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Adiponectin and its receptors are expressed in the chicken testis: influence of sexual maturation on testicular *ADIPOR1* and *ADIPOR2* mRNA abundance

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Abstract

Adiponectin is an adipokine hormone that influences glucose utilization, insulin sensitivity, and energy homeostasis by signaling through two distinct receptors, ADIPOR1 and ADIPOR2. While adipose tissue is the primary site of adiponectin expression in the chicken, we previously reported that adiponectin and its receptors are expressed in several other tissues. The objectives of the present study are to characterize adiponectin, ADIPOR1, and ADIPOR2 expressions in the chicken testis and to determine whether sexual maturation affects the abundance of testicular adiponectin, *ADIPOR1*, and *ADIPOR2* mRNAs. By RT-PCR and nucleotide sequencing, testicular adiponectin, *ADIPOR1*, and *ADIPOR2* mRNAs were found to be identical to that expressed in the abdominal fat pad. Using anti-chicken adiponectin, ADIPOR1, or ADIPOR2 antibodies and immunohistochemistry, adiponectin-immunoreactive (ir) and ADIPOR1-ir cells were found exclusively in the peritubular cells as well as in Leydig cells. However, ADIPOR2-ir cells were found in the adluminal and luminal compartments of the seminiferous tubules as well as in interstitial cells. In particular, Sertoli cell syncytia, round spermatids, elongating spermatids, spermatozoa, and Leydig cells showed strong ADIPOR2 immunoreactivity. Using quantitative real-time PCR analyses, testicular *ADIPOR1* and *ADIPOR2* mRNA abundance were found to be 8.3- and 9-fold higher ($P<0.01$) in adult chickens compared with prepubertal chickens respectively, suggesting that sexual maturation is likely to be associated with an up-regulation of testicular *ADIPOR1* and *ADIPOR2* gene expressions. Collectively, our results indicate that adiponectin and its receptors are expressed in the chicken testis, where they are likely to influence steroidogenesis, spermatogenesis, Sertoli cell function as well as spermatozoa motility.
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Introduction

Adiponectin, which was also termed as adipocyte complement-related protein (ACRP30), adipose most abundant gene transcript 1 (apM1), gelatin-binding protein 28 (GBP28), and AdipoQ (Scherer *et al*. 1995, Hu *et al*. 1996, Maeda *et al*. 1996, Nakano *et al*. 1996), is a 30 kDa adipokine hormone secreted primarily from adipose tissue in several mammalian species. It is composed of four distinct domains that include a signal peptide at the N-terminus followed by a short variable region, a collagenous domain, and a C-terminal globular domain (Shapiro & Scherer 1998). Adiponectin has been found in human and mouse sera as trimeric and hexameric oligomers although heavy molecular weight forms as well as small proteolytic cleavage products have also been detected (Fruebis *et al*. 2001, Waki *et al*. 2003). Plasma adiponectin levels in humans were found to range from 3 to 30 μg/ml, indicating that adiponectin is one of the most abundant hormones in the blood circulation (Arita *et al*. 1999). In addition, adiponectin has been found to affect glucose utilization, lipid synthesis, insulin sensitivity, and energy homeostasis in several mammalian species (Kubota *et al*. 2002, Yamauchi *et al*. 2002). While deletion of the adiponectin gene leads to diet-induced insulin resistance (Maeda *et al*. 2002), adiponectin treatment or overexpression protects overfed rats from gaining weight and cardiovascular disease by suppressing glucose production and enhancing lipid oxidation (Maeda *et al*. 2002, Yamauchi *et al*. 2002). Recently, adiponectin has been shown to negatively influence angiogenesis leading to a suppression of primary tumor growth and activation of apoptotic caspase cascades (Brakenhielm *et al*. 2004).

Adiponectin exerts its action by binding to two specific receptors, ADIPOR1 and ADIPOR2. Both ADIPOR1 and ADIPOR2 are seven transmembrane receptors that are structurally and functionally distinct from G-protein-coupled receptors (Yamauchi *et al*. 2003*b*). ADIPOR1 is abundantly expressed in skeletal muscle, whereas ADIPOR2 is predominantly expressed

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in the liver (Yamauchi *et al*. 2003*b*). ADIPOR1 has been reported to have greater binding affinity to the globular domain of adiponectin while ADIPOR2 binds to both the globular and full-length adiponectin with intermediate affinity (Yamauchi *et al*. 2003*b*). While a low degree of homology exists between *ADIPOR1* and *ADIPOR2* mRNA nucleotide sequences, both ADIPOR1 and ADIPOR2 have been found to be highly conserved molecules in several animals including the chicken (Ramachandran *et al*. 2007).

Through the stimulation of ADIPOR1 or ADIPOR2, adiponectin has been found to activate cyclic adenosine monophosphate-activated protein kinase (PRKAA2; (Yamauchi *et al*. 2002)) resulting in the phosphorylation of various intracellular proteins including acetyl-coenzyme A carboxylase (ACACA) and peroxisome proliferator-activated receptor α (Fruebis *et al*. 2001, Yamauchi *et al*. 2003*a*). Adiponectin stimulates fatty acid oxidation through phosphorylation and inhibition of ACACA and activation of malonyl-coenzyme A (malonyl-CoA) decarboxylase resulting in reduced malonyl-CoA content (Tomas *et al*. 2002). Furthermore, adiponectin has been found to activate the MAP kinase (MAPK) pathway in the placenta (Lappas *et al*. 2005), vascular tissue (Arita *et al*. 2002, Shibata *et al*. 2005), and osteoblasts (Luo *et al*. 2005).

Although adiponectin was once thought to be exclusively secreted from adipose tissue, we have recently detected expression of adiponectin and its receptors in multiple tissues in the chicken (Maddineni *et al*. 2005, Ramachandran *et al*. 2007). However, it is not known whether adiponectin or its receptors are expressed in the chicken testis. Testis in any species, particularly in broiler breeder chickens, become metabolically active for the production of gonadal steroids as well as spermatozoa as the animal undergoes sexual maturation. Therefore, the objectives of the present study are to characterize adiponectin, ADIPOR1, and ADIPOR2 in the chicken testis and to determine whether sexual maturation affects the abundance of mRNA encoding testicular adiponectin, ADIPOR1, or ADIPOR2. Our results provide novel evidence that both adiponectin and its receptors are expressed in the chicken testis and are likely to be involved in multiple functions within the testis.

Results

RT-PCR

Adiponectin, *ADIPOR1*, or *ADIPOR2* partial cDNAs, corresponding to nucleotides 250–599 (GenBank Accession no. AY838798), 389–738 (GenBank Accession no. DQ072275), and 430–774 (GenBank Accession no. DQ072276) respectively, were amplified from single-stranded cDNA reverse transcribed from total RNA extracted from chicken testes (Fig. 1). In addition, full-length cDNA encoding chicken adiponectin, ADIPOR1, and ADIPOR2 were amplified from testicular RNA and their nucleotide sequences were found to be identical to that expressed in chicken adipose tissue (data not shown). The use of either RNA not reversed transcribed (−RT) or water in the place of cDNA failed to amplify any PCR product, confirming the absence of genomic DNA contamination.

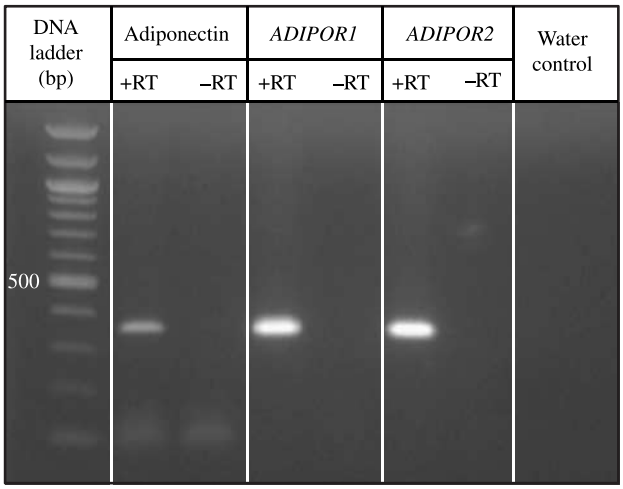

Figure 1 RT-PCR analysis of adiponectin, *ADIPOR1* and *ADIPOR2* gene expressions in sexually mature chicken testis. Approximately 100 ng of cDNA (+RT) was used as template to amplify a 350, 350, and 345 bp chicken adiponectin, *ADIPOR1*, and *ADIPOR2* cDNA respectively. Contamination controls consisted of testis RNA without reverse transcriptase (−RT) or substitution of water for the cDNA template. bp, base pair.

Cellular localization of adiponectin, ADIPOR1, and ADIPOR2 in the chicken testis

Representative distributions of adiponectin-, ADIPOR1-, and ADIPOR2-immunoreactive (ir) cells within the testis of sexually mature chickens are shown in Figs 2–5. Use of two different sets of antibodies against chicken adiponectin, ADIPOR1, or ADIPOR2 generated against different regions of the respective proteins produced identical immunostaining patterns for adiponectin, ADIPOR1, and ADIPOR2 within the chicken testis, further confirming the specificity of immunostaining.

Testicular tissue sections showing adiponectin immunostaining using anti-chicken adiponectin antibody, ADNa, are shown in Fig. 2A–E while that revealed by immunostaining using the anti-adiponectin antibody, ADNb, are shown in Fig. 2G–K. Adiponectin immunostaining was observed exclusively in the peritubular cells surrounding the seminiferous tubules and in the interstitial cells (Fig. 2A, D, G, H, and K). Diffuse adiponectin immunostaining was also noticed throughout the interstitium (Fig. 2K) as well as in the cytoplasm of Leydig cells (Fig. 2H and K). Based on the flattened morphology of cells in the peritubular area that is characteristic of myoid cells, several of the myoid cells appear to contain adiponectin immunoreactivity (Fig. 2J). No adiponectin-specific immunostaining was noticed within the seminiferous tubule.

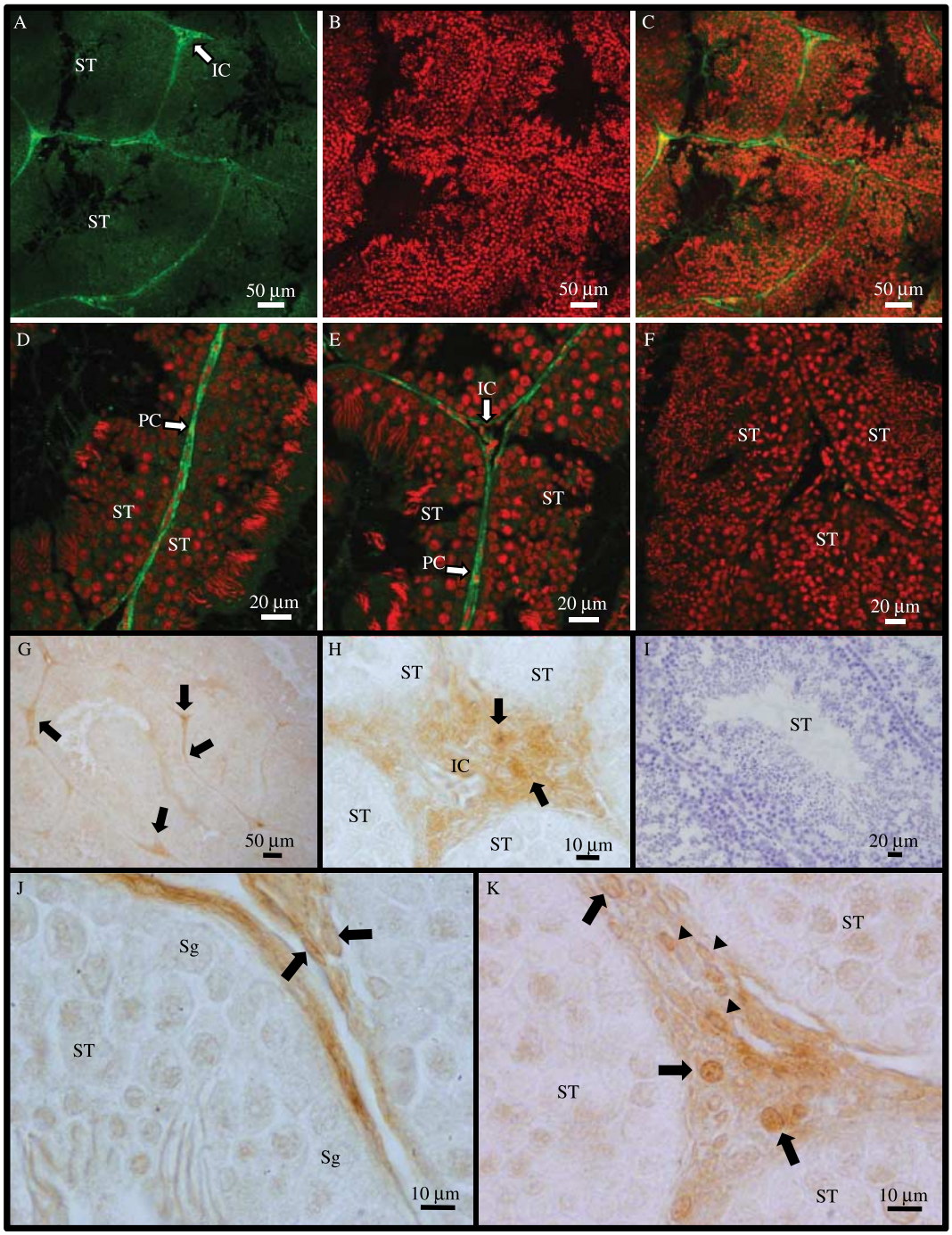

Figure 2 (A–K) Representative photomicrographs of testicular tissue sections from adult chickens showing adiponectin-immunostained cells. Paraformaldehyde-fixed tissue sections were immunostained using (A–E) anti-chicken adiponectin antisera (ADNa) or (G–K) anti-chicken adiponectin IgG (ADNb) as described in Materials and Methods section. (A–C) Confocal images of testicular tissue section showing (A and C) adiponectin immunostaining (green) and (B and C) nuclear staining (red). Note adiponectin immunostaining in the peritubular area and in the interstitium around the seminiferous tubule (ST). (D and E) Confocal images of seminiferous tubules (ST) showing adiponectin immunostaining (green) in the peritubular cells (PC) or interstitial cells (IC) and nuclear (red) staining. (F) Confocal image of testicular tissue sections immunostained with pre-immune rabbit serum as negative control. (G) Adiponectin immunostaining (arrows) around the seminiferous tubules in the peritubular area and in the interstitium. (H) Diffuse adiponectin immunostaining throughout the interstitium (IC) and within the Leydig cells (arrows) around seminiferous tubule (ST). (I) Photomicrograph of a testicular tissue section that is counterstained following immunostaining with anti-chicken adiponectin IgG (ADNb) pre-adsorbed with the immunogen peptide. (J) Adiponectin immunostaining in flattened peritubular cells (arrows). Note the absence of adiponectin immunostaining in spermatogonial cells (Sg) within the seminiferous tubule (ST). (K) Diffuse adiponectin staining in the interstitium, Leydig cells (arrows), and peritubular cells (arrow heads) outside of seminiferous tubules (ST).

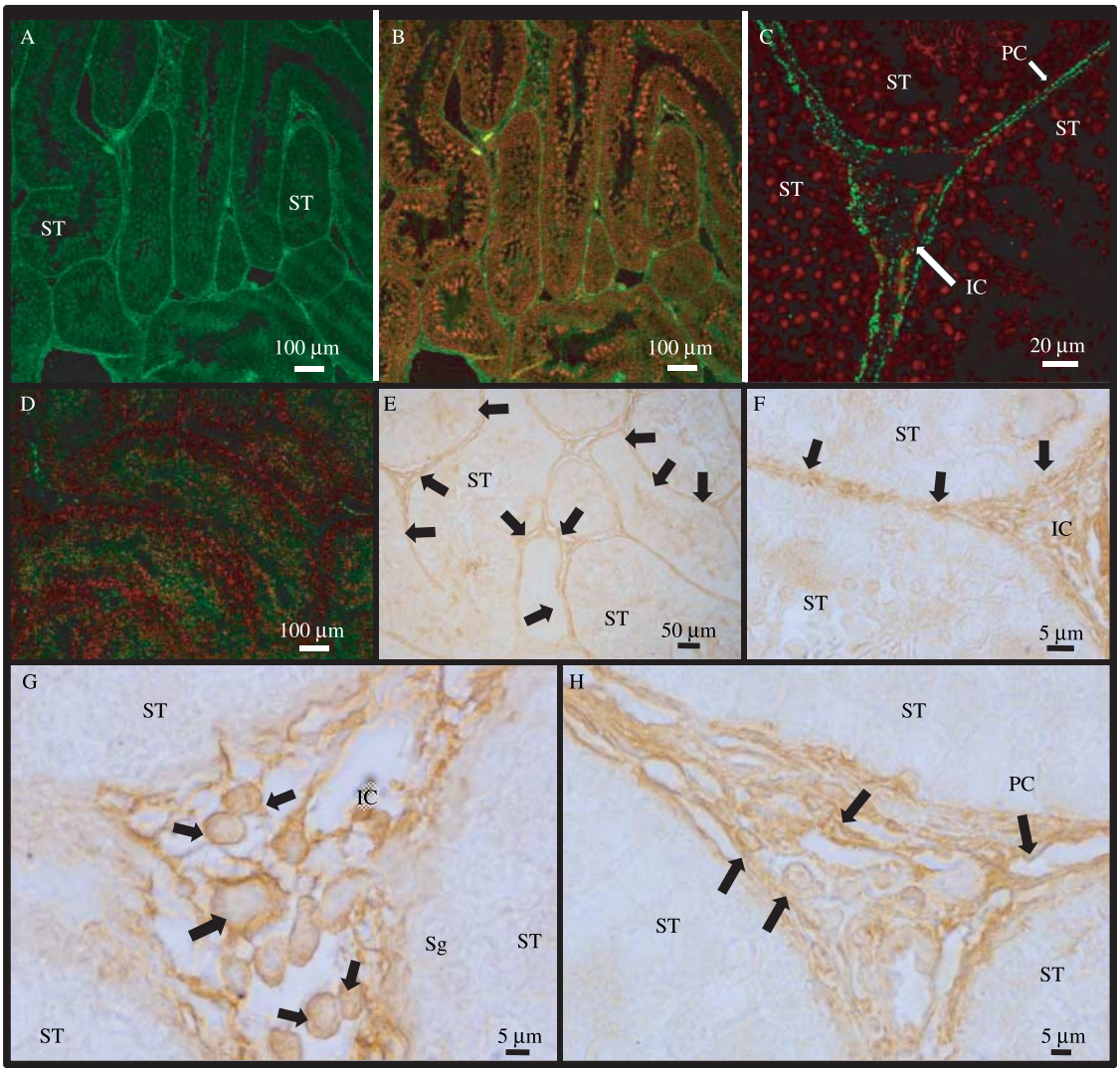

Figure 3 (A–K) Representative photomicrographs of testicular tissue sections from adult chickens showing ADIPOR1-immunostained cells. (A–H) Paraformaldehyde-fixed tissue sections were immunostained using anti-chicken ADIPOR1 IgG (ADNR1a) as described in Materials and Methods section. (A–C) Confocal images of testicular tissue section showing (A and C) ADIPOR1 immunostaining (green) and (B and C) nuclear staining (red) in the peritubular area and in the interstitium (IC) around seminiferous tubule (ST). Note punctuate ADIPOR1 immunostaining (green) in the peritubular cells (PC) or in interstitial cells (IC) in (C). (D) Confocal image of testicular tissue sections immunostained with anti-ADIPOR1 IgG pre-adsorbed with immunogen peptide. (E) ADIPOR1 immunostaining (arrows) around the seminiferous tubules (ST) in the peritubular area and in the interstitium. (F) Diffuse ADIPOR1 immunostaining throughout the interstitium (IC) and in the peritubular area (arrows). (G) ADIPOR1 immunostaining in Leydig cells (arrows) in the interstitium (IC). Note the absence of ADIPOR1 immunostaining in spermatogonial cells (Sg) within the seminiferous tubule (ST). (H) Diffuse ADIPOR1 immunostaining in the interstitium and in Leydig cells (arrows) outside of seminiferous tubules (ST).

Testicular tissue sections showing ADIPOR1 immunostaining as revealed by the use of the anti-chicken ADIPOR1 antibody, ADNR1a, are shown in Fig. 3A–H. Similar to adiponectin, ADIPOR1 immunostaining was noticed in the peritubular cells and in the interstitial cells (Fig. 3A, C and E). ADIPOR1 immunoreactivity appeared punctuate (Fig. 3C), a staining pattern unique to transmembrane receptor proteins. ADIPOR1 immunostaining was noticed both in the cytoplasm and cell membrane of Leydig cells in the interstitium (Fig. 3G and H). ADIPOR1 immunostaining was not observed within the seminiferous tubular cells. Similar to the results observed using the ADNR1a antibody for immunostaining ADIPOR1, the ADNR1b antibody revealed identical staining patterns for ADIPOR1 in the peritubular and interstitial areas around the seminiferous tubule (data not shown).

Testicular tissue sections showing ADIPOR2 immunostaining as revealed by the use of anti-chicken ADIPOR2 antibody (ADNR2a) are shown in Fig. 4A–E while that revealed by immunostaining using the antibody ADNR2b are shown in Fig. 5A–I. In contrast to adiponectin or ADIPOR1, ADIPOR2 immunostaining was observed in several regions within the testicular

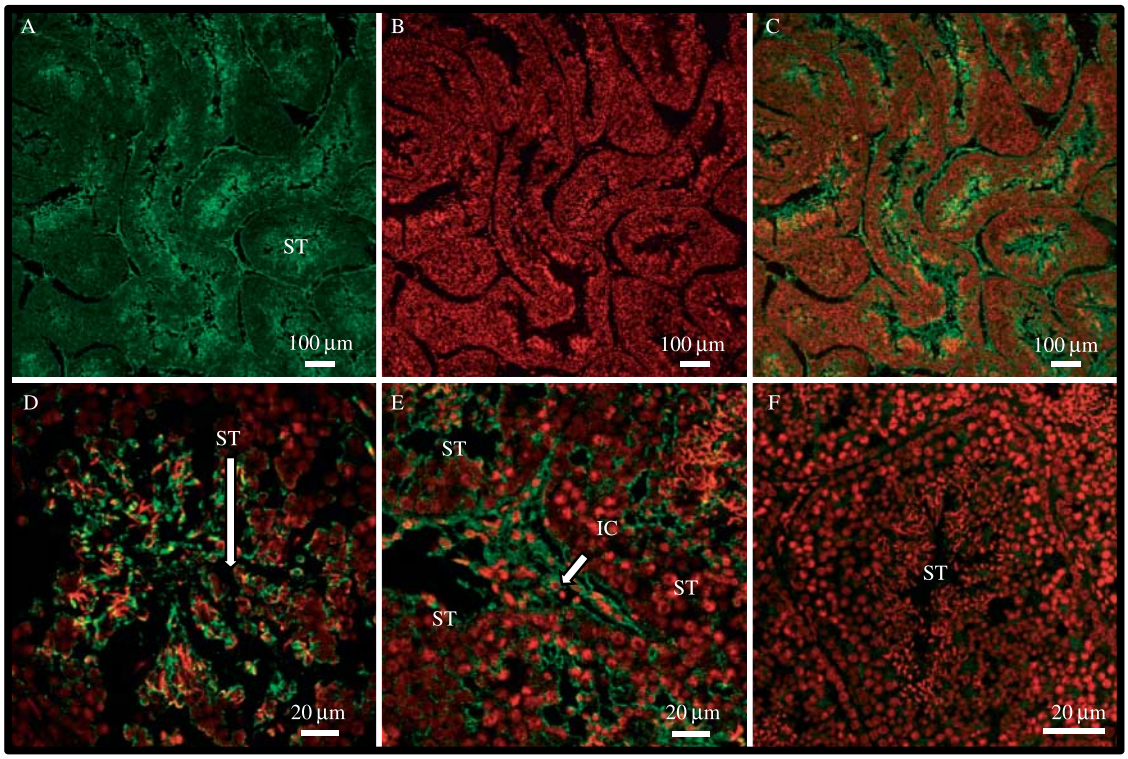

Figure 4 Confocal photomicrographic images of adult chicken testis showing ADIPOR2 immunostaining. Paraformaldehyde-fixed tissue sections were immunostained using anti-chicken ADIPOR2 antiserum (ADNR2a) as described in Materials and Methods section. (A–C) Photomicrographs of testicular tissue section showing (A and C) ADIPOR2 immunostaining (green) and (B and C) nuclear staining (red). Note ADIPOR2 immunostaining in the proluminal region of the seminiferous tubules. (D) Confocal image of seminiferous tubule (ST) showing intense ADIPOR2 immunostaining (green) and nuclear staining (red) in the adluminal and luminal cells within a seminiferous tubule. (E) ADIPOR2 immunostaining (green) and nuclear staining (red) in Leydig cells (arrow). (F) Confocal image of testicular tissue sections immunostained with pre-immune serum as negative control and counterstained to reveal nuclei (red).

tissue. Intensely-stained ADIPOR2-ir cells were found in the testicular interstitium as well as in the proluminal compartment of the seminiferous tubule (Figs 4A–E and 5A–E). Leydig cells within the interstitium showed granular ADIPOR2 immunostaining (Fig. 5D). Within the seminiferous tubule, Sertoli cell syncytia showed intense ADIPOR2 immunostaining while spermatogonial cells appeared not to contain ADIPOR2 immunoreactivity (Fig. 5F–I). Numerous round and elongating spermatids in the adluminal region of the seminiferous tubule showed ADIPOR2 immunoreactivity (Fig. 5E and H). The seminiferous tubular lumen contained ADIPOR2-immunoreactive spermatozoa (Fig. 5E). A small number of peritubular cells also exhibited ADIPOR2 immunostaining (data not shown). When the primary antibody was pre-adsorbed with respective immunogen peptides or when pre-immune serum was used in place of the primary antibodies, adiponectin, ADIPOR1, or ADIPOR2 immunostaining was either abolished or greatly reduced (Figs 2F and I, 3D, 4F and 5J). Furthermore, both sets of antibodies/antisera against chicken adiponectin, ADIPOR1, or ADIPOR2 were validated using western blotting, which detected adiponectin (30 kDa), ADIPOR1 (45 kDa), and ADIPOR2 (45 kDa) in chicken skeletal muscle, adipose tissue, and testis protein extracts (data not shown).

Effect of sexual maturation on adiponectin, ADIPOR1 *and* ADIPOR2 *mRNA quantities*

Quantitative real-time PCR analyses of testicular RNA revealed no significant difference in adiponectin mRNA quantity among 4-, 14-, and 29-week-old chickens (Fig. 6A). However, *ADIPOR1* and *ADIPOR2* mRNA quantities were 8.3- and 9-fold higher in sexually mature 29-week-old chicken testes compared with that of sexually immature 4-week-old chickens respectively (Fig. 6B and C; $n=4$–6/age group; $P<0.01$). However, *ADIPOR1* and *ADIPOR2* mRNA quantities in the testes of 4- and 14-week-old sexually immature chickens were not significantly different ($P>0.05$; Fig. 6B and C). Melting curve analyses revealed the presence of a single amplification product for adiponectin, ADIPOR1, and ADIPOR2, thereby confirming the specificity of reactions (data not shown). Testicular total RNA reverse transcribed in the absence of reverse transcriptase resulted in C_T values not different from the blank (>30 cycles), indicating that genomic DNA was not

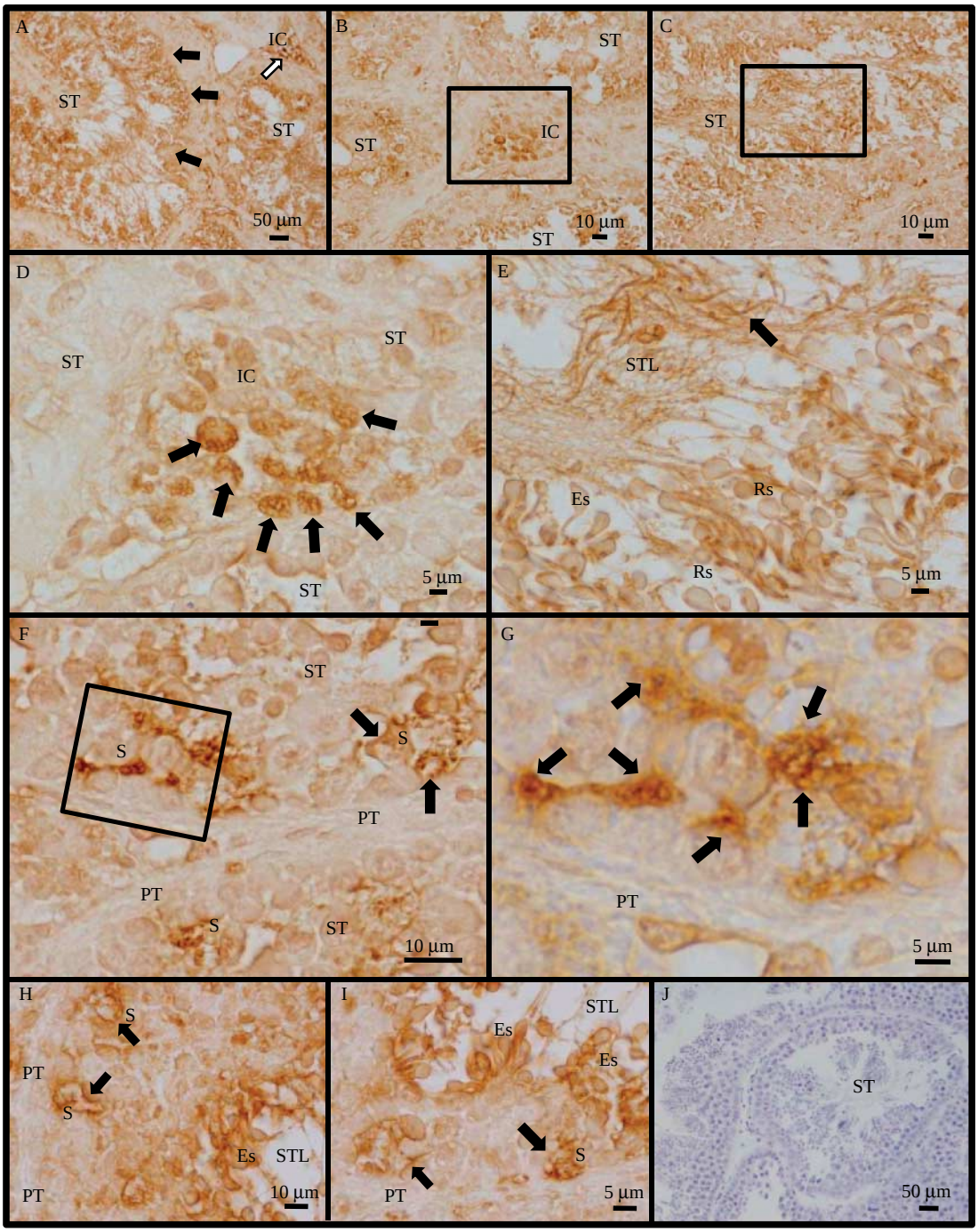

Figure 5 Representative photomicrographs of testicular tissue sections from adult chickens showing ADIPOR2-immunostained cells. (A–I) Paraformaldehyde-fixed tissue sections were immunostained using anti-chicken ADIPOR2 antiserum (ADNR2b) as described in Materials and Methods section. (A) Intense ADIPOR2 immunostaining in the adluminal and luminal compartments (bold arrows) of the seminiferous tubule (ST) and in the interstitium (open arrow). (B) ADIPOR2-immunoreactive (ir) Leydig cells in the interstitium (box, IC; magnified in D) showing granular ADIPOR2 staining in the cytoplasm and on cell membrane (arrows in D). (C) ADIPOR2 immunostaining in the seminiferous tubular (ST) adluminal and luminal compartments (box; magnified in E). Round spermatids (Rs) and elongating spermatids (Es) show ADIPOR2 immunostaining around the cell membrane in (E). Spermatozoa in the seminiferous tubular lumen (STL) show intense ADIPOR2 immunostaining (arrow in E). (F) Intense ADIPOR2 immunostaining in the Sertoli cell (S) syncytia (box; magnified in G; arrows). Peritubular area (PT) is marked to distinguish the two seminiferous tubules (ST). (H and I) Sertoli cells (S; arrows) and elongating spermatids (Es) toward seminiferous tubular lumen (STL) show intense ADIPOR2 immunostaining. Peritubular area (PT) is marked to orient the seminiferous tubular cross section in the photomicrographs. (J) Photomicrograph of testis tissue section immunostained with pre-immune serum in place of anti-chicken ADIPOR2 antiserum as negative control and counterstained as described in Materials and Methods section.

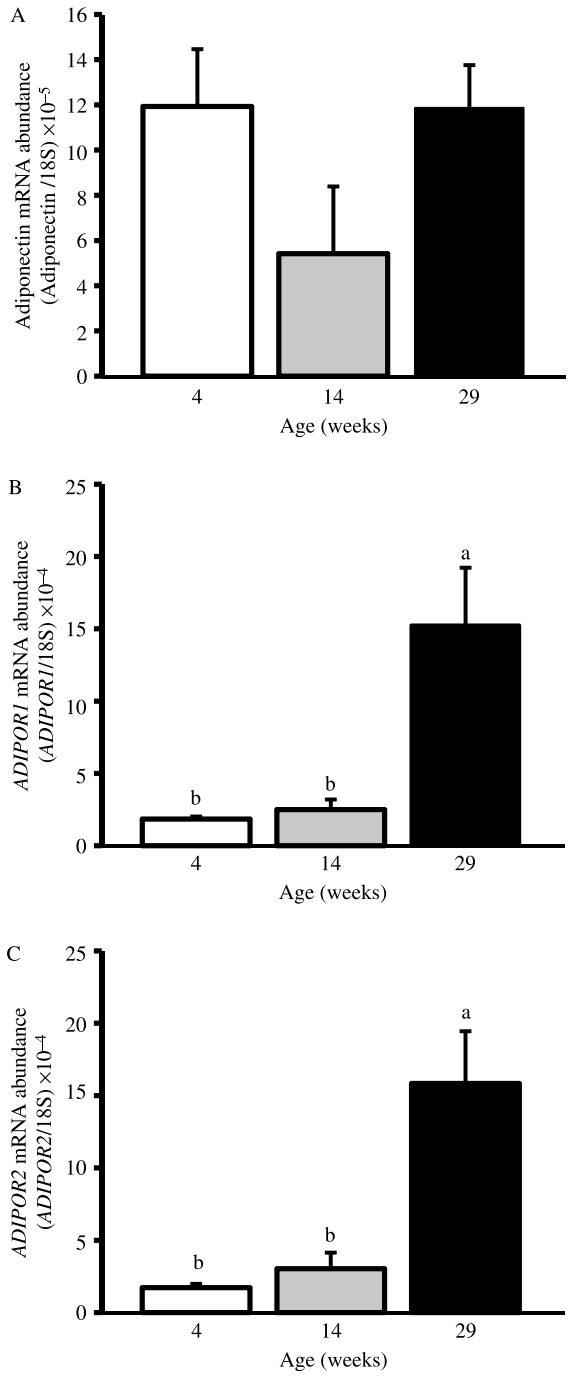

Figure 6 (A) Adiponectin, (B) *ADIPOR1*, and (C) *ADIPOR2* mRNA abundance in the testis of 4-, 14-, and 29-week-old chickens. Total RNA was extracted from the testis and treated with DNASEI. Following RT, ~50 ng of cDNA was used in quantitative real-time PCR using SYBR green as the dye to quantify adiponectin, *ADIPOR1*, *ADIPOR2* mRNAs or 18S mRNA in separate reactions. Each reaction was run in duplicate and the critical threshold (C_T) values and subtracted from that of 18S mRNA, averaged and converted from log-linear to linear term. Different letters above each bar indicate significant difference at $P<0.05$. Data in A–C represent mean±S.E.M. ($n=4$–6).

contributing to adiponectin, *ADIPOR1*, *ADIPOR2* or18S mRNA quantification (data not shown).

Discussion

The present study is the first to describe the expression of adiponectin in the testis of any vertebrate species. We identified the expression of full-length mRNAs encoding chicken adiponectin, ADIPOR1 and ADIPOR2 by RT-PCR analysis and localized adiponectin-, ADIPOR1-, and ADIPOR2-ir cells in the chicken testis. Once thought to be primarily secreted by white adipose tissue, adiponectin has recently been found to be expressed in multiple tissues in the chicken (Maddineni *et al.* 2005). In particular, adiponectin gene expression has been identified in chicken ovarian follicles (Chabrolle *et al.* 2007). Similar to chickens, adiponectin has been found to be expressed in the rat pituitary gland (Rodriguez-Pacheco *et al.* 2007) and oviduct (Archanco *et al.* 2007). In addition, the genes encoding adiponectin receptors, ADIPOR1 and ADIPOR2, are previously found to be expressed in several tissues in the chicken (Ramachandran *et al.* 2007). In support of our present findings, both *ADIPOR1* and *ADIPOR2* mRNAs have been identified in a cDNA library prepared from human testicular RNA (Civitarese *et al.* 2004, Bjursell *et al.* 2007). Furthermore, rat testis has recently been found to express adiponectin, ADIPOR1, and ADIPOR2 (Caminos *et al.* 2008).

The physiological significance of adiponectin or its receptors in testicular function is not known in chickens. The expression of a functional adiponectin receptor, particularly ADIPOR2, appears to be important for the testicular function in mice as ADIPOR2-deficient knockout mice exhibit reduced testis weight characterized by atrophy of the seminiferous tubules and aspermia, while plasma testosterone levels remained unaffected (Bjursell *et al.* 2007). Deletion of the adiponectin gene in mice, however, did not affect fertility in either male or females (Maeda *et al.* 2002), possibly suggesting the presence of adiponectin-like ligands for activating ADIPOR2 receptors and compensating for the lack of adiponectin. However, adiponectin has been found to affect several metabolic functions including glucose utilization, lipogenesis, energy homeostasis, and immunity (Maeda *et al.* 2002, Yamauchi *et al.* 2002, Tilg & Moschen 2006, Schaffler *et al.* 2007). Testicular adiponectin may likely act as a paracrine/autocrine factor thereby supplementing blood-borne adiponectin in order to influence various functions of cells within seminiferous tubules and interstitial cells in the chicken. Since adiponectin has been shown to increase *STAR* mRNA expression and phosphorylation of MAP3K1 in porcine granulosa cells *in vitro* (Ledoux *et al.* 2006), adiponectin is likely to affect steroidogenesis in Leydig cells as well. A recent report has suggested that recombinant adiponectin inhibits basal and human chorionic gonadotropin-stimulated

testosterone secretion from adult rat testis *ex vivo* without affecting selected Sertoli cell-expressed mRNAs (Caminos *et al*. 2008).

In the present study, we found that both adiponectin- and ADIPOR1-ir cells were located in the peritubular cells and Leydig cells surrounding the seminiferous tubules. Similar to our findings, a recent study has suggested that adiponectin immunostaining was found exclusively in Leydig cells and macrophages in the rat testis interstitium (Caminos *et al*. 2008). In addition, the same report (Caminos *et al*. 2008) suggests that *ADIPOR1* mRNA but not *ADIPOR2* mRNA was detected in the seminiferous tubular epithelium isolated from rat testis. In the chicken testis, based on distinguishable flattened cell morphology of the peritubular cells, adiponectin and ADIPOR1 appear to be expressed in peritubular myoid cells. The localization of both adiponectin and ADIPOR1 in peritubular cells indicate that adiponectin is likely to influence myoid cell function. Peritubular myoid cells are involved in the transport of spermatozoa and testicular fluid from the seminiferous tubule (Maekawa *et al*. 1996), secretion of extracellular matrix proteins such as fibronectin (Galdieri & Ricci 1998), and regulation of Sertoli cell function (Tung & Fritz 1980, Maekawa *et al*. 1996). In addition to myoid cells, the peritubular space also contains immune cells such as macrophages (Hutson 1994). It is, therefore, likely that some of the peritubular cells expressing ADIPOR1 protein may be macrophages influenced by adiponectin elsewhere (Yokota *et al*. 2000, Hales 2002). One of the functions of peritubular macrophages is to secrete another adipokine hormone, tumor necrosis factor-α, that plays a major role in controlling Leydig cell function (Hales 2002).

In the present study, ADIPOR2 immunoreactivity was predominantly observed in the Leydig cells as well as in the adluminal and luminal compartments of the chicken seminiferous tubules. Intense ADIPOR2 immunostaining was observed along the Sertoli cell syncytia, suggesting a potential role of adiponectin in regulating Sertoli cell function. Furthermore, ADIPOR2 immunostaining was noticed in the round spermatids, elongating spermatids, and spermatozoa. Based on such widespread ADIPOR2 immunostaining throughout the adluminal and luminal compartments of the seminiferous tubule, we hypothesize that adiponectin is involved in maturation and differentiation of spermatocytes and therefore potentially influences spermatogenesis. Adiponectin is also likely to affect metabolism of cells within the seminiferous tubules, possibly through an activation of PRKAA2 and peroxisome proliferator-activated receptors that have been previously identified in mammalian testis tissues (Cheung *et al*. 2000, Froment *et al*. 2006). A recent study has confirmed that some of the serine/threonine kinases belonging to the family of PRKAA2 are uniquely present in the post-meiotic cells (Xu *et al*. 2007). In addition, the glucose transporters SLC2A1 and SLC2A3, which are expressed in all seminiferous tubular cells in the rat (Burant & Davidson 1994), have been shown to be affected by adiponectin (Ceddia *et al*. 2005). A recent microarray analysis (Pang *et al*. 2006) has provided evidence for greater expression of genes related to the regulation of energy metabolism in mouse spermatids compared with spermatocytes, underscoring the importance of heightened energy metabolism in post-meiotic cells, a process profoundly influenced by adiponectin elsewhere in the body. Adiponectin is also likely to affect sperm motility as our immunohistochemical data suggest that ADIPOR2 is expressed in the flagella of spermatozoa found in the seminiferous tubular lumen.

In the present study, we found that testicular *ADIPOR1* and *ADIPOR2* mRNA quantities were 8.3- and 9-fold higher in adult chickens than prepubertal chickens respectively, suggesting that sexual maturation is associated with up-regulation of *ADIPOR1* and *ADIPOR2* gene expressions. In the present study, prepubertal chickens and adult chickens were raised under different photoperiod and feeding regimen to discourage hyperphagia and rapid growth that are detrimental to reproductive performance. Therefore, the observed up-regulation in *ADIPOR1*, and *ADIPOR2* mRNA levels in adult chicken may have resulted from a combination of factors such as changes in photoperiod, feeding schedule or age. However, in support of our findings, testicular *ADIPOR2* mRNA levels were significantly higher in adult rats compared with prepubertal rats while *ADIPOR2* mRNA expression was found to be elevated in response to CGB5 treatment of hypophysectomized rats (Caminos *et al*. 2008). We hypothesize that the significant elevation of *ADIPOR1* and *ADIPOR2* gene expressions in sexually mature chickens is involved in supporting higher metabolic activity related to spermatogenesis, testicular steroid hormone production, and transport of spermatozoa and testicular fluid. In support of this hypothesis, plasma adiponectin levels were found to be fourfold higher in sexually mature versus sexually immature mice (Combs *et al*. 2003).

Similar to the current identification of adiponectin in the chicken testis, several adipokines have been previously detected in the mammalian testis. Leptin has been found in spermatocytes and Leydig cells in the human testis (Ishikawa *et al*. 2007), while leptin receptor mRNA and protein were detected in the rat, pig, and mouse testis (Xiong & Hales 1993, El-Hefnawy *et al*. 2000, Tena-Sempere & Barreiro 2002). Tumor necrosis factor-α was found to be expressed by testicular macrophages and shown to modulate testosterone secretion by Leydig cells (Xiong & Hales 1993). Furthermore, expression of resistin protein has been demonstrated in interstitial Leydig cells and Sertoli cells within seminiferous tubules (Nogueiras *et al*. 2004).

In conclusion, we provide novel evidence that the chicken testis expresses adiponectin and its receptors,

suggesting that adiponectin either produced locally or from other sources can influence testicular function. We also report that adult chicken testes contained 8.3- and 9-fold greater *ADIPOR1* and *ADIPOR2* mRNA quantities respectively, when compared with those from sexually immature chickens. These findings indicate that sexual maturation is associated with the up-regulation of adiponectin receptor gene expression in the chicken testis. Based on the anatomical localization of cells expressing adiponectin and its receptors within the chicken testis, it is possible that adiponectin influences metabolism in peritubular cells and interstitial cells and may affect spermatogenesis. Therefore, future studies should aim at elucidating the physiological role of adiponectin and its receptors in testicular function.

Materials and Methods

Animals

Broiler breeder male chickens (Ross strain) were maintained by Longnecker Hatcheries (Elizabethtown, PA, USA) according to the recommended maintenance schedule. Prepubertal (4- and 14-week-old) and adult (29-week-old) male chickens were used in this study. Prepubertal chickens (4-and 14-week-old) were maintained under 8 h light:16 h darkness photoperiod and were provided with water and feed *ad libitum*. Adult chickens 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 euthanized by cervical dislocation prior to the removal of testes. One testis from each chicken was snap frozen in liquid nitrogen while the other testis was collected for histology analyses. All animal procedures were carried out in accordance with the Pennsylvania State University Institutional Animal Care and Use Committee.

Reagents

Trizol and RNEasy kits used to extract total RNA were obtained from Invitrogen and Qiagen respectively. Moloney murine leukemia virus (M-MuLV) reverse transcriptase, random primers (RP12), and Taq polymerase used for the RT-PCR were purchased from New England Biolabs (Beverly, MA, USA). Deoxyribonuclease-I (DNASEI) and dNTP mixture was purchased from Qiagen and Roche Applied Sciences respectively. ProLong Gold antifade reagent, TO-PRO-3 iodide, and quantitative real-time PCR reagents (Platinum SYBR Green qPCR Super Mix-UDG, RNAseOut), and Alexa Flour 488 goat anti-rabbit IgG were purchased from Invitrogen. Vectastain ABC Elite kit used for immunohistochemistry was obtained from Vector Laboratories (Burlingame, CA, USA).

RT-PCR analysis

Total RNA was extracted from testes using Trizol and the RNEasy kit following the manufacturer's protocol. The quality and quantity of testicular total RNA were determined by measuring absorbance at 260 and 280 nm using spectrophotometer (ND-1000, NanoDrop, Wilmington, DE, USA). Following on-column DNASEI treatment, first-strand cDNA was synthesized by reverse transcribing 1 μg of total RNA using RP12 and 2U M-MuLV reverse transcriptase in 20 μl reactions. Approximately 100 ng of single-stranded cDNA was used as template to amplify a 350 bp product of adiponectin using ADNFWD1 and ADNREV1 primers, a 350 bp product of ADIPOR1 using R1FWD1 and R1REV1, or a 345 bp product of ADIPOR2 using R2FWD1 and R2REV1 (Table 1). The primers used to amplify adiponectin, ADIPOR1, and ADIPOR2 have been validated previously (Maddineni *et al*. 2005, Ramachandran *et al*. 2007). A touchdown PCR was performed using the following parameters: 94 °C for 1 min, 30 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 11–30 cycles respectively. The PCR products were subjected to agarose gel electrophoresis and ethidium bromide staining for

Table 1 The nucleotide sequences of primers used to amplify chicken testicular adiponectin, *ADIPOR1*, *ADIPOR2* or 18S cDNAs.

Name	Sequence	GenBank accession no.	Product length (bp)
A) Adiponectin			
ADNFwd1	5′-ACAGGTGCAGAAGGACCGAGAGGATT-3′	AY838798	350
ADNRev1	5′-AAGACAGAGCCGCTTGCTTGGTCAAC-3′		
ADNFwd2	5′-GCCAGGTCTACAAGGTGTCA-3′	AY838798	86
ADNRev2	5′-CCATGTGTCCTGGAAATCCT-3′		
B) ADIPOR1			
R1Fwd1	5′-GAATACACACCGAGACGGGCAACATCT-3′	DQ072275	350
R1Rev1	5′-GCCCAAGACGCAGACAATGGAGAGGTA-3′		
R1Fwd2	5′-CCAGGAGAAGGTTGTGTTTG-3′	DQ072275	149
R1Rev2	5′-TGATCAGCAGTGCAATTCCT-3′		
C) ADIPOR2			
R2Fwd1	5′-GAGACTGGCAACATCTGGACGCATCTTC-3′	DQ072276	345
R2Rev1	5′-TGCGATGCCCAGGACACAAATCACAAT-3′		
R2Fwd2	5′-TCATGGCTCTTCCACACAGT-3′	DQ072276	145
R2Rev2	5′-AAGGCTGAGGGTTGCAGTAG-3′		
D) 18S			
18SFwd	5′-GTATGGTTGCAAAGCTGAAACTTA-3′	AF173612	137
18SRev	5′-AAGAGCTCTCAATCTGTCAATCCT-3′		

The GenBank accession number and the amplicon size are also provided.

visualization. For negative controls, RT reactions using 1 μg testicular total RNA without reverse transcriptase (−RT) and water were used as a template in place of single-stranded cDNA. The full-length cDNA encoding chicken adiponectin, ADIPOR1, and ADIPOR2 were also amplified using primers described previously (Maddineni *et al*. 2005, Ramachandran *et al*. 2007) and sequenced (Davis Sequencing, Davis, CA, USA).

Immunohistochemical localization of adiponectin, ADIPOR1, and ADIPOR2 in the chicken testis

Tissue preparation

Chicken testes were fixed by immersion in 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. 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).

Antisera production

Two sets of antisera against chicken adiponectin, ADIPOR1, or ADIPOR2 were made using respective immunogens directed against two different regions of adiponectin, ADIPOR1, or ADIPOR2. The first set of anti-chicken adiponectin antibody (ADNa) was made against a full-length N-terminal histidine-tagged recombinant chicken adiponectin expressed in *Escherichia coli* using chicken adiponectin cDNA (Maddineni *et al*. 2005); (Promab Biotechnologies, Albany, CA, USA). The second set of anti-chicken ADNb was generated using a 15 amino acid peptide immunogen (EMADQADQSDPKMSC) whose sequence was deduced from chicken adiponectin cDNA (Maddineni *et al*. 2005). Two anti-chicken ADIPOR1 antibodies (ADR1a and ADR1b) were made using partial ADIPOR1 peptide sequences (CQWDRFATPKHRQTR for ADNR1a and EQGAAGTASAEDPPC for ADNR1b). Similarly, two anti-chicken ADIPOR2 antibodies (ADR2a and ADR2b) were generated using partial ADIPOR2 peptide sequences (ENSSEDSNHNDESPC for ADR2a and CTTQTAFGEDSSEQR for ADNR2b). The polyclonal antisera were generated by immunizing New Zealand white rabbits with keyhole limpet hemocyanin-conjugated adiponectin, ADIPOR1, or ADIPOR2 peptides (Sigma Genosys, The Woodlands, TX, USA; Covance Research Products, Denver, PA, USA).

Immunofluorescent staining

Testicular tissue sections embedded in paraffin 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 washes in TBS containing 0.50% Triton X-100 (TBSX; Sigma), slides were treated in a blocking solution (2.5% normal goat serum in TBSX) for 1 h. Slides were incubated with primary antibody (1:250 rabbit anti-chicken adiponectin (ADNa), affinity-purified rabbit anti-chicken ADIPOR1 IgG (ADNR1a), or rabbit anti-chicken ADIPOR2 (ADR2a)) for ∼18 h at 4 °C. Following washes in TBS, sections were incubated for 1 h with Alexa Flour 488 goat anti-rabbit IgG (1:400) in blocking solution for 1 h. Coverslips were applied to the slides using ProLong Gold antifade mounting medium with TO-PRO-3 iodide (1:500) following several washes in TBS. To determine the specificity of immunostaining, the primary antibodies were pre-adsorbed with 25 μg/ml of the immunogen for 18 h and centrifuged to pellet the antibody–antigen complexes. As a negative control, non-immune rabbit serum (Vector Laboratories) or primary antibody pre-adsorbed with each respective antigen was substituted in place of the primary antibody. Unless otherwise noted, all steps were performed at room temperature while the washes consisted of three incubations with TBS each 10 min in duration. Green (adiponectin, ADIPOR1, or ADIPOR2) and red fluorescence (nuclear stain) were visualized by exciting fluorophores sequentially with respective lasers using an Olympus FV1000 Laser Scanning Confocal Microscope (Olympus America, Melville, NY, USA).

Chromogenic immunostaining

Testicular tissue sections were prepared for immunostaining as described above. Deparaffinized and hydrated tissue sections were treated with 3% hydrogen peroxide solution to quench endogenous peroxidase. Following several washes in TBSX, slides were treated in a blocking solution (2.5% normal goat serum in TBSX) for 1 h. Slides were incubated with affinity-purified anti-adiponectin IgG (ADNb; 1:400), anti-ADIPOR1 antisera (ADNR1a; 1:250), or anti-ADIPOR2 antisera (ADNR2b; 1:500) for ∼18 h at 4 °C. Following washes in TBS, sections were incubated for 1 h with biotinylated goat anti-rabbit IgG (Vector Laboratories). Tissue sections were washed in TBS, and then treated for 1 h with an avidin peroxidase complex. A brown-colored immunoreaction product was developed using 3′,3′-diaminobenzidine tetrahydrochloride and hydrogen peroxide (Vector Laboratories). After several washes in TBS, tissue sections were dehydrated, cleared in Histoclear, and coverslipped. As a negative control, pre-immune rabbit serum or primary antibody pre-adsorbed with respective antigen was substituted in place of the primary antibody. After immunostaining, the negative control tissue sections were counterstained with hematoxylin to reveal cellular morphology. Unless otherwise noted, all steps were performed at room temperature, with wash steps consisting of three 10-min incubations in TBS buffer. Testicular tissue sections were examined and photomicrographs taken using a Zeiss Axioskop microscope fitted with Olympus DP71 digital camera (Olympus America).

Quantification of testicular adiponectin, ADIPOR1 *and,* ADIPOR2 *mRNA quantities*

Total RNA from the 4-, 14-, and 29-week-old broiler breeder chicken testes (n=4–6/age group) were reverse transcribed using random primers as described above and subjected to quantitative real-time PCR for the determination of adiponectin, *ADIPOR1*, and *ADIPOR2* mRNA quantities. Chicken adiponectin, *ADIPOR1*, and *ADIPOR2* mRNAs and 18S mRNA were quantified

using 2.5 μl of the RT reaction (equivalent to 50 ng single-stranded cDNA) as template in the real-time quantitative PCR. The real-time quantitative PCR consisted of 1×Platinum SYBR Green qPCR Super Mix-UDG (Invitrogen) and 300 nM of forward and reverse primers (ADNFWD2/REV2, R1FWD2/REV2, R2FWD2/REV2; 18SFwd/18SRev; Table 1). Reactions were carried out in the DNA Engine Opticon II (MJ Research, Reno, NY, USA) with the following thermocycle parameters: 50 °C for 2 min, 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 done by heating the PCR products from 65 to 95 °C, held for 15 s at increments of 0.2 °C and the 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 critical threshold (C_T) values for adiponectin, *ADIPOR1*, and *ADIPOR2*, and 18S mRNAs. Adiponectin, *ADIPOR1*, and *ADIPOR2* mRNA quantities were expressed as a proportion of 18S mRNA quantity using the following equation: ΔC_T=(Average $C_{T\text{-target}}$-Average $C_{T\text{-18S}}$). Data were transformed from log-linear to linear terms using the function: $2^{-\Delta C_T}$. For negative controls, RT reactions using 1 μg total RNA with no reverse transcriptase (−RT) were used as template in place of single-stranded cDNA in the quantitative real-time PCR.

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 the analysis of quantitative real-time PCR data, relative adiponectin, *ADIPOR1*, and *ADIPOR2* mRNA quantities to 18S mRNA quantity was first converted from log-linear to linear terms. A probability level of $P<0.05$ was considered statistically significant. Results are presented as arithmetic s.e.m.

Declaration of interest

The authors of this manuscript have nothing to disclose.

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