convincing data has been gathered for any hypothesis to be widely accepted by scientific community. One of the theories suggested that GCPII could serve as a receptor for yet unknown extracellular ligand. This hypothesis is based on the fact that GCPII possesses structural similarity to transferrin receptor[9,11] and is able to internalize upon ligand binding.[28,29] Interestingly, the function of GCPII in the kidney remains completely elusive.

GCPII fulfils the neuromodulatory role within the brain through cleavage and thus deactivation of NAAG, which is the most abundant peptide neurotransmitter in the mammalian brain.[30] During the synaptic transmission, NAAG is released together with excitatory neurotransmitter (such as glutamate) from the presynaptic neuron.[31] Subsequently, glutamate triggers the postsynaptic neuron stimulation through binding into NMDA receptors, while NAAG activates type 3 metabotropic glutamate receptors (mGluR3s) situated on presynaptic neurons and perisynaptic astrocytes.[32-34] The mGluR3s activation evokes a combination of neuroprotective actions resulting in downregulation of the postsynaptic neuron.[31,35]

The involvement of GCPII in the folate metabolism within the small intestine lies in facilitation of folate absorption. The natural folates are usually present in a diet as folylpoly-gamma-glutamates. [36-38] However, the proton-coupled folate transporter responsible for folate absorption from the intestinal lumen is highly specific for monoglutamylated folates.[39,40] The polyglutamylated folates need to be thus first hydrolysed on intestinal brush-border by GCPII.[18] Interestingly, in the rat intestine, polyglutamylated folates are not hydrolysed by GCPII but by γ-glutamylhydrolase.[41] Since GCPII seems not to be present in the mouse intestine[4], it is likely that the folate absorption in mice is facilitated similarly as in rats.

1.5. GCPII-deficient mice

In order to study the physiological function of GCPII in detail, inactivation of *Folh1* gene in mouse seems to be an ideal direction. Indeed, since the enzymatic properties and expression profiles of mGCPII and hGCPII are similar, mice may well approximate humans. Several research groups attempted to produce GCPII deficient mice but the results were rather controversial. Some reports showed that inactivation of *Folh1* gene leads to generation of viable GCPII deficient mice with no obvious phenotype.[42,43] In contrast, others demonstrated that GCPII deficiency is embryonically lethal.[44,45] It remains unclear why such striking differences in outcomes of GCPII-deficient mice preparation have been observed. In all cases, an embryonic stem cells (ESCs) manipulation was chosen for the gene disruption. The methodologies thus mainly differed in the design of the targeting construct.

The first GCPII-deficient mice were reported in 2002 by Bacich and colleagues.[42] The targeting construct for ESCs manipulation lacked intron-exon boundary sequences of exons 1 and 2 of the *Folh1* gene and contained several in-frame stop codons between exon 1 and 2. Produced GCPII-deficient mice displayed dramatically decreased NAAG-hydrolysing activity in the brain and kidney as compared with wild type (WT) littermates. Interestingly, the NAAG and glutamate levels in the brains of GCPII-deficient mice were not significantly impaired. It was thus suggested that deletion of *Folh1* gene might lead to induction of expression of other genes such as Naalad2 gene that encodes GCPIII. GCPIII is one of the closest paralogues of GCPII in both mouse and human proteome and it is the only GCPII paralogue capable of NAAG and $FolGlu_n$ hydrolysis.[46] When performing phenotypic examination of GCPII WT vs. deficient mice, no significant differences in standard neurological behaviour were observed. However, it was shown that GCPII-deficient mice are protected from peripheral neuropathies and traumatic brain injury (TBI).[47]

In 2003, another report regarding preparation of GCPII-deficient mice was published.[44] Surprisingly, in this work, the research group of Joseph T. Coyle showed that manipulation of ESCs cells by deletion of exons 9 and 10 of *Folh1* gene leads to embryonic lethality. The reason for deletion of exons 9 and 10 rather than exons at the beginning of *Folh1* gene lied in the effort to disrupt the active site of GCPII. Indeed, these exons encode zinc ligand domain, which is essential for enzyme activity. The same research group subsequently attempted to reproduce the work of Bacich and colleagues by deletion of exons 1 and 2 of *Folh1* gene in ESCs but was not able to produce viable GCPII-deficient mice.[45]

In 2015, independent research group described production of GCPII-deficient mice by deletion of exon 3 to 5 of *Folh1* gene.[43] In agreement with the report of Bacich and co-workers, Gao and colleagues observed dramatic decrease of NAAG-hydrolysing activity in the GCPII-deficient mouse brains when compared with their WT counterparts. Moreover, GCPII-deficient mice of Gao and co-workers showed similar phenotypic characteristics as these of Bacich and colleagues. Indeed, in comparison with WT mice, GCPII-deficient mice did not display overt behavioural alterations but were less susceptible to TBI and their long-term behavioural outcomes after the TBI were improved.[43]

2. AIMS AND OBJECTIVES

The efforts to inactivate *Folh1* gene in mice by manipulation of the embryonal stem cells have led to rather conflicting results ranging from embryonal lethality to production of viable GCPII-deficient mice with no obvious phenotype. One of the aims of this study was thus to resolve this discrepancy by utilisation of an alternative way for *Folh1* gene inactivation.

The reason why some of the GCPII-deficient mice are viable could relate to residual NAAG-hydrolysing activity detected in these mice. In contrast, the embryonic lethality of other GCPII-deficient mice could be a result of a complete inactivation of *Folh1* gene. The aim was thus to specifically disrupt *Folh1* gene within a sequence encoding active site of GCPII. This would avoid not only potential production of partially active GCPII from alternatively spliced mRNA but also embryonic lethality. We hypothesized that if GCPII fulfils both the receptor and the enzyme functions within the body, GCPII-mutant mice expressing inactive mutant variant of GCPII could be possibly viable.

Finally, even though GCPII has been thoroughly studied for more than three decades, its biological function outside brain and small intestine is still not fully understood. Even in the case of GCPII-deficient mice, researchers mainly focused on its involvement in nervous system. If GCPII-deficient or GCPII-mutant mice were viable, the aim of this study would be to explore the physiological function of GCPII in urogenital system.

To meet the aims, following objectives were set:

1. Perform TALEN-mediated *Folh1* gene disruption in mice within a sequence encoding active site of GCPII
2. Analyse TALEN-mediated mutations and select founder mice carrying both the frame-shift mutations and deletions potentially resulting in only small deletion within the active site of GCPII
3. Establish mutant mouse colonies and develop reliable genotyping protocol
4. Characterize recombinant mouse GCPII in terms of its kinetic properties in NAAG hydrolysis reaction
5. Prepare and characterize recombinant mutant variants of GCPII that would potentially be expressed in GCPII-mutant mice
6. Characterize GCPII-mutant and GCPII-deficient mice
7. Investigate the impact of GCPII disruption on reproductive tissue and renal function by careful examination of GCPII-mutant and GCPII-deficient mice

3. METHODS

GCPII-deficient mice were prepared using TALEN-mediated mutagenesis of mouse zygotes of C57BL/6NCrl genetic background by collaborating laboratory. Tail biopsies from resulting transgenic mice were subjected to phenol-chloroform extraction of chromosomal DNA and the TALEN-mediated mutations within *Folh1* gene were analyzed by sequencing. Selected founder mice were bred with C57BL/6NCrl wild type mice to establish GCPII-mutant mouse colonies. Breeding was monitored by genotyping, which was based on nested PCR.

All *Folh1* gene mutations detected in founder mice were cloned into pTreTight-mGCPII vector using site-directed mutagenesis. Prepared pTreTight-mGCPII-mutant vectors were subsequently used for transient transfection of HEK293 cells. Produced membrane-bound mGCPII mutant variants were characterized by ELISA and Western blotting in terms of their expression yields and by radioactive NAAG-hydrolyzing assay in terms of their activity.

pMT/BiP vectors encoding extracellular parts of mGCPII mutant variants were cloned by restriction cleavage of corresponding pTreTight-mGCPII-mutant variants. Extracellular mGCPII mutant variants were prepared by stable transfection of *Drosophila* S2 cells followed by large scale expression and purification of the recombinant proteins from conditioned media by affinity chromatography. All steps of recombinant protein preparation were monitored by SDS-PAGE followed by silver staining or Western blotting. The protein concentration was measured by Nanodrop. Biochemical properties of rm-GCPIIdel3 were compared to that of rm-GCPII WT by size-exclusion chromatography and Thermofluor assay. rm-GCPII WT was also subjected to enzyme kinetics measurements using NAAG-hydrolyzing activity assay.

Mice used for collection of tissue samples were sacrificed by intraperitoneal injection of anaesthetics followed by cervical dislocation. All dissections were carried out in a maximum of 60 minutes after mice death. All GCPII-mutant mice were characterized by Western blot analysis of brain and kidney lysates. Some of the mutant mice were further characterized by radioactive NAAG-hydrolysing activity assay in brain and kidney lysates. The protein concentration in lysates was measured by Bradford assay.

Mouse intended for phenotypic characterization were sacrificed the same way as mentioned above. Before injection of anesthetics, mice were let to urinate in order to collect urine samples for metabolomics analysis. Later, creatinine level was measured in all urine samples by enzyme-coupled spectrophotometry. The subsequent targeted metabolomics was performed by collaborating laboratory. After the mouse death, gross anatomy of urogenital system was performed followed by collection of seminal vesicles and kidneys for histopathological examination, which was performed by collaborating laboratory.

Where appropriate, statistical analysis was performed using standard statistical methods.

4. RESULTS

4.1. Kinetic characterization of recombinant mouse GCPII

Kinetic parameters of recombinant mouse GCPII (i.e. extracellular part of mGCPII) were published in[4] (see PhD Thesis, Appendix 1), where a direct comparison of rm-GCPII and its human counterpart (rh-GCPII) was performed. Here, only kinetic properties of both enzymes when catalysing NAAG hydrolysis are summarized. The recombinant enzymes of high purity were prepared previously in our laboratory[4,48] by recombinant expression in insect cells. The amount of the enzyme was optimized for each NAAG concentration to ensure that the substrate conversion is below 25% and the kinetic measurements are thus performed within the initial reaction velocity. The kinetic parameters were calculated using GraFit 5.0.4[49] and are summarized in Table 1. It was determined that the catalytic efficiency of rm-GCPII is lower than that of rh-GCPII. Indeed, even though the turnover numbers are very similar, the K_M value of rm-GCPII is almost four times higher than that of rh-GCPII (see Table 1).

Table 1: Kinetic parameters of recombinant mouse GCPII (rm-GCPII) and recombinant human GCPII (rh-GCPII) for NAAG-hydrolysing reaction. The calculations were performed in GraFit 5.0.4.[49]

Enzyme	K_M [nM]	k_{cat} [s^{-1}]	k_{cat}/K_M [× 10^7 s^{-1} M]
rm-GCPII	1900 ± 100	1.44 ± 0.02	0.077 ± 0.001
rh-GCPII	550 ± 60	1.45 ± 0.04	0.265 ± 0.007

4.2. Generation of GCPII-mutant mice using TALENs

In order to generate GCPII-mutant mice, *Folh1* gene disruption using targeted endonucleases was performed. Dr. Petr Kasparek from IMG CAS designed TALENs that targeted exon 11 of *Folh1* gene within the sequence encoding active site of mGCPII (specifically, the sequence encoding zinc-coordinating Glu427 and the catalytic base Glu426, see Figure 3) and performed two independent microinjections into mouse zygotes (for more information see[50]).

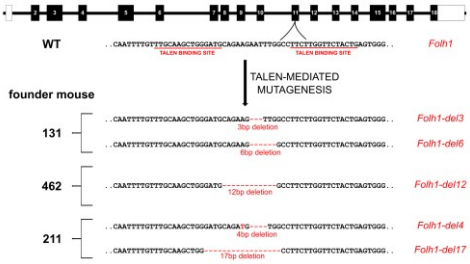

Figure 3: TALEN-mediated *Folh1* gene disruption performed by Dr. Petr Kasparek at IMG CAS. TALEN activity followed by non-homologous end joining generated several different mutations within exon 11 of *Folh1* gene. Three transgenic mice (designated as 131, 462 and 211) in C57BL/6NCrl genetic background were then selected as founders for establishing the GCPII-mutant mouse colonies. The *Folh1* gene and *Folh1*-mutant gene sequences were generated and analysed using Vector NTI.

This resulted in production of F0 generation consisting of 65 transgenic mice in C57BL/6NCrl genetic background. These were analysed for a presence of TALEN-mediated mutations.Three distinct founder mice were then selected for establishing GCPII-mutant mouse colonies – a mouse designated as 131 that carried deletions of 3 bp (del3) and 6 bp (del6) within exon 11 of *Folh1* gene, a mouse 462 with deletion of 12 bp (del12) and a mouse 211 with deletions of 4 bp (del4) and 17 bp (del17).

The potential protein products of the *Folh1*-mutant gene expression are summarized in Figure 4. An expression of *Folh1-del3*, *Folh1-del6* and *Folh1-del12* would lead to production of full-length mGCPII with 1, 2 or 4, respectively, amino acid deletions within the active site of mGCPII (mGCPIIdel3, mGCPIIdel6 and mGCPIIdel12). While mGCPIIdel3 and mGCPIIdel6 lack one from the two active site glutamates, mGCPIIdel12 lacks both of them. The mouse colonies carrying these mutations could thus potentially express inactive variants of mGCPII. Nevertheless, following experiments showed that the biochemical properties of recombinantly prepared mGCPIIdel3, mGCPIIdel6 and mGCPIIdel12 dramatically differ from those of mGCPII wild type (WT) protein (see PhD Thesis, Chapter 4.2.3. and 4.2.4.). The genes *Folh1-del4* and *Folh1-del17* contain frameshift sequences and their potential expression would thus result in shorten protein products (456 and 428, respectively, instead of 752 amino acids).

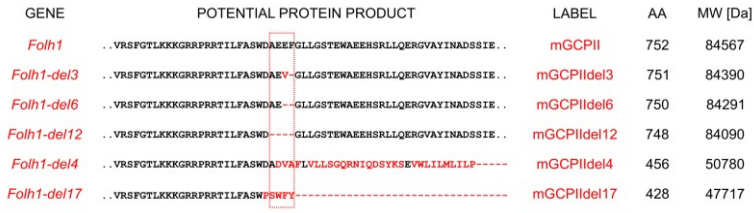

Figure 4: Protein product of *Folh1* gene expression compared to potential protein products of *Folh1*-mutant gene expression. Protein sequences were generated and analysed using Vector NTI.

F1 generations of all five GCPII-mutant mouse colonies were produced by breeding of F0 transgenic mice 131, 462 and 211 with C57BL/6NCrl wild type mice. To monitor the genotypes of newborns, a robust genotyping method based on nested PCR was established (see PhD Thesis, Chapter 4.1.2.). F1 generation of all GCPII-mutant variants bred normally and did not show any obvious phenotype. Similarly, heterozygous mice of F2 and all following generations carrying *Folh1-del3*, *Folh1-del4* or *Folh1-del17* did not show any reproductive or phenotypic abnormalities. The mouse colonies carrying *Folh1-del3*, *Folh1-del4* or *Folh1-del17* were maintained using heterozygote × wild type breeding scheme. The mice homozygous for *Folh1-del3*, *Folh1-del4* or *Folh1-del17* were generated by intercrossing of heterozygous mice of at least F2 generation. Neither embryonic lethality nor any obvious phenotypes of mice

homozygous for *Folh1-del3*, *Folh1-del4* or *Folh1-del17* were observed. Subsequent intercrossing of the homozygous mice was also in correspondence with usual breeding performance of C57BL/6NCrl mice.

In agreement with other GCPII-mutant variants, the heterozygous mice of F2 generation carrying *Folh1-del6* or *Folh1-del12* did not show any obvious phenotype. These colonies were not expanded much further; a reproduction performance was thus not studied in detail. Nevertheless, no reproductive abnormalities were observed in a small cohort of *Folh1-del6* homozygous mice. In addition, mice homozygous for *Folh1-del6* or *Folh1-del12* did not show any obvious phenotype.

4.3. Characterization of GCPII-mutant and GCPII-deficient mice

No GCPII mutant protein variant was detected in any of the GCPII-mutant mice using Western blot analysis of brain and kidney lysates (see PhD Thesis, Chapter 4.3.1.). All prepared GCPII-mutant mice could be thus considered as GCPII-deficient (also referred as Folh1$^{-/-}$), To characterize GCPII-deficient mice thoroughly, brain and kidney lysates from 6 Folh1$^{+/+}$ mice, 3 Folh1$^{+/-}$ mice and 6 Folh1$^{-/-}$ mice were analysed using Western blotting and NAAG hydrolysis assay.

Representative Western blot analysis of brain lysates is depicted in Figure 5A (p. 15). To demonstrate that the loaded amount of total protein was identical in all cases, a detection of β-actin as a loading control was included. To demonstrate that antibody GCPII-04 is able to recognize truncated forms of mGCPII, not only rm-GCPII WT but also rm-GCPIIdel17 served as the protein standards. It was confirmed that no band corresponding to full-length or any truncated variant of mGCPII could be detected in lysates from Folh1$^{-/-}$ mice. Interestingly, the signal around 100kDa corresponding to mGCPII full-length protein displayed lower intensity in case of Folh1$^{+/-}$ mice than in case of Folh1$^{+/+}$ mice. Nevertheless, no truncated variant of mGCPII was observed in the lysates from Folh1$^{+/-}$ mice.

Finally, NAAG hydrolysis analysis was performed in order to confirm that GCPII-deficient mice are indeed devoid of specific GCPII activity (for representative NAAG hydrolysis analysis of brain lysates see Figure 5B, p. 15). While overnight incubation of the brain and kidney lysates from Folh1$^{+/+}$ mice with NAAG led to almost complete hydrolysis of the substrate (NAAG conversion higher than 80%), NAAG conversion in the lysates from Folh1$^{-/-}$ mice did not exceed 6%. In correspondence with Western blotting observations, NAAG hydrolysis activity was decreased in lysates from Folh1$^{+/-}$ mice as compared to lysates from Folh1$^{+/+}$ mice.

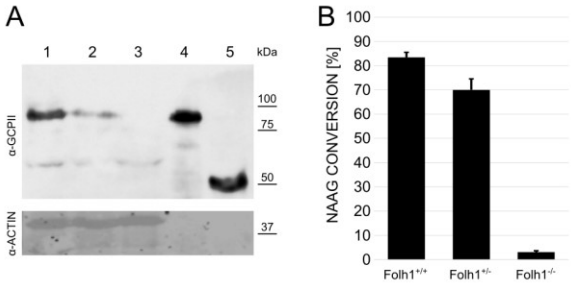

Figure 5: Characterization of GCPII-deficient mice using Western blotting (A) and NAAG hydrolysis analysis (B) of brain lysates. Male mice of age around 8 weeks carrying *Folh1-del17* were used. 100µg of total protein were analysed. **A:** Western blot analysis. To detect mGCPII, antibody GCPII-04 (Exbio) was used – here designated as α-GCPII. To detect β-actin, Monoclonal Anti-β-Actin antibody, clone AC-15 (Sigma-Aldrich) was used – here designated as α-ACTIN. Purified rm-GCPII and rm-GCPIIdel17 served as mGCPII standards. 1 – Folh1$^{+/+}$ mouse; 2 – Folh1$^{+/-}$ mouse; 3 – Folh1$^{-/-}$ mouse; 4 – 5ng of rm-GCPII; 5 – 5ng of rm-GCPIIdel17. **B:** NAAG hydrolysis analysis. Tissue lysates were incubated in 50mM Tris-HCl, 0.1% Tween, pH 7.4 with 100nM NAAG overnight at 37 ºC. Error bars represent standard deviation of 6 biological replicates (for Folh1$^{+/+}$ and Folh1$^{-/-}$ mice) and 3 biological replicates (for Folh1$^{+/-}$ mice).

4.4. Investigation of reproductive tissue phenotype of GCPII-deficient mice

The reproductive tissue phenotype was described in [50] in detail (see PhD Thesis, Appendix 2). The mouse colonies carrying *Folh1-del3*, *Folh1-del4* and *Folh1-del17* were utilized in this study. We found that aged GCPII-deficient mice possess higher propensity for seminal vesicles enlargement than possess their WT counterparts. We explored this phenomenon using cohorts of 20 Folh1$^{+/+}$ mice, 26 Folh1$^{+/-}$ mice and 9 Folh1$^{-/-}$ mice with average age of 70.4 – 70.5 weeks (see Figure 6A).

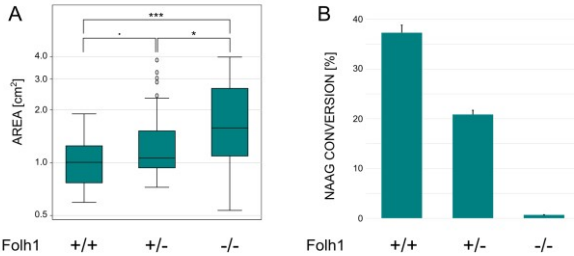

Figure 6: Statistical analysis of enlarged seminal vesicles and NAAG-hydrolysing activity in kidney lysates of aged mice of distinct genotypes. A: Comparison of seminal vesicle areas among Folh1$^{+/+}$, Folh1$^{+/-}$ and Folh1$^{-/-}$ mice. *** indicates p<0.001, * indicates p=0.028 and · indicates p=0.052. Seminal vesicle areas were measured by MUDr. Frantisek Sedlak using ImageJ software (Rueden 2017). Statistical analysis was performed by Dr. Marek Maly at National Institute of Public Health. **B:** Comparison of NAAG-hydrolysing activity (see Chapter 3.2.7., p. 67) in kidney lysates of Folh1$^{+/+}$, Folh1$^{+/-}$ and Folh1$^{-/-}$ mice. NAAG-hydrolysing analysis was carried out in 0.5 µg total protein. Tissue lysates were incubated in 50 mM Tris-HCl, 0.1% Tween, pH 7.4, with 100 nM NAAG at 37 ºC for 6 hours. Error bars represent standard deviation of 2 (for -/-) or 3 (for +/+ and +/-) biological replicates. The figure was adapted from [50].

We showed that the overall difference in seminal vesicle area among these three cohorts is highly statistically significant ($p<0.002$) and not only difference between $Folh1^{+/+}$ and $Folh1^{-/-}$ mice, but also difference between $Folh1^{+/+}$ and $Folh1^{+/-}$ mice is statistically significant ($p<0.001$ and $p=0.028$, respectively). Moreover, we analysed the level of GCPII in kidney lysates of mice of all three genotypes ($Folh1^{+/+}$, $Folh1^{+/-}$ and $Folh1^{-/-}$) and found that the level of GCPII in $Folh1^{+/-}$ kidneys is about a half of the level in $Folh1^{+/+}$ mice as determined by NAAG-hydrolysing activity (see Figure 6B).

We attempt to investigate the possible source of the seminal vesicle enlargement through examination of urogenital system of WT mice using NAAG-hydrolysing activity assay. We found that the NAAG conversion in seminal vesicles and most surrounding urogenital tissues is extremely low. Except of kidney (which served as a positive control), only epididymis and spermatic cord exhibited NAAG conversion at least 50% when 30 µg total protein was measured overnight. These tissues were thus examined for accurate determination of GCPII level. Using tissues from $Folh1^{-/-}$ mice as negative controls, we found out that the level of GCPII in the mouse epididymis was as high as its level in the mouse kidney (see Table 2).

Table 2: Calculated amount of GCPII in the mouse epididymis as compared with previously published GCPII levels in the mouse kidney and brain.[4] The calculation was performed by comparison of NAAG conversion in 0.3 µg of total protein in mouse epididymis with rm-GCPII calibration curve.

mouse tissue	mGCPII [ng/mg]
epididymis	182 ± 12
kidney*	100 – 250
brain*	2.5 – 10

We thus decided to examine whether GCPII is also present in the human epididymis. Indeed, using immunochemistry with 3 different antibodies against GCPII, we detected GCPII-positive cells in all parts of human epididymis (see Figure 7).

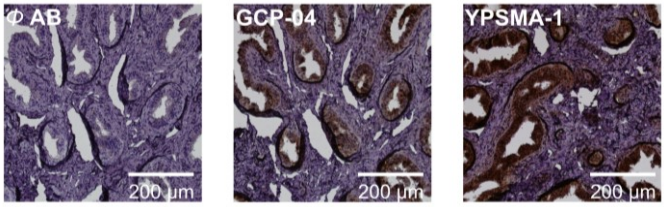

Figure 7: Representative immunohistochemical staining of human epididymis. Formalin-fixed paraffin-embedded human epididymis tissue sections were incubated with the antibodies against GCPII at a concentration of 10 µg/ml to detect GCPII-positive cells. Here, examples of staining using antibodies GCP-04 (Exbio) and YPSMA-1 (Anogen) are shown. As a negative control (designated as *Φ* AB), immunohistochemistry using only secondary antibody Histofine Simple Stain™ MAX PO (MULTI) (Nichirei Biosciences Inc.) was performed. The tissue sections were provided by prof. Josef Zamecnik from Motol University Hospital. The immunochemistry was performed by MUDr. Frantisek Sedlak. The figure was adapted from [50].

4.5. Investigation of potential GCPII involvement in renal function

We investigated potential involvement of GCPII in renal function using histopathological examination of kidney from aged mice and targeted metabolomics in urine. Mouse kidneys were investigated for possible abnormalities by MUDr. Tomas Olejar, Ph.D. at First Faculty of Medicine (see Figure 8). No significant differences in proximal and distal tubules between aged Folh1$^{+/+}$ and Folh1$^{-/-}$ mice were observed.

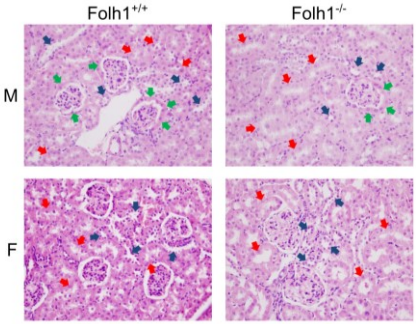

Figure 8: Representative histopathological examination of kidney cross sections of 70 weeks old mice. The kidneys were fixed by formalin, embedded in paraffin, sectioned and stained with haematoxylin and eosin. The red arrows indicate proximal tubules, the blue arrows indicate distal tubules and the green arrows indicate tubularization of Bowman' s capsule. The histopathology was performed by MUDr. Tomas Olejar, Ph.D. at First Faculty of Medicine.

Out of 193 metabolites detected in the mouse urines during targeted metabolomics analysis, only 3 metabolites (inosine, succinyladenosine and NAAG) were found to be significantly discriminating Folh1$^{+/+}$ and Folh1$^{-/-}$ mice (see Figure 9). Interestingly, no folate-related metabolite, including different forms of folate as well as homocysteine, was discriminating between both genotypes. Nevertheless, only monoglutamylated forms of folate were tested since the detection of polyglutamylated forms was not successful. The most significant difference was shown for NAAG since its increased level in Folh1$^{-/-}$ mouse as compared to Folh1$^{+/+}$ mice displayed p-value of 9.28×10^{-18} (alpha level after Bonferroni correction equalled 2.59×10^{-4}).

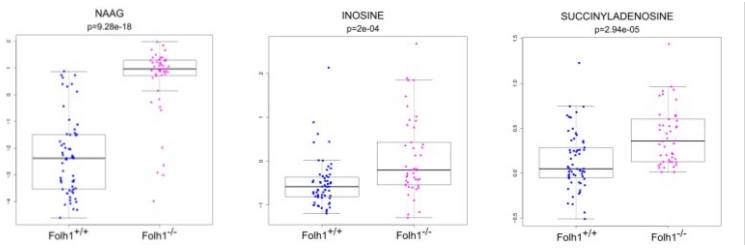

Figure 9: Box plot displays of selected metabolites detected in mouse urine samples. The statistical analysis of metabolomics data was performed by Dr. David Friedecky and colleagues at IMTM.

5. DISCUSSION

Despite extensive research for more than 30 years, the physiological role of GCPII has not been fully determined. The attempts to generate GCPII-deficient mice in order to decipher GCPII function have led to conflicting results ranging from embryonic lethality[44,45] to production of GCPII-deficient mice with no obvious phenotype.[42,43] This dissertation project thus aimed to dissect the discrepancy using gene disruption technique different from ESCs manipulation, which was utilized in all previous studies.

To study GCPII function, mouse model seems to be a suitable approach. Indeed, our direct comparison of recombinant human and mouse GCPII (rh-GCPII and rm-GCPII) revealed that not only the sequences, but also the enzymatic properties of both orthologues are similar.[4] They possess comparable substrate specificities and inhibition profiles; only the catalytic efficiencies towards physiological substrates slightly differ. Indeed, in case of NAAG-hydrolysing activity, the catalytic efficiency of rm-GCPII is about four times lower than that of rh-GCPII. In contrast to the small intestine and prostate, the level of GCPII expression in the brain and kidney seems to be very similar for both orthologues.[51] The mouse model can be thus used for revealing the function of GCPII in the nervous and urogenital system.

For the attempt to inactivate *Folh1* gene in mouse, we used one of the modern gene disruption technique – TALEN technology.[52-54] Recently, simpler-to-design CRISPR/Cas9 technology[55] is being most frequently used for mouse genome modifications. However, at the start of this dissertation project, CRISPR/Cas9 technology was not available. Moreover, off-target activity seems to be a more significant problem for CRISPR/Cas9 than for TALENs.[56]

When inactivating *Folh1* gene by TALENs, we intended to disrupt the active site of mGCPII. This strategy was selected to avoid two main obstacles that had not been addressed in previous reports. First, several publications detected novel alternative splice variants in the knockout mice and showed that these variants may rescue severe phenotype of completely inactivated gene.[57,58] We hypothesized that the viable GCPII-deficient mice published by Bacich and colleagues could contain alternatively spliced variants that enabled partial preservation of GCPII activity.[42] This was strengthen by the facts that the authors saw residual NAAG-hydrolysing activity in the brains of GCPII-deficient mice and that the attempt of Tsai and colleagues to generate GCPII-deficient mice, in which the disruption of the mGCPII active site was performed, led to embryonic lethality.[44]

Second, for the purpose of disrupting the active site of GCPII, Tsai and colleagues replaced exons 9 and 10 of *Folh1* gene encoding zinc ligands by PGK-Neo.[44] Therefore, the authors did not only disable GCPII activity, but also GCPII 3D structure. In case GCPII fulfils both the enzyme and the receptor functions, disrupting both functions at the same time could lead to embryonic lethality. We thus hypothesized that together with greater disruptions leading to complete inactivation of *Folh1* gene, TALEN technology may also generate small deletions within the active site of mGCPII. These modifications could possibly only disable mGCPII activity but not its structure and the potential receptor function would be thus preserved.

From two independent attempts to generate mice with TALEN-mediated indel mutations of *Folh1* gene, we selected 3 founder mice that altogether contained 2 frame-shift deletions (*Folh1-del4* and *Folh1-del17*) and 3 small deletions within the mGCPII active site (*Folh1-del3*, *Folh1-del6* and *Folh1-del12*). To establish all 5 mutant mouse colonies, we bred the founder mice with C57BL/6NCrl wild type mice and monitored the resulting genotypes by developed genotyping method. By heterozygous mating of all 5 established GCPII-mutant mouse colonies, we obtained offsprings with typical Mendelian inheritance. We did not observe embryonic lethality of mice homozygous for *Folh1* mutant gene.

We thus set out to investigate whether the mutant variants of GCPII are expressed in the GCPII-mutant mice. We performed Western blot analysis of two distinct tissues that normally express the highest level of GCPII in mice – brain and kidney. The signal corresponding to GCPII was not observed in either the soluble fractions or the insoluble fractions of tissue lysates from GCPII-mutant mice. In these mutant mice, GCPII is either expressed and immediately degraded or not expressed at all. This assumption strengthens the fact that recombinantly prepared mGCPII mutant variants dramatically differ from rm-GCPII WT in terms of their biochemical properties. The mutant mice bearing any of the *Folh1* mutant variant could be thus considered as GCPII-deficient mice.

To thoroughly characterize GCPII-deficient mice (also referred as Folh$^{-/-}$), we utilized two independent methods – Western blotting and NAAG-hydrolysing activity assay – to analyse brain and kidney lysates from the number of 8 weeks old mice that was sufficient for statistical significance. Western blot analysis did not detect GCPII full-length protein or GCPII truncated variant, which could possibly be a product of expression of *Folh1-del4* and *Folh1-del17* gene, in any lysate from GCPII-deficient mice. Moreover, NAAG-hydrolysing activity was dramatically decreased in all lysates from GCPII-deficient mice. From these reasons, we are confident that our attempt to inactivate *Folh1* gene in mice through GCPII active site disruption led to generation of viable GCPII-deficient mice.

We thus confirmed the results of the original publication of Bacich and colleagues[42] and disproved that GCPII deficiency is embryonically lethal as was repeatedly reported by the research group of Joseph T. Coyle.[44,45] Moreover, during the time when this dissertation project has been in the process, another publication reporting viable GCPII-deficient mice emerged.[43] Recently, International Mouse Phenotyping Consortium (IMPC) also released information about preparation of viable GCPII-deficient mice.[59] It remains unclear why any strategy of Coyle's research group for inactivation of *Folh1* gene did not result in production of live GCPII-deficient mice. However, thanks to the work of four independent laboratories (us including), it seems to be evident that disruption of GCPII encoding gene does not lead to embryonic lethality of the mice.

Interestingly, when comparing tissue lysates from GCPII WT mice (also referred as Folh$^{+/+}$) and mice bearing both *Folh1* and *Folh1*-mutant alleles (also referred as Folh$^{+/-}$), the intensity of Western blot signal of GCPII differed. Indeed, we observed that the amount of GCPII in lysates from Folh$^{+/-}$ heterozygous mice was about a half the amount of GCPII in lysates from Folh$^{+/+}$ mice. This observation was

further confirmed by NAAG-hydrolysing activity assay, which displayed NAAG conversion in the lysates from Folh$^{+/-}$ mice being around a half the NAAG conversion in the lysates from Folh$^{+/+}$ mice. Such observation is in accordance with the reports published previously.[42,43] In contrast, while residual NAAG-hydrolysing activity in the brains of our GCPII-deficient mice seems to be negligible since the NAAG conversion in saturation did not exceed 4% of the values obtained for WT mice, others showed NAAG conversion in GCPII-deficient mice within the linear range of reaction velocity ranging from 6% to 18% of the values obtained for WT mice.[42,43] Since none of the viable mice produced by others was generated by disruption of active site, it cannot be excluded that these mice indeed contain the alternatively spliced variants that partially preserve GCPII activity.

In agreement with other studies[42,43,59], our GCPII-deficient mice did not show any reproductive or obvious phenotypic abnormalities. Since the publications focusing on GCPII-deficient mice phenotype have mainly investigated the nervous system[43,47,60], we decided to explore the impact of *Folh1* gene inactivation on the mouse urogenital system. We found that aged GCPII-deficient mice possess higher propensity to seminal vesicle enlargement. Interestingly, this phenotype was not observed only in Folh1$^{-/-}$ mice but to a lesser extent also in Folh1$^{+/-}$ mice. Therefore, it is possible that the enlargement of seminal vesicle is somehow connected with GCPII activity since not only the young Folh1$^{+/-}$ mice but also the aged Folh1$^{+/-}$ mice display decreased expression level of GCPII when compared to Folh1$^{+/+}$ mice.

The seminal vesicle dilation in our GCPII-deficient mice seemed to originate solely from accumulation of luminal fluid.[50] This phenotype of C57 mice has been previously reported as one of the signs of aging.[61,62] Nevertheless, the mice, which showed enlargement of seminal vesicles caused by accumulation of luminal fluid, were at least 24 months old[61], i.e. much older than the mice investigated in our study. Enlarged seminal vesicles were also reported in aged C57 mice with lower age limit of 16 months.[62] However, in this case, the seminal vesicle dilation was a result of either an infection or a presence of abdominal tumour, neither of which we saw during our histopathological examination.[50]

In order to research the possible source of seminal vesicle dilation in GCPII-deficient mice, we investigated the expression level of GCPII in the seminal vesicles and most surrounding tissues of WT mice using NAAG-hydrolysing activity assay. Surprisingly, GCPII seemed to be only present in the epididymis and spermatic cord since no other urogenital tissue displayed a pronounced NAAG conversion. Nevertheless, it cannot be excluded that GCPII is present in the seminal vesicles in an amount sufficient for execution of a specific function. Indeed, 60-75% of the seminal vesicle mass is represented by the seminal fluid[61] and thus only around 35-40% of the seminal vesicle mass remains for the cells. Since protein concentration in the seminal fluid can be as high as 86 mg/ml[63], the amount of GCPII expressed in the seminal vesicle cells can be dramatically underestimated.

Although the NAAG-hydrolysing activity assay was performed using the reaction conditions highly specific for GCPII[64], enzymes such as GCPIII, which could be possibly also present in the studied tissue, may influence the outcome of the analysis. It has been shown that GCPIII is expressed in considerable amounts in the

mouse testis, kidney and bladder.[65] No other tissues of male urogenital system have been explored though. To exclude a potential participation of other enzymes including GCPIII, we investigated NAAG-hydrolysing activity of the spermatic cord and epididymis (as the only tissues displaying pronounced NAAG conversion) from both WT and GCPII-deficient mice.[50] As a result, apart from kidneys[4], only epididymis may be additionally considered as a GCPII-expressing tissue in mice.

To put our findings into the context of human physiology, we investigated a potential presence of GCPII in the human epididymis using immunochemistry. Based on the results, we concluded that not only GCPII mRNA[66], but also GCPII protein is expressed in the human epididymis. In humans, ejaculatory duct obstruction is rather uncommon but has been indicated as one of the causes of male infertility.[67] It may be only speculated whether the absence of GCPII in the epididymis is somehow associated with increased occurrence of enlarged seminal vesicles. The reason why the inactivation of *Folh1* gene leads to seminal vesicles dilation in aged GCPII-deficient mice thus remains enigmatic and further research is desirable.

Surprisingly, the function of GCPII in the kidney has not been studied yet, though this tissue express one of the highest levels of GCPII in both the humans and mice.[2,4,20] We hypothesized that inactivation of *Folh1* gene in mice could somehow impair the kidney function and would thus point out to the possible role of GCPII in this tissue. To investigate this assumption, we performed histopathological examination of the kidney and metabolomic analysis of mouse urine samples.

Kidneys of aged GCPII-deficient mice did not display any pathological abnormalities. Moreover, from 193 tested metabolites present in mouse urine, only 3 metabolites discriminated between Folh1$^{+/+}$ and Folh1$^{-/-}$ mice. This would suggest that the function of GCPII in the kidney is not crucial. Such conclusion would be particularly profitable for small-ligand based targeting of anti-cancer drug since the GCPII inhibition in the kidney would probably not cause much harm. A nephrotoxicity caused by kidney intake of anti-cancer drug could be then avoided by serial co-medication of one of the potent GCPII inhibitor such as PMPA.[68] From the three metabolites that were shown to significantly discriminate Folh1$^{+/+}$ and Folh1$^{-/-}$ mice, NAAG is particularly interesting. It has been shown that NAAG level in the brains of GCPII-deficient mice do not significantly differ from that of GCPII WT mice.[42] Therefore, it seems that GCPII-deficient mice control the amount of NAAG by its increased excretion. The two other metabolites discriminating Folh1$^{+/+}$ and Folh1$^{-/-}$ mice – inosine and succinyladenosine – have not been yet studied in terms of why their levels are sensitive to the presence of GCPII. Further investigation is thus necessary before coming to a conclusion of the necessity of GCPII in the kidney.

It remains unclear, why none of the generated GCPII-deficient mice shows any pronounced phenotype. It is possible that the lack of the strong phenotype is connected with inappropriate experimental settings. Indeed, in case GCPII-deficient mice need to be first challenged before the phenotype would be obvious, the standard housing conditions would not reveal it. In addition, as already suggested by others[42], other proteins, such as GCPIII, could at least partially compensate the GCPII action. As a follow-up of this dissertation project, it would be thus interesting to attempt preparation of mice that would be not only GCPII-deficient, but also GCPIII-deficient.

6. CONCLUSION

1. TALENs were utilized to disrupt *Folh1* gene in mice within a sequence encoding active site of GCPII.
2. All 65 transgenic mice of F0 generation were analysed for TALEN-mediated mutations. Three founder mice carrying altogether two frame-shift deletions (*Folh1-del4* and *Folh1-del17*) and three small in-frame deletions (*Folh1-del3*, *Folh1-del6* and *Folh1-del12*) within the active site of GCPII were used to establish GCPII-mutant mouse colonies.
3. For facile monitoring of mouse breeding, reliable genotyping protocol based on nested PCR was developed.
4. Recombinant mouse and human GCPII were compared in terms of their kinetic properties for NAAG-hydrolysing reaction. The catalytic efficiency of rm-GCPII was more than 3 times lower than that of rh-GCPII, the difference mainly resulted from distinct K_M values.
5. Recombinant extracellular parts of all GCPII mutant variants were expressed and variants rm-GCPIIdel3, rm-GCPIIdel6 and rm-GCPIIdel12 were purified. Their characterization revealed that none of the variants displays biochemical properties similar to rm-GCPII.
6. GCPII-mutant mice were characterize in terms of the expression of the potential GCPII mutant variants using Western blotting. Since none of the variants was detected in the brains or kidneys of GCPII-mutant mice, these mice may be considered as GCPII-deficient mice. NAAG-hydrolysing activity in the brain and kidney lysates from GCPII-deficient mice was almost completely abolished.
7. GCPII-deficient mice were viable, bred normally and did not show any obvious phenotype.
8. Closer examination of urogenital system revealed increased propensity to seminal vesicle enlargement in aged GCPII-deficient mice as compared with their WT counterparts. The source of this phenotype is to be determined since in WT mouse urogenital system, GCPII seems to be present in substantial levels only in the epididymis and kidney. Nevertheless, this finding is relevant to human physiology since GCPII was also detected in the human epididymis.
9. No pronounced phenotype was seen in the kidneys of aged GCPII-deficient mice as investigated by histopathology and metabolomic analysis of urine. This observation could be beneficial in cancer treatment using drugs conjugated to small ligand targeting GCPII in tumours. Since the inhibition of GCPII in the kidney would probably not cause much harm, patients could obtain GCPII inhibitor as a co-medication to avoid nephrotoxicity caused by anti-cancer drug.

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8. LIST OF PUBLICATIONS

8.1. Publications related to dissertation project

a) Research articles

1. **Vorlová B.**, Sedlák F., Kašpárek P., Šrámková K., Malý M., Zámečník J., Šácha P., Konvalinka J.: A novel PSMA/GCPII-deficient mouse model shows enlarged seminal vesicles upon aging. *Prostate*, 2018; In press, doi: 10.1002/pros.23717. **IF=3.347**
2. Knedlík T., **Vorlová B.**, Navrátil V., Tykvart J., Sedlák F., Vaculín Š., Franěk M., Šácha P., Konvalinka J.: Mouse glutamate carboxypeptidase II (GCPII) has a similar enzyme activity and inhibition profile but a different tissue distribution to human GCPII. *FEBS Open Bio*, 2017; **7**(9): 1362-78. **IF=1.782**

b) Review articles

Vorlová B., Knedlík T., Tykvart J., Konvalinka J.: GCPII and its close homolog GCPIII: from a neuropeptidase to a cancer marker and beyond. Invited review to *Front Biosci (Landmark Ed)*, 2018; Submitted. **IF=2.349**

c) Meeting abstracts

1. **Vorlová B.**, Kašpárek P., Šácha P., Sedláček R., Konvalinka J.: Generation and basic characterization of glutamate carboxypeptidase II knock-out mice. *Transgenic Res*, 2016; 25(2): 267
2. **Vorlová B.**, Kašpárek P., Šácha P., Sedláček R., Konvalinka J.: Revealing novel functions of glutamate carboxypeptidase II using knock-out mice. *FEBS J*, 2017; 284: 358
3. **Vorlová B.**, Sedlák F., Kašpárek P., Sedláček R., Šácha P., Konvalinka J.: Revealing novel functions of glutamate carboxypeptidase ii, a diagnostic and therapeutic target in neuropathologies and prostate cancer. *Mol Biol Cell*, 2017; 28

8.2. Other publications

1. **Vorlová B.**, Nachtigallová D., Jirásková-Vaníčková J., Ajani H., Jansa P., Rezáč J., Fanfrlík J., Otyepka M., Hobza P., Konvalinka J., Lepšík M.: Malonate-based inhibitors of mammalian serine racemase: kinetic characterization and structure-based computational study. *Eur J Med Chem*, 2015; 89:189-97. **IF=4.816**
2. Jirásková-Vaníčková J., Ettrich R., **Vorlová B.**, Hoffman H.E., Lepšík M., Jansa P., Konvalinka J.: Inhibition of human serine racemase, an emerging target for medicinal chemistry. *Current Drug Targets*, 2011; 12(7): 1037-55. **IF=3.112**