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Abstract

Objective: Type 1 diabetes onset is preceded by a pre-inflammatory stage leading to insulitis and followed by targeted destruction of the insulin-producing beta cells of the pancreas. Osteopontin (OPN) is a secreted phosphoprotein with cytokine properties, implicated in many physiological and pathological processes, including infection and autoimmunity. We have previously identified up-regulated osteopontin transcripts in the pancreatic lymph nodes of the NOD (Non-Obese Diabetic) mouse at the pre-diabetic stages. Investigating the underlined disease initiating mechanisms may well contribute to the development of novel preventive therapies. Our aim was to construct *opn* null mice in a NOD autoimmune-prone genetic background and address the pathogenic or protective role of the osteopontin molecule in the early stages of type 1 diabetes.

Methods: We generated *opn* null mutant mice in a NOD genetic background by serial backcrossing to the existing C57BL/6 *opn* knockout strain. The presence of *opn* wild type or null alleles in the congenic lines was evaluated by PCR amplification. We used NOD *opn*-null mice to assess the phenotypic evolution of type 1 diabetes. The presence of OPN in the serum was evaluated by ELISA and by immunostaining on the mouse tissues. The primary gene structure of the NOD *opn* encoding gene and protein sequences were compared to the known alleles of other mouse strains. Evaluation of Single Nucleotide Polymorphisms (SNPs) variation between *opn* alleles of the *opn* gene is reported.

Results: In the absence of OPN, type 1 diabetes is accelerated, suggesting a protective role of this cytokine on the insulin-producing cells of the pancreatic islets. Conversely, in the presence of the *opn* gene, an increase of the OPN protein in the serum of young NOD mice indicates that this molecule might be involved in the immune regulatory events taking place at early stages, prior to disease onset. Our data support that OPN acts as a positive regulator of the early islet autoimmune damage, possibly by a shift of the steady-state of T1D pathogenesis. We report that the OPN protein structure of the NOD/*Shi*LtJ strain corresponds to the a-type allele of the osteopontin gene. Comparative analysis of the single nucleotide polymorphisms between the a-type and b-type alleles indicates that the majority of variations are within the non-coding regions of the gene.

Conclusions: The construction of *opn* null mice in an autoimmune genetic background (NOD.B6.Cg-*spp1*$^{-/-}$) provides important tools for the study of the implication of the OPN in type 1 diabetes,

offering the possibility to address the significance of this molecule as an early marker of the disease and as a therapeutic agent in preclinical studies.

Key words: autoimmune diabetes; osteopontin; congenic; knockout mice; NOD

Abbreviations: BB rat: BioBreeding rat;

NOD: Non Obese Diabetic;

OPN: osteopontin,

Th1: T helper 1;

GWAS: Genome Wide Association Studies;

SPP1: secreted phosphoprotein 1;

Eta-1: Early T lymphocyte activation-1

1. Introduction

Autoimmune diabetes is a multifactorial disease with genetics and environmental factors implicated in its aetiology. While over 40 loci are associated with the human and the mouse type 1 diabetes phenotype and a multitude of studies contributed to a comprehension of its pathomechanisms, the exact molecular events involved in its initiation remain elusive. Studies have addressed three major elements, all contributing to type 1 diabetes: i) the genetic predisposition, ii) the immune component and iii) the target tissue, *i.e.* the insulin-secreting beta cells in the pancreatic islets. Recently the implication of the target tissue, the beta cells of the pancreas, was extensively studied (James et al., 2018). The initial loss of self-tolerance and the immune mediated destruction of the beta cells indicate that local events are also important in the physiopathological equilibrium of the target organ. It has been proposed that beta cell senescence is part of type 1 diabetes pathogenesis and demonstrated that elimination of senescent beta cells prevents autoimmune destruction, thereof preventing disease (Thompson et al., 2019).

The existence of animal models, spontaneously developing type 1 diabetes, the BB rat (Hornum et al., 2002) and the NOD mouse (Anderson and Bluestone, 2005; Leiter, 1993; Wicker et al., 1995) were and continue to be fundamental to the advancement of our understanding of the disease. The NOD mouse is the experimental model of choice in the study of the genetics and immune physiopathology of type 1 diabetes (de Gouyon et al., 1993; Garchon et al., 1994; Melanitou et al., 2003; Melanitou et al., 1998; Reed and Herold, 2015). These mice spontaneously develop a disease with similar characteristics to the human type 1 diabetes (Makino et al., 1980). Interestingly, NOD mice develop autoimmune conditions against several organs and cells, other than the pancreatic islets, including the salivary, thyroid and adrenal glands, testis, thymus and red blood cells (Johansson et al., 2003). They also are susceptible to a Multiple sclerosis-like disease (Experimental autoimmune encephalomyelitis, EAE) after immunization with MOG_{35-55} peptide (Slavin et al., 1998). A common aetiology in autoimmune diseases at the genetic and immune regulation level has been suspected and explored (Baxter, 1997; Ramsdell and Ziegler, 2003; Rosenblum et al., 2015; Wicker et al., 1992). Specific HLA alleles are a key component of autoimmune disease aetiology. The particular MHC

Class II haplotype carried by the NOD genome is a major contributing risk factor for the development of type 1 diabetes (Bhatnagar et al., 2001; Johansson et al., 2003; Wicker et al., 1992). In particular the properties of the I-Ag^7 molecules of the NOD MHC are responsible for a defect in the negative selection of islet-antigen-specific T cells (Bhatnagar et al., 2001; Ridgway et al., 1998). Several genetic studies by us and others have identified non-MHC susceptibility loci in a pattern similar to human GWAS studies, using crosses of the NOD mouse with non-susceptible strains (de Gouyon et al., 1993; Driver et al., 2012; Melanitou et al., 2003; Melanitou et al., 1998). Due to its extensive use in the studies of type 1 diabetes, the strengths and pitfalls of the NOD mouse model are the subject of several discussions and reviews, mainly pointing out the advantages of this animal model in the study of the disease (Driver et al., 2012; Reed and Herold, 2015).

The multigenic nature of type 1 diabetes aetiology renders difficult the study of a single gene. Yet a single gene modification in the NOD genetic background offers a valuable model in the study of the disease-associated impact of the mutated gene.

In our previous work we have identified an increase of transcripts coding for OPN at an early age, prior to inflammation of the pancreatic islets in the NOD mice, by transcriptome analysis (Regnault et al., 2009). This observation ~~was~~ is in accordance with the early presence of insulin autoantibodies (Melanitou et al., 2004). OPN, also named SPP1 or Eta-1 is an extracellular matrix protein implicated in the regulation of a variety of biological events i.e. tissue morphogenesis and homeostasis, tissue regeneration with properties in wound healing and tissue repair, including immune responses. It is a Th1 cytokine expressed by a large variety of tissues and cells including immune-related cells and in particular activated T cells, B cells, macrophages (MF) (Weber et al., 2002) and dendritic cells (DC) (Shinohara et al., 2006) and it is highly induced during inflammation (Lund et al., 2009; Lund et al., 2013; Scatena et al., 2007). OPN is found to play a role in a variety of pathological processes including autoimmune diseases, certain cancers and infections (Fan et al., 2008; Wirestam et al., 2017; Wong et al., 2005). *Opn* deficient mice show severely impaired type-1 immunity to bacterial (*Listeria monocytogenes*) and to viral infections (herpes simplex virus-type 1) (Ashkar et al., 2000).

Several of these activities were attributed to the secreted form of OPN (sOPN) which acts through its receptors $\alpha_v\beta_3$ and CD44 by promoting Th1 development (Cantor, 2000; Shinohara et al., 2005)

and cellular resistance to apoptosis (Hur et al., 2007; Khan et al., 2002; Scatena et al., 1998) by inhibiting IL-10 and enhancing IL-6 responses (Weber et al., 2002). Alternative translation of a non AUG site downstream of the canonical AUG start codon generates intracellular OPN molecules (iOPN) (Shinohara et al., 2008a), identified to be part of cellular processes, including migration and motility (Junaid et al., 2007; Zhu et al., 2004). iOPN expression in plasmacytoid dendritic cells (pDCs) induces IFNα expression (Shinohara et al., 2006) and in conventional DCs promotes differentiation of Th17 cells (Shinohara et al., 2008a; Shinohara et al., 2008b). Th17 and Th1 cell components in type 1 diabetes patients affect the balance of T effector /T regulatory cells with an impact on disease outcome (Bradshaw et al., 2009; Shinohara et al., 2008b).

In order to investigate the role of OPN in the autoimmune diabetes phenotype, we constructed congenic mice in a NOD genetic background lacking the *opn* gene (NOD.B6.Cg-*spp1*^{tm1Blhj}/Pas) and we investigated the impact of OPN on type 1 diabetes. We investigated the primary sequence of the NOD *opn* gene and the single nucleotide polymorphisms contained in the gene locus. The implication of the osteopontin in the host response to various infectious microorganisms represents a possible breakthrough towards the study of the impact of infections on type 1 diabetes. This new knockout strain opens many interesting perspectives both for fundamental studies aiming to comprehend the early mechanisms leading to the disease and for preclinical evaluation of OPN-related preventive therapies.

2. Materials and Methods

2.1. Mice and Ethical statement

Pathogen-free NOD/ShiLtJ and the *opn* (*spp1*) null congenic B6.Cg-*Spp1*$^{tm1Blh/J}$ mice (Liaw et al., 1998) were purchased by the Jackson Laboratories (Bar Harbor, ME). All mice were bred under specific pathogen-free conditions, in the animal facility of the Institut Pasteur. All experiments were performed under the ethical approval for animal welfare of the Institutional committee and under strict accordance with the European guidelines (Directive 2010/63/EU) for animal care and use. Protocols were approved by the Institut Pasteur Ethics Committee (N°: 2013-0014) and all efforts were made to

minimize suffering. EM is authorized to perform experiments on vertebrates (Paris Department of Veterinary Services, DDSV).

2.2. Construction of opn null congenic lines

The NOD.B6.Cg-*spp1*$^{tm1Blh/J}$/Pas mice (abbreviated in the text as NOD.B6.Cg.*opn*$^{-/-}$) were created by serial backcrossing of the F1 B6.Cg-*Spp1*$^{tm1Blh/J}$ x NOD/ShiLtJ mice carrying the null *opn* gene into the NOD genetic background for up to generation BC14 (N15). The null *opn* allele was selected by PCR genotyping of the mice at each generation and mice carrying this allele were used for breeding. At the BC7 (N8) and at the BC14 (N15) generations the NOD *opn* knockout strain was established by brother/sister matting and the null allele was fixed at homozygocity. Internal control animals hemizygous or homozygous for the wild type allele also were selected by intercrossing at the BC7 generation and assessed for glycaemia.

2.3. DNA preparation and genotyping

DNA was prepared from mouse tail biopsies (0.5 mm) under standard methods, with the minor modifications as previously described (Melanitou et al., 1998), yielding a highly purified DNA preparation. The presence of wild type and null *opn* alleles was assessed by genotyping using the PCR primers previously described (Liaw et al., 1998). Briefly, a 600 bp endogenous fragment (wild type allele) was amplified by the following primers: OPNi3: 5'CCATACAGGAAAGAGAGACC3'; OPNi4: 5'AACTGTTTTGCTTGCATGCG3'. For detecting the null allele a 500 bp fragment was generated by PCR using the OPNi3 together with the Neo cassette primer: 5'CGTCCTGTAAGTCTGCAGAA3' as described (Liaw et al., 1998) (Fig. S1).

2.4. Phenotypic assessment of diabetes

Wild type (NOD) and *opn* mutant mice (NOD.B6-*opn*$^{-/-}$) were monitored from 4 to over 30 weeks of age, on a weekly basis, for the development of spontaneous diabetes. Blood glucose measurements were evaluated using an Elite glucometer (Bayer, Elkhardt, IN) and Glucoflex-R Blood Glucose test strips. Diagnosis of diabetes established by at least two consecutive no fasting glucose measurements, with values greater than 250 mg/dl. Throughout the construction of the knockout mice, animals were

regularly monitored for blood glycaemia. Representative data shown are from 53 BC7 (N8) mice (29 females and 24 males), 44 BC14 (N15) mice (24 females and 20 males), and 17 NOD wild type mice (10 females and 7 males) (Fig. 1A-C and Fig. S3). Animals were monitored for glycaemia measurements in the morning.

2.5. OPN protein quantification in the serum by enzyme-linked immunosorbent assay (ELISA)

Sera OPN protein concentrations from wild type NOD/ShiLtJ mice kept in our facility were evaluated by a quantitative sandwich immunoassay method using Quantikine mouse osteopontin ELISA kit (R&D Systems, Cat N° MOST00) as described (Giraud et al., 2019). The colour intensity corresponding to the OPN concentrations was read at OD 450 wavelength in an absorbance microplate reader (ELx800TM). Reconstitution of mouse OPN standard was used for the standard curve preparation by serial dilutions at eight concentrations (0, 39, 78, 156, 312, 625, 1250, 2500 pg/ml of OPN standard protein), all in duplicates (Fig. S2~~1~~). Samples were diluted at 300 fold. Buffers used were as supplied by the kit (https://www.rndsystems.com/products/mouse-rat-osteopontinopn-quantikine-elisa-kit_most00).

2.6. Histology

Histology analysis of mouse tissues (pancreas, pancreatic lymph nodes, thymus and kidney) were performed as previously described (Giraud et al., 2019; Melanitou et al., 2004) after tissue fixation by 10% formalin and embedded in paraffin. 5 µM sections were obtained and stained with H&E. The presence of OPN in the various tissues was evaluated by immunohistochemistry using anti-OPN specific antibodies (AF808, R&D systems, Minneapolis, MN, USA).

2.7 Preparation of T-RNA, PCR amplification and cDNA sequencing

The sequence of the *opn* cDNA from the NOD mice was confirmed in our laboratory. Total RNA was prepared from various tissues of the NOD mice as previously described (Giraud et al, 2019). Briefly the RNeasy Plus mini Kit (Qiagen) was used for RNA extractions from various NOD mouse tissues according to the instructions of the manufacturer (https://www.qiagen.com/us/). Reverse transcribed

T-RNA (10 µg) to first strand cDNA obtained by using random hexamers (Roche Diagnostics) and MMLTV-RT reverse transcriptase (Moloney Murine Leukemia Virus Reverse Transcriptase CAS N°: 9068-38-6). cDNA fragments were prepared for sequencing by PCR (polymerase chain reaction) using the *In Vitrogen* Taq polymerase kit (https://www.thermofisher.com/fr/, Cat N°: 10342053) with annealing temperature at 58°C. Two pairs of oligonucleotide primers were designed and used for the preparation of the NOD *opn* cDNA (Invitrogen, life technologies): OPN-6F: TGACCCATCTCAGAAGCAGA, OPN-6R: GGCTTTGGAACTTGCTTGAC (613 bp fragment) and OPN-7 F: CTGATGCCACAGATGAGGAC, OPN-7 R : CAGAAGCAAAGTGCAGAAGC (596 bp fragment). PCR cDNA fragments were prepared after separation in 2% low melt agarose gels, run in TBE buffer using the QIAEX II, (Qiagen) agarose extraction Kit. DNAs were eluted from the agarose fragments by adding 20 µl of 10mM Tris-HCl, pH 8.5, suspended by vortexing and incubate at 50°C for 5 min. After brief centrifugation (30 sec) the supernatants, containing the DNA fragments were collected. Optical Density (DO) of DNA concentration and DNA integrity by electrophoresis of 3 µl on agarose gels were evaluated. Purified PCR products (20ng/100 bases) were sent for sequencing at MilleGen^{R} Biotechnologies, by the Sanger method (http://www.millegen.com).

2.8 Comparative analysis of sequence variation of the NOD osteopontin gene.

cDNA and aminoacid sequences corresponding to the osteopontin gene of various strains of mice were retrieved from the Mouse Genome Informatics Databases (http://www.informatics.jax.org/). The public primary sequence of the NOD osteopontin gene and protein (MGP_NODShiLtJ_G0029615) was compared with the sequences of b-type allele carried by the C3H/J (MGP_C3HHeJ_G0029512), of a-type allele carried by C57Bl/6 (NP_001191130.1; MGI_C57BL6J_98389) and c-type allele of the DBA/2 strain (MGP_DBA2J_G0029627). The Clustal Omega software was used for sequences alignments (Sievers et al., 2011).

The *opn* gene locus on Chr5 spans 9,933 base pairs (chr5 position: 104,433,118 bp - 104,443,050 bp) (http://www.informatics.jax.org/marker/MGI:98389). For single nucleotide polymorphisms (SNPs) within the locus region all the above mentioned mouse strains were compared. Phylogenetic analysis

showed a close in-group proximity of the *Mus musculus* strains used in our analysis (by the Clustal Omega software (https://www.ebi.ac.uk/Tools/msa/clustalo/). Row data for SNPs characteristics are shown on Table S2.

2.9 Statistical analysis

The *p* values for significance between groups of animals were calculated by the two-tailed Z t-test using XLSTAT 7.5.2 version (P values are as indicated on legends of Figures). Survival curve analysis was performed by the Kaplan-Meier method and compared by the long rank test (Wilcoxon test) with PRIZM software (GraphPad, version 5.0).

3. Results

3.1. Construction and phenotypic characterisation of opn mutant NOD line

NOD congenic mice carrying a non-functional *opn* gene were constructed by introgressing the targeted mutation of the *opn* gene carried by the C57BL/6.Cg-*spp1*$^{-/-}$ strain (Liaw et al., 1998) into the NOD genetic background at the F1 generation and by repeated backcrossing thereafter into the NOD strain. Selection of the presence of the mutated allele performed in the breeders at each generation, up to backcross 14 (N15 generation, see Methods and Fig. S1). At the BC14 generation animals were intercrossed to generate homozygocity for the *opn* null alleles. The genetic background at this generation of the NOD.B6.Cg-*spp1*$^{-/-}$/*Pas* strain carries over 99.99% NOD genome.

Opn knockout mice were first evaluated for type 1 diabetes at the N8 generation (Fig. 1A). Both females and males showed a significant acceleration of type 1 diabetes in the absence of *opn* in comparison with the wild type strain (Fig. 1A; P<0.001 and P=0.003, respectively). Type 1 diabetes is observed as early as at 16 weeks of age in the knockout *versus* 20 weeks in the wild type females, (permanent glycaemia >250nmol/L; P<0.001, Fig. 1A). Similarly, a disease acceleration was observed in the male mutant strain, even though, compared to the females, the earliest appearance of glycaemia, was delayed at 24 weeks of age (P<0.003, Fig. 1A). Male versus female differences in disease onset are indeed a characteristic of the diabetes phenotypes in the NOD strain (Fig. S3) and as expected this

difference also was significant in the *opn* knockout strain (P<0.0001, Fig. 1B). Similar type 1 diabetes phenotypes were obtained at the N13 generation (Table 1). At this generation we established an internal control strain, homozygote for the wild type alleles (NOD.B6.Cg-*spp1*$^{+/+}$) and we compared type 1 diabetes onset to the NOD original strain. As expected no significant differences were observed in females nor in the males (P=0.052 and P=0.36 respectively, Table 1), indicating that the genetic information at least for type 1 diabetes, carried by the recipient strain was identical to the full congenic NOD.B6.Cg-*spp1*$^{+/+}$ (Table 1). Yet, while it cannot be excluded that remaining small chromosomal segments of a C57BL/6 origin may be present at this generation, they apparently have none or negligible impact to the disease.

Type 1 diabetes phenotypic assessment was finally addressed at the N15 generation in which the knockout transgene is in a nearly homogeneous NOD genetic background. We established four phenotypic windows according to the age of the mice and evaluated for disease onset (Fig. 1C). Comparative analysis between the *opn* wild type and knockout females demonstrated significant disease differences for all phenotypic windows studied (Fig. 1C, P<0.0001), whilst in the male animals type 1 diabetes differences were observed after 26 weeks of age (Fig. 1C, 26-30 wks: P=0.019; >31 wks: P<0.0001). As expected, these differences also remained significant when male versus female knock out animals were compared (Fig. 1D, P<0.0001) as well as when male versus female knockout hemyzygotes carrying one *opn* null allele were compared (p=0.036, Table 1).

The presence of one *opn* allele delayed the onset of Type 1 diabetes in the N13 female mice when compared to the knockout animals (Fig. 2). To note however that i) *opn* gene hemyzygocity attenuated the differences between female and male animals (Table 1) and ii) the type 1 diabetes phenotypic differences observed between the NOD wild type strain and the presence of one or two *opn* alleles are compatible with an OPN dose effect on the disease phenotype (Fig. 2).

3.2. Early opn gene expression in the NOD mouse

Opn transcripts were previously identified in the pancreatic lymph nodes (PLN) of the NOD mice and correlated with the early presence of Insulin Autoantibodies (E-IAA) (Melanitou et al., 2004; Regnault et al., 2009). We identified OPN protein in the sera of female NOD mice (Fig. 3 ~~2~~ and Fig.

S2). Higher concentrations of the protein were observed at 5 weeks of age followed by a decrease at older ages (Fig. 3 ~~2~~). However the presence of OPN in the sera remained at approximately 200 ng/ml throughout life (Fig. 3). Male mice did not show the same pick of osteopontin at 5 weeks of age. This is not surprising as i) type 1 diabetes onset is observed at a later age in males than in females and ii) type 1 diabetes follows the presence of E-IAA which is also an early type 1 diabetes-related phenotype in the female mice (Melanitou et al., 2004).

We then examined the presence of OPN protein in various tissues of the NOD wild type mice (Fig. 4A-F). OPN protein is detectable by immunostaining as early as at 3 weeks of age in the pancreatic islets of the NOD mice and remains detectable up to 9 weeks (Fig. 4A-C). The pancreatic ducts also stain for OPN protein (Fig. ~~4~~B, at 5 weeks, blue arrows).

Discreet OPN protein staining was detected in the B and T cell zones and in the medullary cords of the PLN (Fig. 4D), in the medullary cortex of the thymus, probably due to OPN expression from small numerous lymphocytes (Fig. 4E) and in the cells lining the lumen of the kidney tubules (Fig. ~~4~~F). While small lymphocytes are present in the normal thymic medulla, they are less dense than in the cortex. O*pn* gene expression in the kidney was previously reported, with a renoprotective action in renal injury (Wuthrich, 1998; Xie et al., 2001), in contrast *opn* gene expression in the thymus, to our knowledge, has not been reported previously.

3.3 The NOD strain carries the a-type alleles of osteopontin

Osteopontin is implicated in the host response to several infectious microorganisms (Table S1a) (Sampayo-Escobar et al., 2018) as well as in several autoimmune conditions (Clemente et al., 2016; Kaleta and Boguska, 2017; Rittling and Singh, 2015). Due to its pleiotropic properties, OPN may be an ideal potential molecular link between the environment, with an emphasis on pathogenic microorganisms and the autoimmune response. Moreover several autoimmune diseases, including type 1 diabetes, are triggered or even protected by various pathogenic agents (Table S1b) (Ercolini and Miller, 2009). Several alleles have been reported coding for the osteopontin gene and are associated with various autoimmune diseases (Trivedi et al., 2011). Gene polymorphisms were found to be associated with various forms of cancer (Kamal et al., 2017) and infections in animals (Bissonnette,

2018; Giraud et al., 2019) and in human populations as well as with autoimmune diseases (Lavi et al., 2017), including type 1 diabetes (Marciano et al., 2009). We compared the NOD osteopontin amino acid (Fig. 5 & Fig. S4) and nucleotide sequence (Fig. S5) with the published sequences of various strains of mice, specifically the ones commonly used in the studies of the host response to infections. In addition, we sequenced the NOD cDNA and demonstrated that this strain contains the a-type alleles of osteopontin (Ono et al., 1995). Our sequence is in agreement with the publicly available *opn* sequence of the NOD strain (Fig. 5). Aiming to address the implication of genetic variations of the osteopontin gene locus in type 1 diabetes in the NOD mouse, we also searched for variations of single nucleotide polymorphisms (SNPs) between the NOD and the commonly used strains of mice, especially in studies of the host response to infections (Table S2). Several SNPs were identified in the NOD locus on mouse chromosome 5 containing the *spp1* gene (a-type allele) coding for osteopontin, when compared with the C3H (b-type allele) and DBA (c-type allele). The *opn* alleles of the C57Bl/6 and the BALB/c strains are similar to the NOD a-type allele (Fig 5 and Table S1). Comparison of SNPs between a-type and b-type alleles showed that the majority of the polymorphisms are located within the non-coding regions of the gene locus (Fig 6). Phylogenetic analysis confirmed the predicted small in-group variation rate (0.00017%) between strains NOD and C57BL/6 (a-type alleles), while the a-type strains are relatively more genetically distant to the b-type alleles containing strain C3H (0.01468%) (Fig. 6). The amino acid differences between alleles of the protein are shown on figure 5. In particular, three amino acid differences are located **i**) near the RGD (Arg-Gly-Asp) integrin binding site responsible for the interaction with several integrin receptors (Christensen et al., 2012); **ii**) near a phosphorylation site which is important for posttranslational modifications of the OPN protein (Jono et al., 2000), conferring pro-apoptotic and pro-inflammatory properties to the protein (Gao et al., 2016) and **iii**) adjacent to the heparin binding site at the C-terminal part of the protein. In the human, OPN heparin- binding domains are associated with internalization signals (Clemente et al., 2016). The heparin binding domain maintains the OPN in the full-length form by blocking the cleavage of the protein by the thrombin (Platzer et al., 2011). At present, it is not known if these amino acid differences impact any of the OPN disease-related phenotypes. However, the type 1 diabetes differences reported herein, using the NOD.B6.Cg-*spp1*^{tm1BlhJ}/Pas strain are clearly related to the

absence of the protein. The contribution of the osteopontin a-type alleles to additional autoimmune diabetes-related molecular mechanisms cannot be excluded. Additional studies should take in consideration the role, if any, of these allelic differences in the fine tuning of the implication of this protein in the disease, including in the target tissue responses, particularly in the β insulin-producing cells of the pancreatic islets.

4. Discussion

Our initial data of the expression patterns of the OPN protein in the sera of the NOD mice (Fig. 2) have suggested a link of OPN with the early pathophysiological changes leading to type 1 diabetes and are in agreement with published reports that have identified OPN to be up regulated in the serum of type 1 diabetes patients (Berezin and Kremzer, 2013; Talat et al., 2016) and prediabetic children (our unpublished observations). However several questions remained unclear: i) the cell or tissue origins of the peripheral protein as it cannot be attributed with certainty to gene expression from the pancreatic lymph nodes and ii) the role of OPN, protective or aggressive for type 1 diabetes. Indeed OPN is a secreted phosphoprotein expressed by a variety of cells and tissues and it was shown that it is also expressed by the pancreatic islets (Gong et al., 2009). In one study the presence of OPN autoantibodies identified in the sera of human type 1 diabetes patients found to be directed against OPN expressed in the islet somatostatin cells (Fierabracci et al., 1999), indicating that the exact cell cross-talk of the pancreatic islets and the implication of OPN is not clearly defined. A vital role of OPN was suggested (Aspord et al., 2004). These authors also identified early expression of *opn* transcripts in the pancreatic islets of diabetic mice at the early phases of the disease. The correlation of the presence of OPN in the pancreatic lymph nodes, in the pancreatic islets and in the sera at the early pre-inflammatory stages indicates the implication of this molecule at the initiation and progression of type 1 diabetes. However, as mentioned above, it was not clear if a protective or inversely a role in promoting disease kick off may be attributed to this molecule.

We hypothesized that at this pre-inflammatory stage possible initial repair mechanisms may take place. Indeed tissue homeostasis may be regulated by a balance between pro- or anti-inflammatory mechanisms implicating i) the genetic predisposition of the NOD genetic background to

the disease or ii) an inherent resistance of the organism to the evolution of the disease. OPN may be implicated in both conditions due to its described cell survival and anti-apoptotic properties (Khodavirdi et al., 2006; Lee et al., 2007). It is also a pro-inflammatory cytokine associated with the local accumulation of lymphocytes and macrophages (Ashkar et al., 2000; Wang and Denhardt, 2008). These properties are compatible with a protective or inversely with an aggressive pro-disease role of this molecule.

Recently it was reported that deletion of *opn* in the C57BL/6 genetic background, results in various minor β-cells defects that in a healthy organism can be compensated (Wendt et al., 2017). While our data reported herein, together with other published observations (Berezin and Kremzer, 2013; Gong et al., 2009; Talat et al., 2016), are in agreement with an association of the OPN with type 1 diabetes, the exact contribution of the OPN molecule to T1D was pending. Aiming to clarify this question we constructed *opn* knockout mice in the NOD autoimmune-prone genetic background. Phenotypic evaluation of these mice demonstrated that in the absence of *opn*, type 1 diabetes is accelerated, therefore establishing a protective role of the OPN in the islets. One hypothesis is that the presence of the protein in the pancreatic islets as identified by immunostaining (Fig. 43A-C), may protect the β cells from senescence and eventually apoptosis at the early stages of the disease prior to the immune attack by T cells and other lymphocytes. This is supported by the type 1 diabetes differences observed between *opn* wild type and *opn* null NOD mice at the early ages (15-25 weeks) of the female mice, whilst after 26 weeks these differences are less pronounced (Fig 1A). Indeed OPN plays a regulatory role on phagocytosis and enhances cell survival by inhibiting apoptosis (Wang and Denhardt, 2008). It is interesting to note that the protective role of OPN in type 1 diabetes is in contrast with the implication of this molecule in multiple sclerosis. OPN is found to participate in the relapsing stages of the disease, whilst disease remission is observed by anti-OPN antibodies (Clemente et al., 2017). It was reported that the intracellular isoform of osteopontin is involved in inhibition of the Th17 cell response in vitro and in vivo, while mice carrying osteopontin-deficient dendritic cells develop a dominant pathogenic Th1 response at least in the central nervous system (CNS) (Shinohara et al., 2008b). The pleiotropic properties of this protein may account for a pro-inflammatory role in multiple sclerosis (Ashkar et al., 2000; Jansson et al., 2002; O'Regan and Berman, 2000; O'Regan et

al., 2000a; O'Regan et al., 2000b), but inversely for an immunoprotective role in type 1 diabetes. Osteopontin may also participate in wound healing mechanisms in the beta cells of the pancreas.

Our observations concerning the variations of the primary structure of the *opn* gene and OPN protein, showed i) a high conservation of the protein between the different strains of the laboratory mice; ii) confirmed the presence of the a-type allele in the NOD, BALB/c and C57Bl/6 mice and iii) underlined the amino acid variations between the NOD a-type and the b-type alleles.

The functional properties of the OPN protein, in particular its role in inflammatory processes and in immune modulation as well as its implication in cell survival and wound healing certainly represent an interest in the study of the pathogenic mechanisms involved in type 1 diabetes. Moreover OPN is a pluripotent molecule found to be associated with the host response to various infectious microorganisms.

Additional studies using our NOD *opn* null mice are required especially in defining the OPN-related mechanisms implicated in type 1 diabetes protection. Moreover OPN addition therapy in type 1 diabetes and OPN inhibition in multiple sclerosis, may represent potential therapeutic strategies for these diseases and should be evaluated using our mouse model in preclinical studies.

5. Conclusions

The construction of *opn* null NOD mice, reported herein, represents an interesting tool in the studies aiming to address the pathological mechanisms in type 1 diabetes, as well as in deciphering the implication of the environment and in particular of infectious microorganisms on this disease. Additional studies of the allelic differences of the OPN protein and *opn* gene locus between the NOD and the commonly used strains of mice are required to address their relation, if any with the pleiotropic properties of this protein.

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Data availability

The data generated and described in the study are included in the Manuscript and in the supplementary file. All data related to this study are available from the author by reasonable request.

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Declaration of competing interest

The author declares that there is none competing interests associated to this study.

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Legends to Figures

Fig. 1. T1D onset is accelerated in the absence of OPN at the N8 generation of the NOD.B6-opn$^{-/-}$ congenic strains. (A) Representative data of survival curves comparison between NOD wild type (n=17) and *opn* knockout mice (n=44), females (NOD.B6-opn$^{-/-}$: n=20; NOD: n=10; P<0.0001) and males (NOD.B6-opn$^{-/-}$: n=24; NOD: n=7; P=0.003). **(B)** Survival curves comparison between females (n=20) and males (n=24) NOD.B6-opn$^{-/-}$ mice (P<0.0001). **(C)** Phenotypic windows according to the age of T1D onset in the presence or in the absence of OPN at the N15 generation. Ages are as indicated on the x axis. Comparative analysis of T1D between NOD/*LtJ* and NOD.B6-*opn*$^{-/-}$ females (n=10 and n=24 respectively) and between males (n=7 and n=29 respectively). **(D)** Phenotypic windows of T1D in male and females NOD.B6-*opn*$^{-/-}$ knockout mice at N15 generation. Representative data are from 24 F and 29 M at the N15 generation. Wilcoxon test statistics are: ***P<0.0001, **P=0.0004; *P=0.019.

Fig. 2. Contribution of osteopontin alleles in T1D phenotype. Representative data of survival curves between female NOD wild type (n=17) and mice homozygous (n=44) or hemyzygotes (n=10) for the *opn* knockout allele at the N13 generation. P=0.4671 (ns) for the *spp1*$^{+/-}$ hemyzygotes versus NOD wild type (Gehan-Breslow-Wilcoxon Test).

Fig. 3. Longitudinal analysis of OPN concentrations in the NOD/*LtJ* mouse serum. Osteopontin knockout C57 BL6.Cg-*spp1*$^{-/-}$ mice were used for negative control (green triangle). Mean values+/- SD are shown on the table. For comparison C57Bl/6 serum OPN content is: 287, 53 ng/ml and C57Bl/6.Cg. *Spp1*$^{-/-}$ is: 0 as expected. Representative data are from the mean concentrations of the sera of at least 3F mice (+/- STDEVA).

Fig. 4. OPN immunostaining in NOD mouse tissues. (A) pancreas from NOD/*Shi/LtJ* female mice at 3 weeks; **(B)** at 5 weeks and **(C)** at 9 weeks of age. **(A-C)** OPN protein is detected in the pancreatic ducts (blue arrows) and in the islets (black arrows) is shown. **(D)** OPN immunostaining in PLN is

shown in brown and specific parts of the tissue are indicated; **(E)** in the thymus, OPN is apparent as discrete dark brown staining of the connective tissue at the thymic cortex areas. **(F)** OPN is expressed in the kidney, in cells lining the lumen of the tubules**. (A-C)** female NOD/*LtJ* mice at 8-9 weeks of age.

Fig. 5. Primary structure of the amino acid sequence of the osteopontin protein. **A.** Sequence comparison of the NOD protein with the ostepontin a-type and b-type alleles. Amino acid differences are highlighted; integrin binding, phosphorylation and heparin binding sites are indicated by the boxes. The OPN sequence of the NOD shown is from our cDNA sequence which is identical to the published sequence (OPNa: MGP_NODShiLtJ_G0029615 or MGI_C57BL6J_98389). OPNb corresponds to MGP_C3HHeJ_G0029512. **B.** Description of the amino acid changes of the OPN protein.

Fig. 6. Comparative analysis of Single Nucleotide Polymorphisms (SNPs) between a-type (NOD/*ShiLtJ*) and b-type (C3H/*HeJ*) alleles of the *spp1* gene locus. **A.** The locus on Chr5 spans from 104,433,118-104,443,050 bp (9,933 bp) (http://www.informatics.jax.org/marker/MGI:98389). Polymorphisms (SNPs) for each region of the locus are indicated as a % versus the total number (149 SNPs). **B.** Phylogenetic Tree showing the in-group distance of the *Mus musculus* strains used in our analysis (Clustal Omega software https://www.ebi.ac.uk/Tools/msa/clustalo/) (Madeira et al., 2019; Sievers et al., 2011). Close in-group distance between NOD/*ShiLtJ* and C57Bl/6J 0 (0.00017%); NOD/ShiLtJ and C3H/*He*J (0.01468%). Row data for SNPs characteristics are shown on Table S2.

Supporting information

Legends to Figures

Fig. S1. Construction of NOD.B6-*opn*$^{-/-}$ mice. (A) The seven exons of *opn* are designated in the blue boxes. Positions of the opn primers amplifying the wild type alleles are OPn3 and Opn4 and the mutant alleles OPn3 and Neo1360. (**B)** Agarose gel electrophoresis (1.5%) of PCR fragments for wild type (WT and knockout (KO) alleles as described by Liaw et al, 1998 (Reference (Liaw et al., 1998) in the text).

Fig. S2. Standard curve for OPN ELISA concentrations of OPN in the NOD mouse serum. Dilutions: 0, 39, 78, 156, 312, 625, 1250, 2500 pg/ml of OPN standard protein, each point in duplicate as described in the Methods section.

Fig. S3. Type 1 diabetes phenotypes in NOD/*Shi/LtJ* wild type mice. Males (7 animals) and females (10 animals) from our colony. Data are presented as percentages of T1D.

Fig. S4. Comparative sequence analysis of the entire OPN (SPP1) proteins between strains of mice. C3H/J: MGP_G0029512; C57Bl/6: MGI_98389; NOD: G0029615; DBA/2: MGP_G0029627 and NOD sequence issued from the thymus cDNA sequence. Signal peptide of the secreted form of the protein is underlined in yellow. Highly conserved peptide region located at the N-terminal extremity of the mature protein is underlined in blue. Cell attachment sequence RGD at position 159. *Thrombin cleavage site: R168-S169 and **Calcium Binding domain: D216-S228. ****Heparin binding site.

FIG. S5. Single Nucleotide Polymorphisms (SNPs) comparative analysis of the *Spp1* gene locus between NOD/*ShiLt*J and C3H/*He*J & DBA/2J strains of mice. **A.** SNPs were extracted from the *spp1* locus on Chr5 as mentioned in figure 4 (http://www.informatics.jax.org/marker/MGI:98389). SNPs percentages for each region of the locus are shown. **B.** Phylogenetic Tree showing the close in-group proximity of the *Mus musculus* strains used in our analysis (Clustal Omega software (https://www.ebi.ac.uk/Tools/msa/clustalo/) (Madeira et al., 2019). Scales are represented by the number of differences between sequences: NOD/*ShiLt*J and C57Bl/6J, BALB/cJ & DBA: 0 (0.00017%); C3H/*He*J versus all four other strains: 0.01468%. Row data for SNPs characteristics are shown on Table S2).

Table 1

Table 1: Summary of the contribution of the *opn* gene in Type 1 Diabetes phenotypes. Cumulative T1D frequency by the ages of the mice. F: Females; M: Males

Crosses	**Gender (age)**	**Number of animals (T1D vs Total)**	**P value for T1D differences**
NOD/LtJ	F vs M (up to 25 wks)	2/10 vs 1/7	P=0.068 (ns)
NOD/LtJ	F vs M (26-30 wks)	5/10 vs 2/7	P=0.002**
NOD/LtJ	F vs M (31-40 wks)	8/10 vs 2/7	P<0.0001***
N8 NOD.B6-opn$^{-/-}$ vs NOD/LtJ	F (up to 25 wks)	12/20 vs 2/10	P<0.001***
N8 NOD.B6-opn$^{-/-}$ vs NOD/LtJ	M (up to 25 wks)	5/24 vs 2/7	P=0.003***
N8 NOD.B6-opn$^{-/-}$	F vs M	17/20 vs 15/24	P<0.0001***
N13 NOD.B6-opn$^{+/+}$ vs NOD	F (>30 wks)	5/6 vs 8/10	P=0.0529 (ns)
N13 NOD.B6-opn$^{+/+}$ vs NOD	M (>30 wks)	4/6 vs 5/7	P=0.3699 (ns)
N13 NOD.B6-opn$^{+/-}$	F vs M	9/10 vs 7/7	P=0.036*
N15 NOD.B6-opn$^{-/-}$ vs NOD/LtJ	F (<20 wks)	12/24 vs 2/10	P<0.0001***
N15 NOD.B6-opn$^{-/-}$ vs NOD/LtJ	F (>31 wks)	23/24 vs 8/10	P=0.0004***
N15 NOD.B6-opn$^{-/-}$ vs NOD/LtJ	M (<20 wks)	4/29 vs 1/7	P=0.183 ns
N15 NOD.B6-opn$^{-/-}$ vs NOD/LtJ	M (>31 wks)	17/29 vs 2/7	P<0.0001***
N15 NOD.B6-opn$^{-/-}$	F vs M	23/24 vs 17/29	P<0.0001***

Figure 1

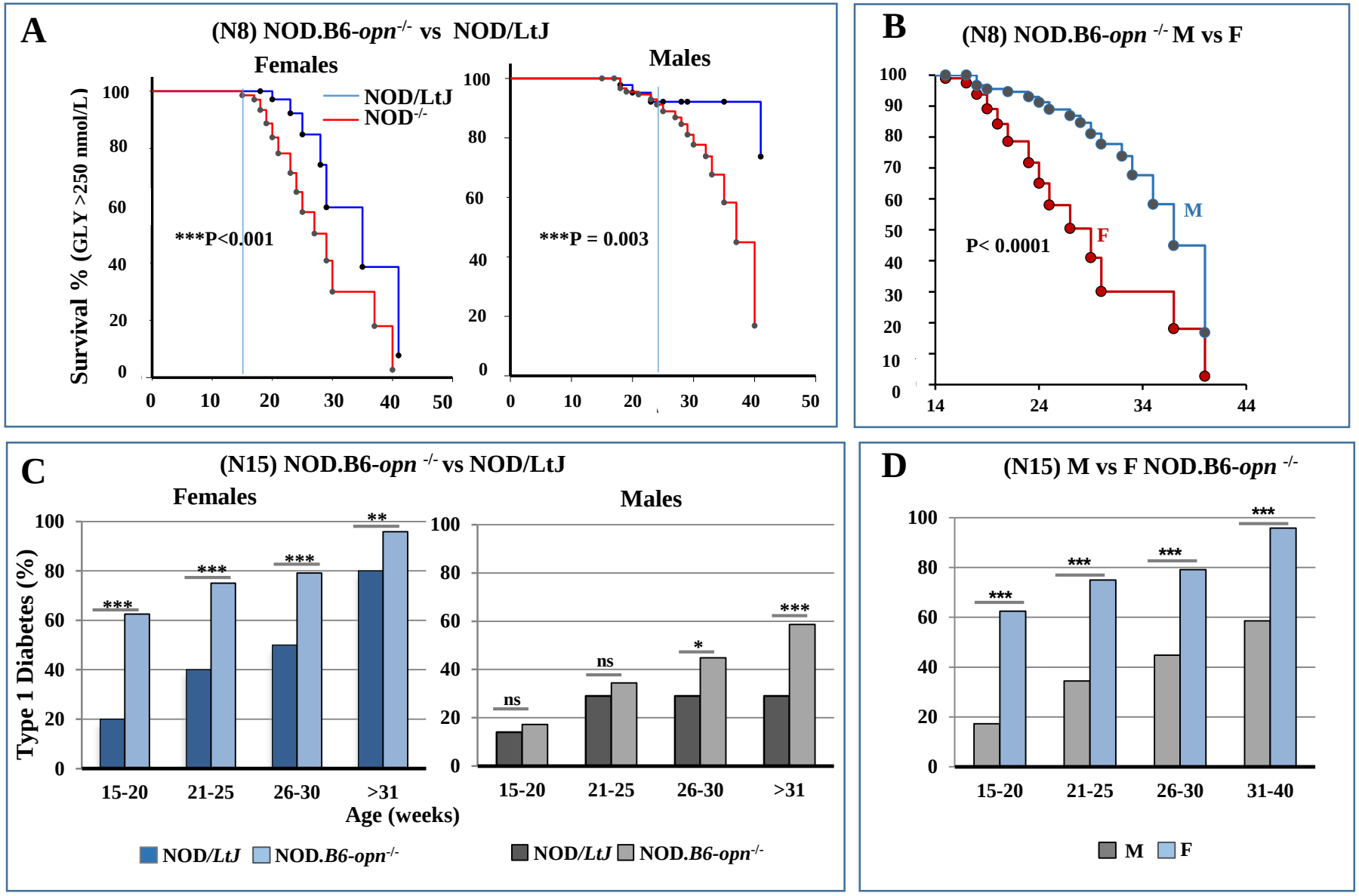
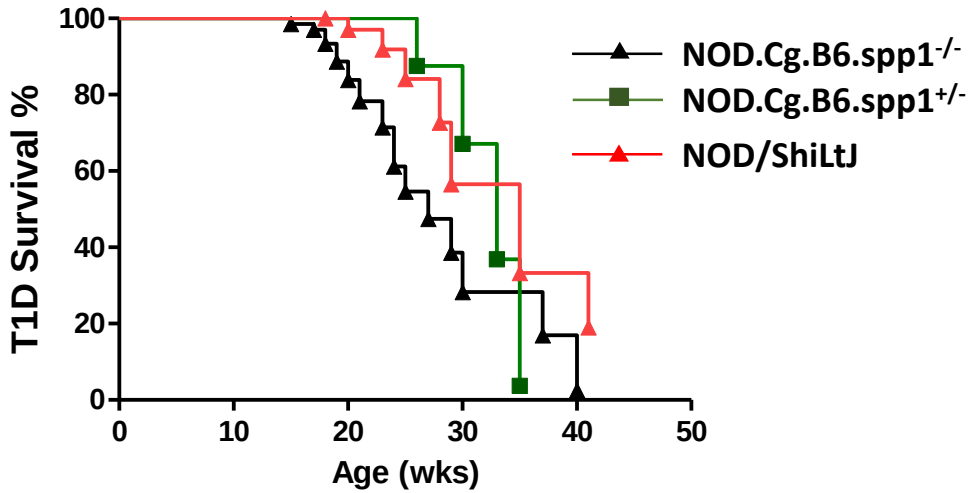
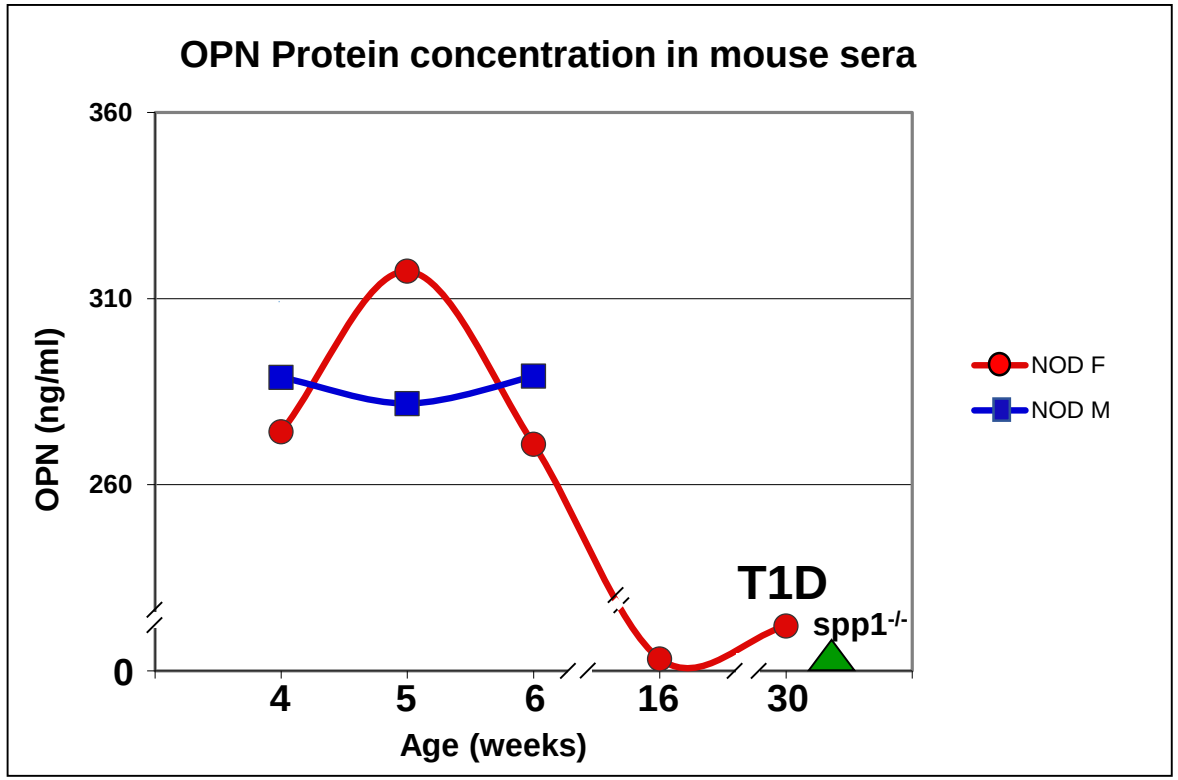

A
(N8) NOD.B6-opn -/- vs NOD/LtJ
Females
Males
Survival % (GLY >250 nmol/L)
NOD/LtJ
NOD -/-
***P<0.001
***P = 0.003
B
(N8) NOD.B6-opn -/- M vs F
M
F
P< 0.0001
C
(N15) NOD.B6-opn -/- vs NOD/LtJ
Females
Males
Type 1 Diabetes (%)
15-20
21-25
26-30
>31
Age (weeks)
NOD/LtJ
NOD.B6-opn -/-
ns
D
(N15) M vs F NOD.B6-opn -/-
31-40
M
F

Figure 2



Strain	P value
NOD.Cg.B6.spp1$^{-/-}$ *vs* **NOD.Cg.B6.spp1$^{+/-}$**	P<0.0001***
NOD.Cg.B6.spp1$^{+/-}$ *vs* NOD	ns
NOD.Cg.B6.spp1$^{-/-}$ *vs* NOD	P<0.0001***

Figure 3



Sample	4 weeks OPN [ng/ml]	5 weeks OPN [ng/ml]	6 weeks OPN [ng/ml]	16 weeks OPN [ng/ml]	30 weeks OPN [ng/ml]
NOD F (n=3)	274,19+/-52	317,36+/-30	270,86+/-23	213,19+/-20	222,03
NOD M (n=5)	288,83+/-52	281,83+/-36	289,15+/-29		

Figure 4

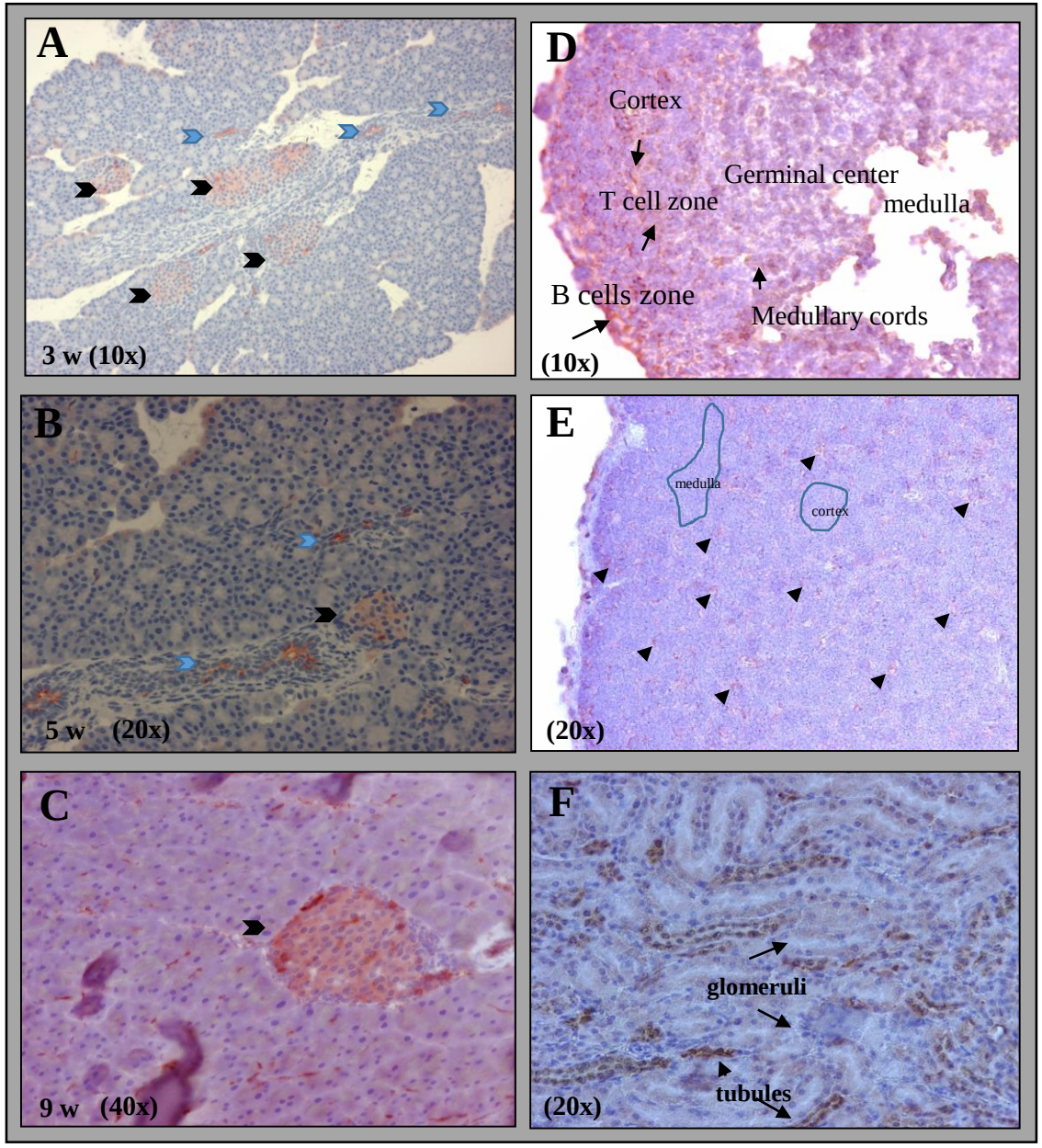

A
3 w (10x)
D
Cortex
Germinal center
T cell zone
medulla
B cells zone
Medullary cords
(10x)
B
5 w (20x)
E
medulla
cortex
(20x)
C
9 w (40x)
F
glomeruli
tubules
(20x)

Figure 5

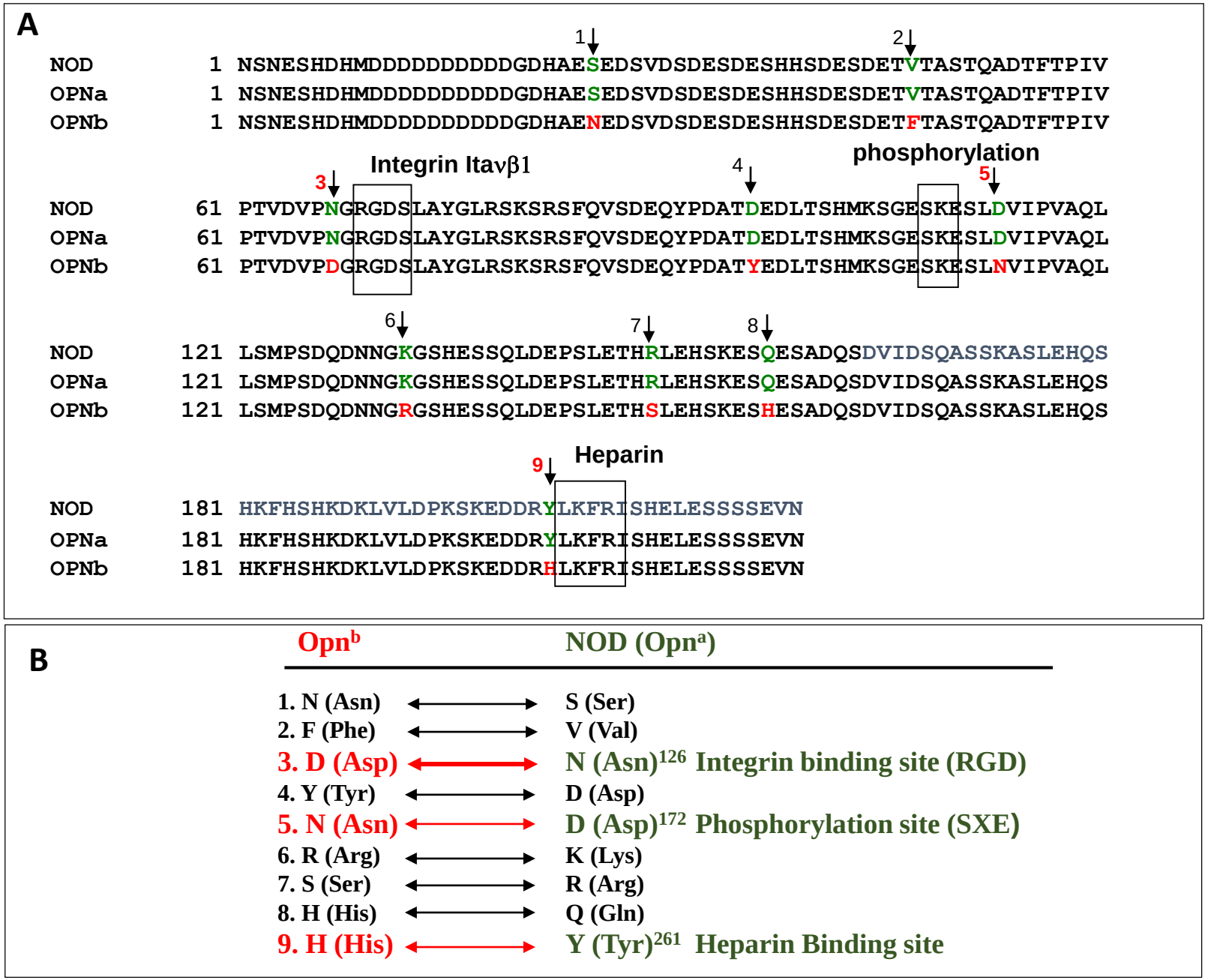
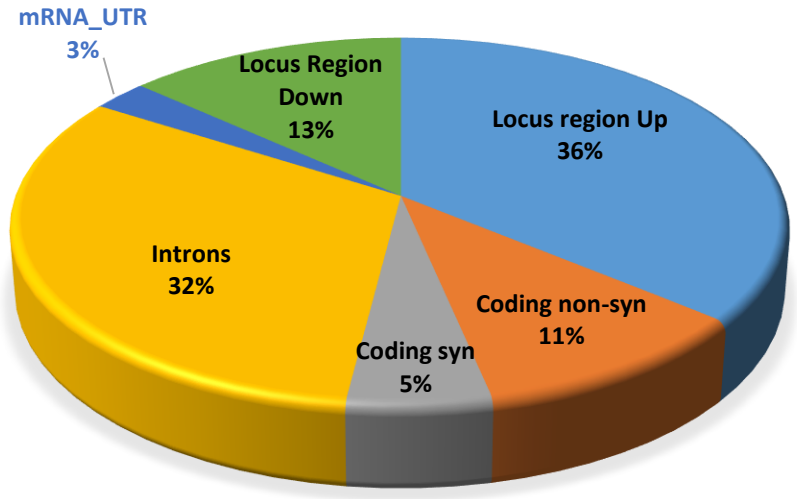
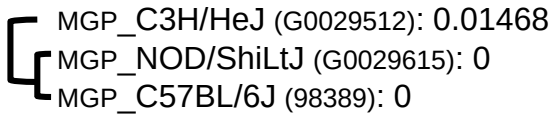

A
1
2
NOD 1 NSNESHDHMDDDDDDDDDDGDHAESEDSVDSDESDESHHSDESDETVTASTQADTFTPIV
OPNa 1 NSNESHDHMDDDDDDDDDDGDHAESEDSVDSDESDESHHSDESDETVTASTQADTFTPIV
OPNb 1 NSNESHDHMDDDDDDDDDDGDHAENEDSVDSDESDESHHSDESDETFTASTQADTFTPIV
Integrin Itavβ1
phosphorylation
3
4
5
NOD 61 PTVDVPNGRGDSLAYGLRSKSRSFQVSDEQYPDATDEDLTSHMKSGESKESLDVIPVAQL
OPNa 61 PTVDVPNGRGDSLAYGLRSKSRSFQVSDEQYPDATDEDLTSHMKSGESKESLDVIPVAQL
OPNb 61 PTVDVPDGRGDSLAYGLRSKSRSFQVSDEQYPDATYEDLTSHMKSGESKESLNVIPVAQL
6
7
8
NOD 121 LSMPSDQDNNGKGSHESSQLDEPSLETHRLEHSKESQESADQSDVIDSQASSKASLEHQS
OPNa 121 LSMPSDQDNNGKGSHESSQLDEPSLETHRLEHSKESQESADQSDVIDSQASSKASLEHQS
OPNb 121 LSMPSDQDNNGRGSHESSQLDEPSLETHSLEHSKESHESADQSDVIDSQASSKASLEHQS
Heparin
9
NOD 181 HKFHSHKDKLVLDPKSKEDDRYLKFRISHELESSSSEVN
OPNa 181 HKFHSHKDKLVLDPKSKEDDRYLKFRISHELESSSSEVN
OPNb 181 HKFHSHKDKLVLDPKSKEDDRHLKFRISHELESSSSEVN
B
Opnb
NOD (Opna)
1. N (Asn) S (Ser)
2. F (Phe) V (Val)
3. D (Asp) N (Asn)126 Integrin binding site (RGD)
4. Y (Tyr) D (Asp)
5. N (Asn) D (Asp)172 Phosphorylation site (SXE)
6. R (Arg) K (Lys)
7. S (Ser) R (Arg)
8. H (His) Q (Gln)
9. H (His) Y (Tyr)261 Heparin Binding site

Figure 6

A. SNPs: *spp1* gene locus a-type vs b-type alleles



B. Phylogenetic Tree



Supplementary material for on-line publication only

Click here to download Supplementary material for on-line publication only: Supplement FIG S1-S5.pdf

Table S1

Click here to download Supplementary material for on-line publication only: Table S1a & 1b.pdf

Table S2

Click here to download Supplementary material for on-line publication only: Table S2.xlsx

CRediT author statement

Evie Melanitou conceptualize the project, design the genetic methodology, performed the experiments, analyze the data and wrote and edit the manuscript.

***Declaration of Interest Statement**

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

*Title page (contains Article title, author name, affiliation, etc)

Investigation of type 1 diabetes in NOD mice lacking the osteopontin gene

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