REPRODUCTION

NAMPT (visfatin) in the chicken testis: influence of sexual maturation on cellular localization, plasma levels and gene and protein expression

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Abstract

Nicotinamide phosphoribosyltransferase (NAMPT) is a cytokine hormone and rate-limiting enzyme involved in production of NAD and therefore affects a variety of cellular functions requiring NAD. Spermatogenesis and testicular steroidogenesis are likely to depend on NAD-dependent reactions and may therefore be affected by changes in testicular NAMPT expression. The objectives of the present study are to investigate testicular NAMPT expression as well as plasma NAMPT levels in prepubertal and adult chickens. By RT-PCR, *NAMPT* cDNA expression was detected in prepubertal and adult chicken testes. Using immunohistochemistry, NAMPT was predominantly localized in the nucleus of myoid cells, Sertoli cells, and Leydig cells in the prepubertal chicken testis. In adult chickens, however, NAMPT-immunostaining was observed in the cytoplasm of Leydig cells, Sertoli cells, primary spermatocytes, secondary spermatocytes, round spermatids, and elongated spermatids, but not in the spermatogonial cells. Using real-time quantitative PCR, adult chicken testis was found to contain fourfold greater *NAMPT* mRNA quantity compared with prepubertal chickens. Testicular NAMPT protein quantities determined by western blotting were not significantly different between adult and prepubertal chicken testes. Using immunoblotting, NAMPT was detected in the seminal plasma and sperm protein extracts obtained from chicken semen. Plasma NAMPT levels, determined by enzyme immunoassay, were at least 28-fold higher in the adult chickens compared with prepubertal male chickens. Taken together, sexual maturation is associated with several changes in testicular NAMPT expression indicating that NAMPT is likely to play a significant role in testicular functions such as spermatogenesis and steroidogenesis.

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Introduction

Nicotinamide phosphoribosyltransferase (NAMPT, also known as visfatin) a cytokine hormone and an enzyme, has recently received much attention due to its potential role in obesity, dyslipidemia, type II diabetes mellitus, and immune disorders (Chen *et al*. 2006, Haider *et al*. 2006, Brentano *et al*. 2007, Varma *et al*. 2007). Originally identified 40 years ago as NAMPT (Dietrich *et al*. 1966, Powanda *et al*. 1969, Streffer & Benes 1971), it was found to act as an intracellular enzyme that catalyzes the rate-limiting conversion of nicotinamide to nicotinamide mononucleotide (NMN), a substrate in the biosynthesis of NAD. Several years later, the peptide was rediscovered as pre-B cell colony-enhancing factor, a presumptive cytokine that enhances maturation of B-lymphocyte precursors in the presence of interlukin-7 and stem cell factor (Samal *et al*. 1994). Although lacking a signal peptide, NAMPT is believed to be secreted in an as yet uncharacterized pathway (Tanaka *et al*. 2007), and has been detected in both milk (Yonezawa *et al*. 2006) and in blood circulation. Elevated plasma NAMPT levels have been associated with a variety of conditions including type II diabetes (Chen *et al*. 2006, Yilmaz *et al*. 2008) and polycystic ovarian syndrome (Chan *et al*. 2007). The biologically active form of NAMPT appears to be essential for life, as homozygous mice carrying a mutant *NAMPT* allele are embryonic lethal at day 10.5 (Revollo *et al*. 2007). Conversely, haplodeficiency of the *NAMPT* gene in female mice resulted in defects in NAD biosynthesis and glucose-stimulated insulin secretion from pancreatic islets due to a decrease in both intracellular and plasma NAMPT levels (Revollo *et al*. 2007). Interestingly, these results were sex specific, as such a decrease in NAMPT levels were not observed in the male $NAMPT^{+/-}$ mice (Revollo *et al*. 2007). While previous studies have

shown NAMPT expression in a variety of tissues including testes (Rongvaux *et al.* 2002, Revollo *et al.* 2007), the possible contribution of testicular NAMPT has not yet been explored.

Recently, we have shown that chicken *NAMPT* cDNA and protein are highly homologous to mammalian NAMPT (Krzysik-Walker *et al.* 2008). In addition, NAMPT protein levels were elevated with age in the skeletal muscle and plasma of male chickens. Similar to mammalian NAMPT expression, our preliminary studies indicated that NAMPT is ubiquitously expressed in various tissues of the chicken, including the testes. In order to further characterize testicular NAMPT expression during sexual maturation, broiler breeder male chickens were utilized for the current study. Broiler breeder males are genetically selected for production of rapidly growing progenies that are able to attain a 40-fold increase in body weight within 42 days post-hatch. Consequently, broiler breeder chickens are an excellent model to study the influence of NAMPT, a molecule that is important for energy metabolism, on testicular function. During sexual maturation, we presume that NAD biosynthesis would be vital to support spermatogenesis and produce energy to enable sperm survival and mobility. Therefore, based on the role of NAMPT in NAD biosynthesis, we hypothesized that testicular NAMPT expression would increase when broiler breeder male chickens undergo sexual maturation.

The objectives of the current study were to characterize testicular NAMPT gene and protein expression and determine plasma NAMPT levels in prepubertal and adult chickens. We provide novel evidence that *NAMPT* gene expression is significantly increased in the testis of adult male chickens compared with prepubertal chickens and is accompanied by a 28-fold increase in plasma NAMPT levels. Furthermore, our data indicate that NAMPT expression within the testis undergoes a dramatic shift from nuclear localization in myoid cells and Leydig cells in prepubertal chickens to cytoplasmic localization in primary spermatocytes, Sertoli cells, Leydig cells, and postmeiotic cells within the seminiferous tubules of adult chicken testis. We also present evidence that NAMPT is detectable in seminal plasma and sperm protein extracts, suggesting that NAMPT could play an important role in sperm physiology.

Results

Detection of NAMPT cDNA in testis by RT-PCR

A 309-bp partial cDNA encoding *NAMPT* was amplified from total RNA extracted from prepubertal (4- or 14-week-old) and adult (29-week-old) chickens (Fig. 1). The nucleotide sequence of the resultant product was found to be identical to that of *NAMPT* cDNA
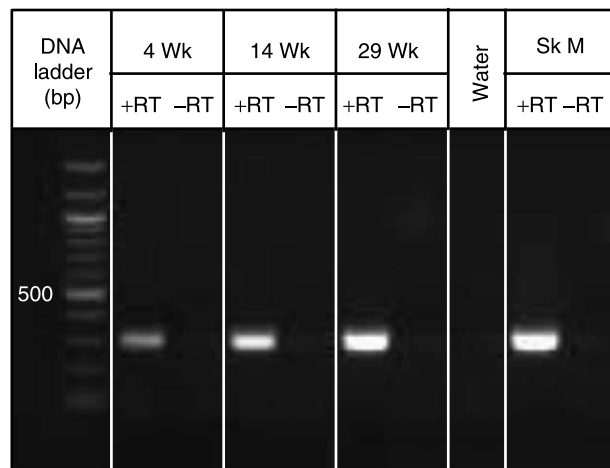

Figure 1 RT-PCR analysis of *NAMPT* gene expression in the testes of prepubertal and adult chickens. Total RNA was extracted, DNase-I digested, and RT (+RT). Approximately 50 ng of cDNA was used as template to amplify a 309-bp chicken *NAMPT* cDNA. Skeletal muscle cDNA was included as a positive control. Contamination controls consisted of RNA from each tissue without RT addition (−RT) or substitution of water for the cDNA template. 4 Wk, 4-week-old prepubertal chicken; 14 Wk, 14-week-old prepubertal chicken; 29 Wk, 29-week-old adult chicken, Sk M, skeletal muscle.

expressed in chicken adipose and skeletal muscle. Furthermore, a 1482-bp full-length *NAMPT* cDNA was also amplified from the chicken testis (data not shown). The use of either water in place of cDNA template or cDNA that was not reverse transcribed did not produce any PCR product (Fig. 1), confirming the absence of genomic DNA contamination.

Localization of NAMPT-immunoreactive cells in the testis of prepubertal and adult chickens

Prepubertal (14-week-old) chicken testis

NAMPT immunolabeling within the prepubertal chicken testis is shown in Fig. 2A–D. Around the seminiferous tubules, NAMPT immunostaining was found in the myoid cells that appeared flattened (Fig. 2A, C and D). In addition, several cells in the interstitial space revealed NAMPT immunostaining in the nucleus, possibly representing Leydig cells (Fig. 2A and D). Within the seminiferous tubules, NAMPT immunostaining was found in the nucleus of Sertoli cells and primary spermatocytes (Fig. 2A and C). Preadsorption of the NAMPT antibody with the immunogen greatly reduced or completely abolished NAMPT immunostaining in the prepubertal chicken testis indicating that the immunostaining was specific (Fig. 2B).

Adult (29-week-old) chicken testis

NAMPT immunostaining within the adult chicken testis is shown in Fig. 3A–I. NAMPT-immunostained cells
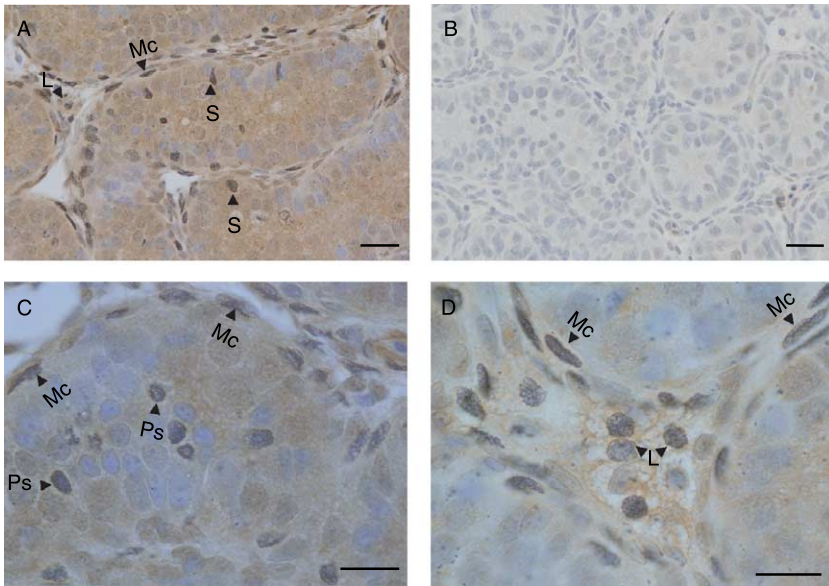
Figure 2 (A–D) Representative photomicrographs of testicular tissue sections from prepubertal chickens showing NAMPT-immunostained cells. Testicular tissue sections were immunostained using anti-human NAMPT antibody and counterstained with hematoxylin. (A, C, and D) NAMPT-immunostaining in the nuclei of myoid cells (Mc), Sertoli cells (S), primary spermatocytes (Ps), and Leydig cells (L). (B) Negative control using preadsorbed antibody in place of primary antibody. Scale bar, 20 μm.

were predominantly observed in the seminiferous tubular adluminal and luminal compartments as well as in the interstitium (Fig. 3A, C, D and F). In contrast to the prepubertal chicken testis, NAMPT immunostaining was noticed in the cytoplasm but not in the nucleus (Fig. 3G–I). In addition, NAMPT immunostaining was not observed in the peritubular myoid cells or in the spermatogonial cells (Fig. 3C, E and G). However, NAMPT immunostaining was noticed in the cytoplasm of primary spermatocytes (Fig. 3G and H), secondary spermatocytes (Fig. 3G and I), round spermatids (Fig. 3D, F and G), and elongated spermatids (Fig. 3D, G and I). Intense NAMPT immunostaining was observed in the seminiferous tubular lumen (Fig. 3F), which is likely to be part of Sertoli cell syncytia and/or spermatozoa. Leydig cells in the interstitium (Fig. 3C) and Sertoli cell cytoplasm in the adluminal compartment (Fig. 3C and D) exhibited strong NAMPT immunostaining. This pattern of NAMPT immunostaining was similar in seminiferous tubules of different development stages within the adult chicken testis. Preadsorption of the NAMPT antibody with the immunogen greatly reduced or completely abolished staining indicating that the immunostaining was specific in the adult chicken testis (Fig. 3B).

Relative quantity of testicular NAMPT mRNA by real-time quantitative PCR

Testicular *NAMPT* mRNA quantity was significantly greater in adult chickens compared with prepubertal chickens (Fig. 4; $P < 0.01$). Melting curve analyses showed the presence of a single PCR product for *NAMPT* mRNA or 18S mRNA, confirming the specificity of the reactions (data not shown).

Detection and quantification of testicular NAMPT protein from prepubertal and adult chickens

In order to detect NAMPT protein in the chicken testis and determine whether NAMPT protein quantity is different between prepubertal and adult chicken testes, a western blot analysis was performed. Our previous study had established the specificity of an anti-human NAMPT antibody to detect chicken NAMPT (Krzysik-Walker *et al.* 2008). A 52-kDa NAMPT protein was detected in the protein extracts obtained from both prepubertal (14-week-old) and adult (29-week-old) chicken testes (Fig. 5A). The molecular weight of testicular NAMPT protein was identical to that expressed in chicken skeletal muscle, adipose tissue, and liver (Krzysik-Walker *et al.* 2008). NAMPT protein quantity relative to ACTB quantity was not significantly different between prepubertal and adult chicken testes (Fig. 5B; $P > 0.05$). Similar to testicular protein, a 52-kDa NAMPT protein was detected in the seminal plasma and sperm protein extracts obtained from chicken semen (Fig. 6). In addition, we observed a lower molecular weight ($\sim$28 kDa) NAMPT protein in both sperm protein extract and in seminal plasma. Chicken adipose and liver protein extracts contained a similar 28-kDa NAMPT protein (Krzysik-Walker *et al.* 2008), which may have resulted from proteolytic degradation (Samal *et al.* 1994) or due to alternative splicing, as tissue-specific visfatin splice variants have been previously reported in the pig (Chen *et al.* 2007).

Quantification of plasma NAMPT levels in prepubertal and adult chickens

In order to determine whether sexual maturation affects plasma NAMPT levels, we quantified plasma NAMPT in prepubertal (4- and 14-week-old) and adult

(29-week-old) chickens. Plasma collected from adult chickens contained significantly greater levels of NAMPT when compared with that of prepubertal chicken plasma (Fig. 7; $P<0.02$). The intra-assay coefficient of variation (CV) was found to be 13.85% using pooled chicken plasma as reference standard.

The same reference standard yielded an intra-assay CV of 6.46% in a previous study (Krzysik-Walker *et al.* 2008). However, the greater intra-assay CV observed in the present study should not affect our interpretation as the plasma visfatin levels were higher by 28-fold in adult chickens compared with that of prepubertal chickens.
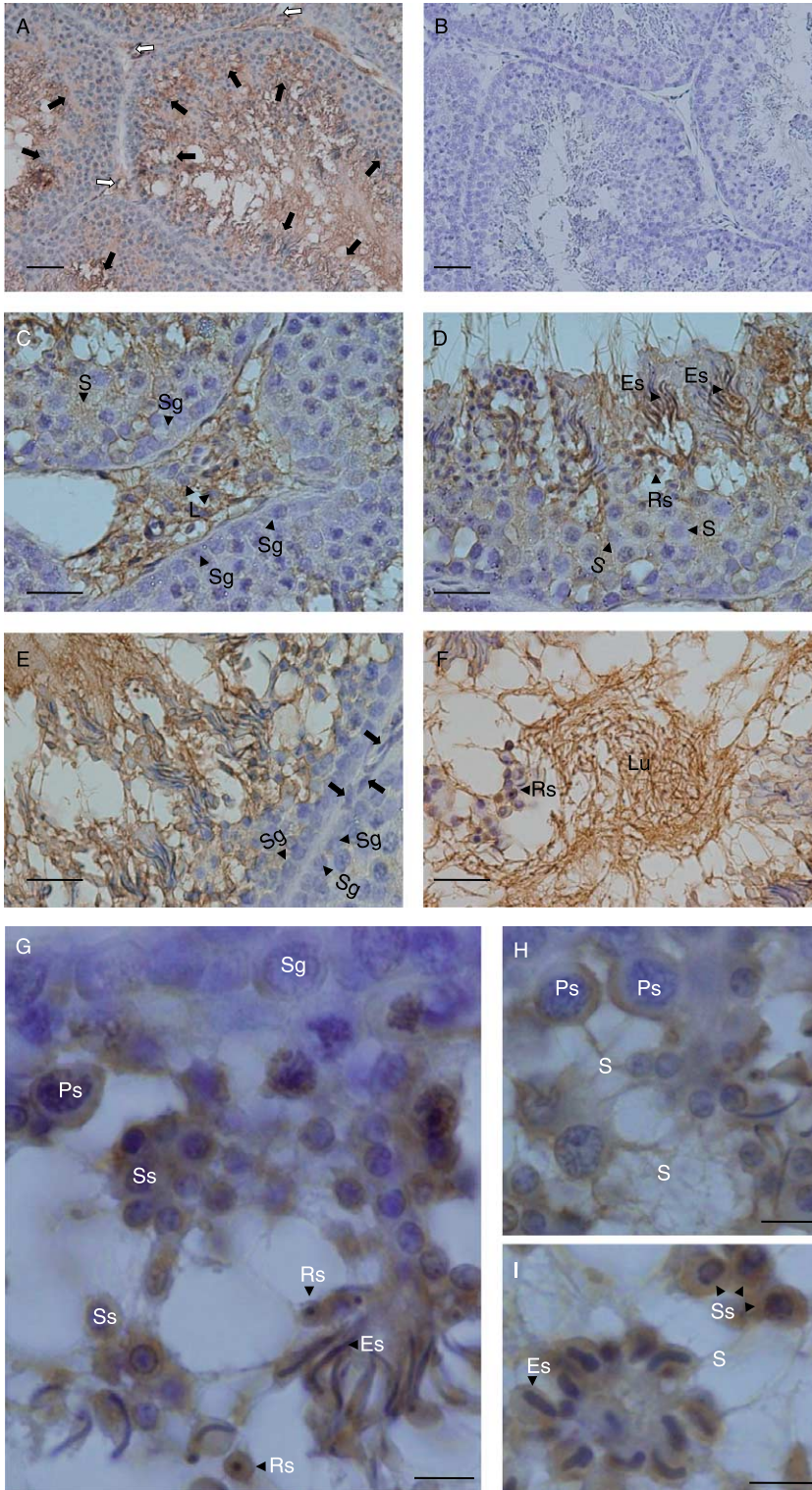

Figure 3 (A–I) Representative photomicrographs of testicular tissue sections from adult chickens showing NAMPT immunostaining. Testicular tissue sections were immunostained using anti-human NAMPT antibody and counterstained with hematoxylin. Seminiferous tubules showing NAMPT immunostaining (black arrows) in the proluminal compartment and in the interstitium (white arrows) (A). NAMPT immunostaining was observed in Sertoli cells (S; C, D, H and I), Leydig cells (L; C), primary spermatocytes (Ps; G and H), secondary spermatocytes (Ss; G and I), round spermatids (Rs; D, F and G), and elongated spermatids (Es; D, G and I). Seminiferous tubular lumen (Lu; F) showed intense NAMPT immunolabeling representing Sertoli cell syncytia and/or spermatozoa. Negative control using preadsorbed antibody in place of primary antibody showed no specific immunostaining (B). NAMPT immunostaining was not observed in spermatogonial cells (Sg; C, E and G) or in myoid cells (black arrows; E). Scale bar: 20 μm (A–F); 10 μm (G–I).
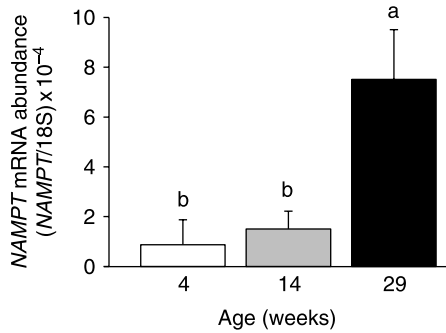
Figure 4 *NAMPT* mRNA abundance relative to 18S mRNA in prepubertal and adult chicken testes. Total RNA from prepubertal (4- or 14-week-old) and adult (29-week-old) chicken testis was extracted, DNase-I digested, and reverse transcribed. Approximately 50 ng of cDNA was used in real-time quantitative PCR. Data are represented as mean±s.e.m. Different letters above each bar indicate significant difference at $P<0.05$ ($n=4$–6/age group).

Discussion

The present study is the first to characterize NAMPT expression within the testis of avian species. We provide novel evidence to suggest that testicular NAMPT expression and plasma NAMPT levels significantly increase as the chicken becomes sexually mature. Using RT-PCR and western blot analysis, we found that NAMPT is expressed in the prepubertal and adult chicken testis. This is consistent with previous reports on the detection of NAMPT in the testis of pigs (Chen *et al*. 2007) and mice (Revollo *et al*. 2007).

The present study suggests that the subcellular localization of NAMPT varied with sexual maturation in the chicken testis. This shift in NAMPT subcellular localization with sexual maturation may reflect either the metabolic or proliferative state of the cells. A previous study had observed that NAMPT was localized to the nucleus of nondividing PC-12 cells, while subcellular NAMPT localization was found in the cytoplasm of proliferating PC-12 cells (Kitani *et al*. 2003). In addition to altered subcellular localization, expression of NAMPT in myoid cells was influenced by sexual maturation. *NAMPT* gene expression appears to be mechanically sensitive in amniotic epithelial cells, with expression increasing in response to acute stretching (Nemeth *et al*. 2000, Ognjanovic *et al*. 2005). Furthermore, stretching-induced NAMPT expression protected the amniotic epithelial cells from apoptosis (Kendal-Wright *et al*. 2008). Based on these findings, we hypothesize that the expanding seminiferous tubules in adult testis may have compressed the myoid cells and led to the absence of NAMPT expression in these cells.

A physiological role of testicular NAMPT is currently unknown in any species. Previous studies have shown that NAMPT is a rate-limiting enzyme in the biosynthesis of NAD (Revollo *et al*. 2004). Therefore, NAMPT

expression in the testicular cells may augment NAD production from nicotinamide, which is then used to support the heightened metabolic activity of Sertoli cells and postmeiotic cells. In addition, NAD is an essential cofactor for the activity of a group of deacetylases called sirtuins (SIRT). In mouse fibroblasts, NAMPT treatment lead to a dose-dependent increase in both cellular NAD levels as well as SIRT1 expression (Revollo *et al*. 2004). SIRT1 itself appears to be an important enzyme for testicular function as SIRT1 deficiency has been found to attenuate spermatogenesis and germ cell function in mice (Coussens *et al*. 2008). Moreover, male *Sirt1*$^{-/-}$ knockout mice had increased proportion of mature sperm with elevated DNA damage, while a microarray analysis of SIRT1-deficient testis revealed dysregulated expression of 85 genes that are involved in spermatogenesis and protein sumoylation (Coussens *et al*. 2008). In addition to increasing NAD production, NAMPT has been found to increase expression of vascular endothelial growth factor (VEGFA) and VEGFA receptor-2 in vascular endothelial cells through activation of the MAP kinase (MAPK1) and phosphatidylinositol-3-kinase/AKT1 pathways (Adya *et al*. 2008). VEGF increases
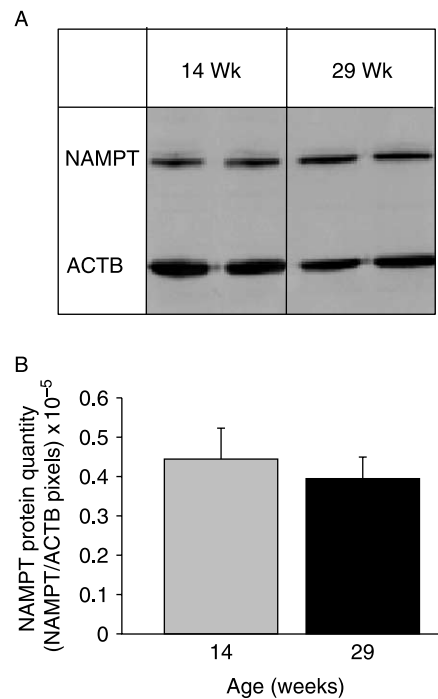

Figure 5 (A and B) Detection and quantification of NAMPT protein in the prepubertal and adult chicken testis. (A) Representative blot from two prepubertal (14-week-old) and two adult (29-week-old) chicken testes showing NAMPT and ACTB immunostaining. Approximately 10 μg of total protein extract from each testis was electrophoresed and blotted onto PVDF membrane. NAMPT was detected by sequential immunostaining using a rabbit anti-human NAMPT antibody and mouse anti-chicken ACTB antibody. (B) Quantity of NAMPT protein relative to ACTB in prepubertal (14-week-old) and adult (29-week-old). Densitometric data are represented as mean±s.e.m. ($n=5$/age group).
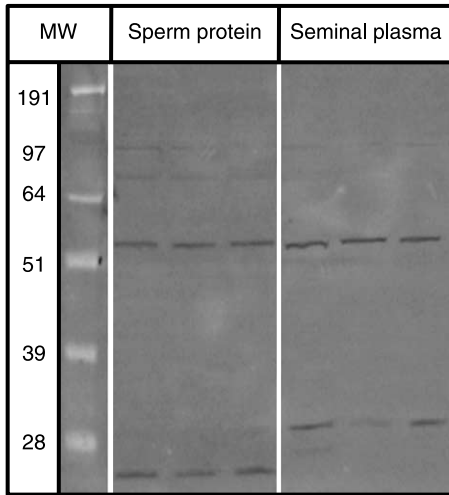
Figure 6 Detection of NAMPT protein in chicken sperm cell protein extract and seminal plasma. Sperm cells and seminal plasma from semen ejaculates from three breeder chickens were separated by centrifugation. Approximately 20 μg of total protein from seminal plasma or sperm protein extract from each chicken was electro-phoresed and blotted onto PVDF membrane. NAMPT was detected by immunostaining using a rabbit anti-human NAMPT antibody.

vascular endothelial cell proliferation in the testis (Rudolfsson *et al*. 2004) and can therefore affect testicular vascularization. VEGF expression has been detected in mouse Sertoli cells, whereas its receptors are located in spermatocytes and spermatids (Nalbandian *et al*. 2003). Hypoxia increases testosterone release in the mouse TM3 cell line mediated by increased VEGFA production (Hwang *et al*. 2007). Interestingly, hypoxia leads to an increase in *NAMPT* gene expression in adipocytes and in a breast cancer cell line (Bae *et al*. 2006, Segawa *et al*. 2006). Collectively, it appears that NAMPT can play a major role in testicular cellular metabolism, vascularization, and in Sertoli cell and Leydig cell functions.

The present study found that plasma NAMPT levels were 28-fold higher in adult chickens compared with prepubertal chickens. This elevated plasma NAMPT in the adult chicken may be due to several factors such as body weight, muscle mass, age, hormones associated with sexual maturation, and/or chronic feed/calorie restriction. For optimal reproductive performance, the broiler breeder males used in this study were provided with a restricted amount of feed (2 h of feeding to an equivalent of 350 calories per day) according to the feeding schedule recommended by the breeder company (Longnecker Hatcheries, Elizabethtown, PA, USA). In a previous study, plasma NAMPT levels did not change in 8-week-old broiler chickens that were fasted for 48 h (Krzysik-Walker *et al*. 2008). However, 48-h fasting did appear to increase NAMPT expression in the rat, suggesting that NAMPT is functioning as a stress- and nutrient-sensitive molecule (Yang *et al*. 2007). While the NAMPT protein lacks a cleavable signal peptide and is

thought to be released from cells by a nonclassical pathway (Tanaka *et al*. 2007), the source and function of circulating NAMPT are still unknown. Nonetheless, the NAMPT activity of NAMPT may catalyze NAD synthesis from nicotinamide in the blood circulation, and produce precursors that are then available for cellular uptake and homeostasis of intracellular NAD levels (Billington *et al*. 2008). Indeed, one such precursor, NMN, has been detected in the blood circulation and extracellular fluid of mice (Revollo *et al*. 2007).

We found that testicular *NAMPT* mRNA quantity was fourfold greater in adult chickens compared with prepubertal chickens. Testicular NAMPT protein quantity, however, was not different between adult and prepubertal chickens. Such a discordance in NAMPT protein versus mRNA quantity may be due to several factors, such as lack of translation of *NAMPT* mRNA, rapid turnover of NAMPT protein, and/or a change in localization of NAMPT in myoid cells to germ cells within the seminiferous tubules in the adult testes or secretion of NAMPT. It is also likely that some of the *NAMPT* mRNA is contained in the sperm that did not undergo translation.

In summary, sexual maturation is associated with an increase in testicular NAMPT expression and circulating NAMPT levels in broiler breeder chickens. Further studies are required to elucidate the role of testicular NAMPT in spermatogenesis and steroidogenesis.

Materials and Methods

Animals

Broiler breeder male chickens (Ross strain) were maintained by Longnecker Hatcheries according to the recommended maintenance schedule. Prepubertal (4- and 14-week-old) and adult (29-week-old) male chickens were utilized in this study. Prepubertal chickens were maintained under 8 h light:16 h darkness photoperiod and were provided water and feed
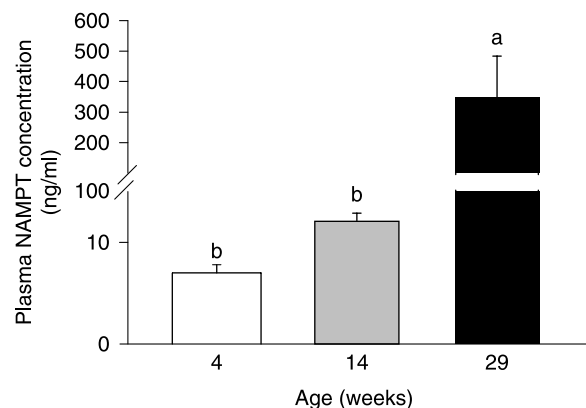

Figure 7 Plasma NAMPT levels in prepubertal (4- or 14-week-old) and adult (29-week-old) as determined by enzyme immunoassay. Different letters above each bar indicate significant difference at $P<0.05$. Data are represented as mean±s.e.m. ($n=6$/age group).

ad libitum. Adult chickens (29-week-old) were maintained under 16 h light:8 h darkness photoperiod and provided with water *ad libitum* but were feed restricted following a feeding program recommended for broiler breeder males (Aviagen, Huntsville, AL, USA). Chickens were killed by cervical dislocation prior to the removal of testis. One testis from each chicken was snap frozen in liquid nitrogen, while the other testis was collected for histological analyses. All animal procedures were carried out in accordance with the Pennsylvania State University Institutional Animal Care and Use Committee.

RNA extraction and cDNA production

Total RNA was extracted from prepubertal and adult chicken testes (*n*=6/age group) using Trizol (Invitrogen) and RNEasy kit (Qiagen) following manufacturer's protocol. The epididymal region was not dissected out of the testes, and therefore, testicular RNA may contain epididymal RNA as well. The quality and quantity of testis total RNA were determined by measuring absorbance at 260 and 280 nm using a spectrophotometer (ND-1000, NanoDrop, Wilmington, DE, USA). Following on-column DNase-I (Qiagen) treatment, first strand cDNA was synthesized by reverse transcribing 1 µg total RNA using random primer 12 and 2U Moloney murine leukemia virus RT (New England Biolabs, Beverly, MA, USA) in 20 µl reactions.

RT-PCR analysis

Approximately 50 ng of single-stranded testis cDNA, or skeletal muscle cDNA as a positive control, was used as template to amplify a 309-bp product of *NAMPT* with the following primer sequences: forward 5′-CAG-GAATAGGAGCTTCAGCCCATTTGGT-3′ and reverse 5′-GTGCCTCTGGACTTCGGCCTTCAATTAT-3′. A touchdown PCR was performed using 300 mM forward and reverse primers, 300 mM dNTP (Roche Biochemicals), and 1 U Taq polymerase (New England Biolabs) with the following thermocycle parameters: 94 °C for 1 min, 32 cycles of 94 °C for 5 s, and 72–68 °C for 3 min. Annealing and primer extension were done at 72, 70, and 68 °C during 1–5, 6–10, and 11–32 cycles respectively. The PCR products were subjected to agarose gel electrophoresis and ethidium bromide staining for visualization. For negative controls, RT reactions using 1 µg total RNA from each tissue with no RT ($-$RT) or water were used as a template in place of RT reactions that contained RT ($+$RT). The resultant partial *NAMPT* cDNA, as well as the full-length *NAMPT* cDNA amplified using primers previously described (Krzysik-Walker *et al*. 2008), was sequenced (Davis Sequencing, Davis, CA, USA) to confirm authenticity.

Immunohistochemical localization of NAMPT in prepubertal and adult chicken testes

Tissue preparation

Prepubertal (14-week-old) and adult (29-week-old) chicken testes (*n*=3/age group) were fixed by immersion in fresh 4% paraformaldehyde for 2–3 h at room temperature, cut into smaller equal-sized pieces, and fixation continued in 4% paraformaldehyde at 4 °C for ∼18 h. Testicular pieces were then washed in PBS (pH 7.4), dehydrated, cleared, and embedded in paraffin using an automated tissue processor (Thermo Fisher, Kalamazoo, MI, USA). Tissue sections (4 µm) were cut using a rotary microtome (Microm, Walldorf, Germany), and serial sections were mounted on Superfrost Plus glass slides (VWR, West Chester, PA, USA).

Immunostaining

Paraffin tissue sections were deparaffinized in Histoclear (National Diagnostics, Atlanta, GA, USA) and hydrated in descending concentrations of ethyl alcohol in water and rinsed in Tris-buffered saline (TBS; 0.01 M Tris–HCl and 0.15 M sodium chloride solution, pH 7.4). Following several washes in TBS containing 0.1% Triton X-100 (TBSX; Sigma), slides were treated in a blocking solution (2% normal goat serum in TBSX) for 1 h. Slides were incubated with rabbit anti-NAMPT antibody (1:400; Phoenix Pharmaceuticals, Belmont, CA, USA) at 4 °C for ∼18 h. The rabbit anti-human NAMPT antibody used for immunostaining was raised against a 51-amino acid synthetic human NAMPT peptide (amino acid residues between 400 and 450), which is 98.1% homologous to the corresponding chicken NAMPT sequence. Following washes in TBS, sections were incubated for 1 h with biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA). Tissue sections were washed in TBS and then treated for 1 h with an avidin peroxidase complex (Vector ABC Elite kit, Vector Laboratories). A brown colored NAMPT immunostain was developed using 3′,3′-diaminobenzidine tetrahydrochloride and hydrogen peroxide (Vector Laboratories). After several washes in TBS, tissue sections were lightly counterstained with hematoxylin, dehydrated, cleared in Histoclear, and coverslipped. To determine the specificity of immunostaining, the primary antibody (anti-human NAMPT antibody) was preadsorbed with 25 µg/ml immunogen (51 amino acid synthetic human NAMPT peptide; Bethyl Laboratories, Montgomery, TX, USA) for 18 h and centrifuged to pellet the antibody–antigen complexes. As a negative control, the supernatant was substituted in place of the primary antibody during immunohistochemical staining. Unless otherwise noted, all steps were performed at room temperature, with wash steps consisting of three ten-minute incubations in TBS buffer. NAMPT-immunostained testicular tissue sections were examined, and photomicrographs were taken using an Olympus BX61 microscope fitted with Olympus DP71 digital camera (Olympus America, Melville, NY, USA).

Determination of NAMPT *mRNA quantity by real-time quantitative PCR in prepubertal and adult chicken testes*

Total RNA extracted from prepubertal (4- and 14-week-old) and adult (29-week-old) chicken testes (*n*=4–6/age group) was reverse transcribed using random primers as described above and subjected to real-time quantitative PCR. Chicken *NAMPT* mRNA and 18S mRNA were quantified separately

utilizing 2.5 µl of the RT reaction (equivalent to 50 ng single stranded cDNA) as template. The real-time quantitative PCR consisted of 1× Platinum SYBR Green qPCR Super Mix-UDG (Invitrogen) and 300 nM of forward and reverse primers containing the following sequences: *NAMPT*, forward 5′-AGTCCAGAGGCACCACTCAT-3′ and reverse 5′-CTGAGATATGGTGGCAGCAA-3′, 18S, forward 5′-GTATG GTTGCAAAGCTGAAACTTA-3′ and reverse 5′-AAGAGCTCT-CAATCTGTCAATCCT-3′. The reactions were carried out in a DNA Engine Opticon II (MJ Research, Reno, NV, USA) with the following thermocycle parameters: 50 °C for 2 min (to incubate with uracil DNA glycosylase and prevent amplification of deoxyuracil-containing PCR products), 95 °C for 2 min, followed by 35 cycles of 95 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s. At the end of amplification, a melting curve analysis was performed by heating the PCR products from 65 to 95 °C, held for 15 s at increments of 0.2 °C, and fluorescence was detected to confirm the presence of a single amplification product. Samples from each animal were run in duplicate to obtain average log-linear threshold (C_t) values for *NAMPT* and 18S mRNA. The log-linear threshold values during the exponential phase of the PCR for *NAMPT* were subtracted from that of 18S mRNA. *NAMPT* mRNA quantity was expressed as a proportion of 18S mRNA quantity. For negative controls, equivalent amounts of RT reactions using 1 µg total RNA with no RT were used as template in place of single stranded cDNA in the real-time quantitative PCR.

Quantification of NAMPT protein in prepubertal and adult chicken testes by western blot analysis

Approximately 0.5 g of prepubertal (14-week-old) and adult (29-week-old) chicken testes ($n=5$/age group) were homogenized using the Tekmar Tissumizer (Tekmar Company, Cincinnati, OH, USA) in 3 ml lysis buffer (10 mM Tris–HCl, 150 mM NaCl, pH 8.0, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS), containing protease inhibitor cocktail and phosphatase inhibitor cocktail 2 (Sigma–Aldrich). The homogenate was then passed through a 22-gauge needle and shaken in a thermomixer (Eppendorf, Westbury, NY, USA). The lysate was centrifuged at 14 000 ***g*** for 15 min at 4 °C, and the supernatant was collected. Total protein concentration was estimated by a protein dye-binding assay (Bradford 1976) using a commercial kit (Bio-Rad), with chicken ovalbumin as the standard. Aliquots of testicular protein extracts were stored at −80 °C until analyzed by western blotting.

One-dimensional gel electrophoresis was performed with the NuPAGE Novex minigel system (XCell *SureLock* Mini Cell; Invitrogen) according to manufacturer's recommendations. Samples were prepared by combining ∼10 µg total protein extract with 4× NuPAGE LDS sample buffer and 10× NuPAGE reducing agent, and heated for 10 min at 70 °C prior to electrophoresis. Proteins were separated on a 10% Bis-Tris polyacrylamide gel (Invitrogen) with MOPS running buffer under denaturing conditions for 50–60 min, and electrotransferred onto Immun-Blot PVDF membranes (0.20 µm; Bio-Rad) at 30 V for 60 min with the XCell II blot module (Invitrogen). Membranes were incubated in blocking

buffer (1× casein solution; Vector Laboratories) for 2 h at room temperature, followed by an overnight incubation in rabbit anti-human NAMPT antibody (1:15 000 in blocking buffer; Bethyl Laboratories) at 4 °C with gentle agitation. The membranes were washed once for 15 min in 10 mM Tris, 150 mM NaCl, 0.1% Tween-20 (TBS–T), followed by three washes for 5 min each in TBS–T. Immunoreactive proteins were detected by incubation in HRP conjugated to goat anti-rabbit IgG (1:10 000; Pierce, Thermo Fisher Scientific, Rockford, IL, USA) for 1 h at room temperature. The membrane was washed as previously described and treated with ECL Plus Chemiluminescence Detection Reagent (Amersham Biosciences). Chemiluminescent signals were detected using the Storm 860 optical scanner (Amersham Biosciences), and the signals were analyzed using Image Quant TL software (Amersham Biosciences). The chemiluminescence signal intensity of each band was calculated using a local average background correction. In order to normalize for protein load on immunoblots comparing NAMPT protein quantities in prepubertal and adult chicken testes, membranes were reprobed using mouse anti-β-actin (ACTB) antibody (1:4000 Sigma–Aldrich), followed by incubation in HRP conjugated to goat anti-mouse IgG (1:10 000; Pierce), and the chemiluminescence signal was determined as previously described. NAMPT protein quantity was expressed as a proportion of ACTB, and the relative amounts of testicular NAMPT protein were then compared.

Detection of NAMPT in seminal plasma and sperm protein extract by western blotting

Approximately 250–500 µl of semen was collected through manual massage from each breeder male chicken ($n=3$) as a single ejaculate and kept on ice. Sperm motility was confirmed by microscopic examination. Semen from each animal was centrifuged at 600 ***g*** for 20 min at 4 °C, and the seminal plasma was recovered. The resultant sperm pellet was centrifuged one more time to remove residual seminal plasma. The sperm pellet was resuspended in 500 µl RIPA lysis buffer containing protease inhibitor cocktail and phosphatase inhibitor cocktail 2 (Sigma–Aldrich). Protein from the sperm pellet was extracted as described above. Protein concentration of the seminal plasma and sperm protein extract were determined by protein dye-binding assay. Twenty microgram of seminal plasma or sperm protein was subjected to electrophoresis and western blotting for detection of NAMPT as described above.

Quantification of plasma NAMPT levels in prepubertal and adult chickens

Blood samples from prepubertal (4- and 14-week-old) and adult (29-week-old) chickens ($n=7$/age group) were collected from the wing vein into syringes coated with 10% EDTA. Blood samples were centrifuged at 1500 ***g*** for 15 min at 4 °C, and plasma was collected and stored at −80 °C until further analysis. Plasma NAMPT concentrations were determined using a human NAMPT (COOH-terminal) enzyme immunoassay kit (Phoenix Pharmaceuticals), which had been previously

validated for quantifying plasma NAMPT levels in chickens (Krzysik-Walker *et al.* 2008). All samples were run in triplicate, with sample comparisons run in the same assay to eliminate between assay variation.

Statistical analyses

All analyses were carried out by ANOVA using the general linear model procedure of the Statistical analysis system (SAS Institute, Cary, NC, USA). Differences between individual means were partitioned by Tukey's pairwise comparisons analysis in SAS. For analysis of real-time quantitative PCR data, relative *NAMPT* mRNA quantity to 18S mRNA quantity was first converted from log-linear to linear terms. All data are represented as mean±s.e.m. A probability level of $P < 0.05$ was considered statistically significant. Results are presented as arithmetic means±s.e.m.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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