YWK-II protein as a novel G_o-coupled receptor for Müllerian inhibiting substance in cell survival

Xueqian Yin[1,*], Songying Ouyang[1,*], Wenming Xu[2,*], Xiaopeng Zhang[1], Kin Lam Fok[2], Hau Yan Wong[2], Jiaping Zhang[2], Xiaobo Qiu[1], Shiying Miao[1], Hsiao Chang Chan[2,‡] and Linfang Wang[1,‡]

[1]National Laboratory of Medical Molecular Biology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, Peking Union Medical College, 5 Dong Dan San Tiao, Beijing 100005, China
[2]Epithelial Cell Biology Research Center, Li Ka Shing Institute of Health Sciences, Department of Physiology, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, Hong Kong, China

*These authors contributed equally to this work
‡Authors for correspondence (e-mail: wanglf@ms.imicams.ac.cn; hsiaocchan@cuhk.edu.hk)

Accepted 6 March 2007
Journal of Cell Science 120, 1521-1528 Published by The Company of Biologists 2007
doi:10.1242/jcs.001230

Summary

Müllerian inhibiting substance (MIS) has recently been implicated in multiple cellular functions including promotion of cell survival, but the receptor(s) and signaling pathways involved remain elusive. We have investigated the possibility of YWK-II protein, previously shown to interact physically with MIS and G_o protein, being a receptor mediating the cell survival effect of MIS. In YWK-II-overexpressing CHO cells, MIS activates the G_o-coupled ERK1/2 signaling pathway and promotes cell survival with altered levels of p53 and caspase-3. YWK-II antibody is found to interfere with the ability of MIS to promote viability of mouse sperm and affect MIS-activated ERK1/2 phosphorylation. In vivo studies involving injection of YWK-II antibody into the seminiferous tubule of the mouse testis, where MIS is known to be produced, show significant reduction in the sperm count with accumulation of p53 and cleaved caspase-3 in testicular nuclei. Taken together, the present study has demonstrated a new G_o-coupled receptor for MIS in mediating ERK1/2 activation leading to anti-apoptotic activity or cell survival.

Key words: MIS, YWK-II protein, APLP2, CHO, Sperm viability, G_o, Extracellular signal-regulated kinase (ERK), Cell survival

Introduction

Müllerian inhibiting substance (MIS), also known as anti-Müllerian hormone (AMH), has long been recognized for its signature effect on development, causing regression of the Müllerian ducts, the anlagen of the Fallopian tubes, uterus and upper vagina in mammalian species, and thus its key role in sex determination (Teixeira et al., 2001). Signaling by MIS is traditionally believed to be propagated by the MIS type II receptor, a transmembrane serine threonine kinase expressed in high levels in the Müllerian duct, Sertoli cells, granulose cells and uterus, which, upon binding to MIS, recruits a type I receptor into the receptor complex (Teixeira et al., 2001). Interestingly, MIS has been shown to promote sperm viability but yet no MIS type II receptor has been found in sperm (Teixeira and Donahoe, 1996). More recently, studies have also implicated MIS in mammary tumor growth in vivo by inducing cell cycle arrest and apoptosis (Gupta et al., 2005). However, conflicting results have also been obtained indicating MIS as a survival factor for neurons in vitro (Wang et al., 2005). Taken together, it appears plausible that MIS may have multiple actions depending upon the nature of the receptors present in different cells. However, receptors, apart from the MIS type II and I receptors, and related signaling pathways involved in mediating different effects of MIS, especially on cell survival, have not been elucidated.

YWK-II protein is a type I membrane protein and the target antigen to a monoclonal antibody raised against human sperm proteins (Yan et al., 1990). YWK-II protein contains a segment with high homology (70.6%) to the transmembrane-cytoplasmic region of the amyloid protein precursor (APP) found in brain plaques of suffers of Alzheimer's disease (Yan et al., 1990). The YWK-II protein has been subsequently found to be homologous to the human placenta amyloid protein precursor homologue (APPH) (Sprecher et al., 1993) and is related to the rat amyloid-precursor-like protein 2 (APLP2) (Sandbrink et al., 1994). The human homologous gene coding the YWK-II protein (also now known as APLP2) has been mapped to chromosome locus 11q24-25 and is expressed ubiquitously in various tissues. YWK-II protein/APLP2 appears to be a multifunctional protein although the mechanism remains to be clarified. In neuronal PC12 cells undergoing programmed cell death following trophic factor withdrawal, the synthesis of APLP2 was upregulated (Araki and Wurtman, 1998), indicating its link to apoptosis or cell survival.

In a previous study, we showed that the cytoplasmic segment of the YWK-II protein binds G_o protein (Huang et al., 2000). When the extracellular segment of YWK-II protein was used as bait in a yeast two-hybrid system, a recombinant active MIS (303-376; rMIS) was found binding to YWK-II protein and the interaction was verified by a GST pull-down assay and by surface plasmon resonance (SPR) (Tian et al., 2001). On the other hand, rMIS has been shown to facilitate the motility and sustain the viability of human sperm (Tian et al., 2001); however, the mechanism of action of MIS on sperm remains unclear since an MIS type II receptor has not

been shown to be present in germ cells (Teixeira and Donahoe, 1996; Baarends et al., 1994). We thus hypothesized that YWK-II protein may be a G_o-coupled receptor involved in mediating the observed cell survival enhancing effect of MIS in sperm.

Extracellular signal-regulated kinases 1 and 2 (ERK1/2) are important members of the mitogen-activated protein kinase (MAPK) family known as key effectors of activation by G-protein-coupled receptors (Werry et al., 2005). The G-protein-coupled activation of the ERK/MAPK phosphorylation cascades have been thought to play crucial roles in the regulation of cell cycle progression (or cell growth) and apoptosis (or cell survival) in diverse types of cells (Chang et al., 2003; Gudermann, 2001). The observed effects of MIS in promoting cell survival in sperm (Tian et al., 2001) and neurons (Wang et al., 2005), the demonstrated ability of YWK-II protein to interact with both MIS and G_o protein, and the observed link between YWK-II protein/APLP2 and apoptosis and/or cell survival (Ariazi and Gould, 1996; Jarvis and Grant, 1999) suggest that YWK-II protein may serve as a G-protein-coupled receptor for MIS in cell survival.

In the present study, we tested the above hypothesis and demonstrated the involvement of YWK-II protein in G_o-coupled ERK signaling pathways that mediate the effect of MIS on cell survival in a model cell line, CHO, overexpressing exogenous YWK-II protein, and in mouse sperm in which YWK-II protein is abundantly expressed.

Results

Localization of YWK-II protein on CHO cell membrane

In order to demonstrate the ability of YWK-II protein to act as a receptor, its localization on cell membrane was examined. A eukaryotic expression vector containing mouse IgGκ signal peptide sequence, a cDNA segment of the YWK-II protein, the extracellular region previously used as a bait protein to detect interaction with rMIS in the yeast two-hybrid system (Tian et al., 2001), and enhanced green fluorescence protein (EGFP) for detection purpose, was constructed (pEGFP-N1-YWK-II). When CHO cells were transiently transfected with the control vector pEGFP-N1 and the nuclei stained with propidium iodide (PI), EGFP was found, by confocal microscopy, to distribute evenly throughout the cells including the cytoplasm and nuclei (Fig. 1a-c). However, when CHO cells were transfected with pEGFP-N1-YWK-II, a green ring of fluorescence was detected (Fig. 1d-f), suggesting a membrane location. To confirm that the exogenously expressed EGFP-YWK-II protein is the proper YWK-II protein, immunofluorescence with YWK-II antiserum as primary antibody and red fluorescent TRITC-conjugated goat anti-mouse IgG as second antibody was performed and resulted in rings of both green and red fluorescence that overlapped, as compared to a ring emitting only the green fluorescence when preimmune rabbit serum was used as the primary antibody, in the control (Fig. 1g-l). The nature of the EGFP-tagged recombinant protein was further demonstrated by western blotting using YWK-II antiserum which showed a 67 kDa protein in CHO cells stably transfected with pEGFP-N1-YWK-II but the endogenous YWK-II protein was not detected in the pEGFP-N1-transfected cells (Fig. 2). These results proved that the EGFP-tagged recombinant protein is YWK-II protein and that the EGFP-YWK-II protein is localized to the membrane in CHO cells, fulfilling the prerequisite of a receptor. Therefore, the EGFP-YWK-II-overexpressing cells can be utilized as a model to investigate the role of YWK-II protein as a receptor for MIS and its related signaling pathways.

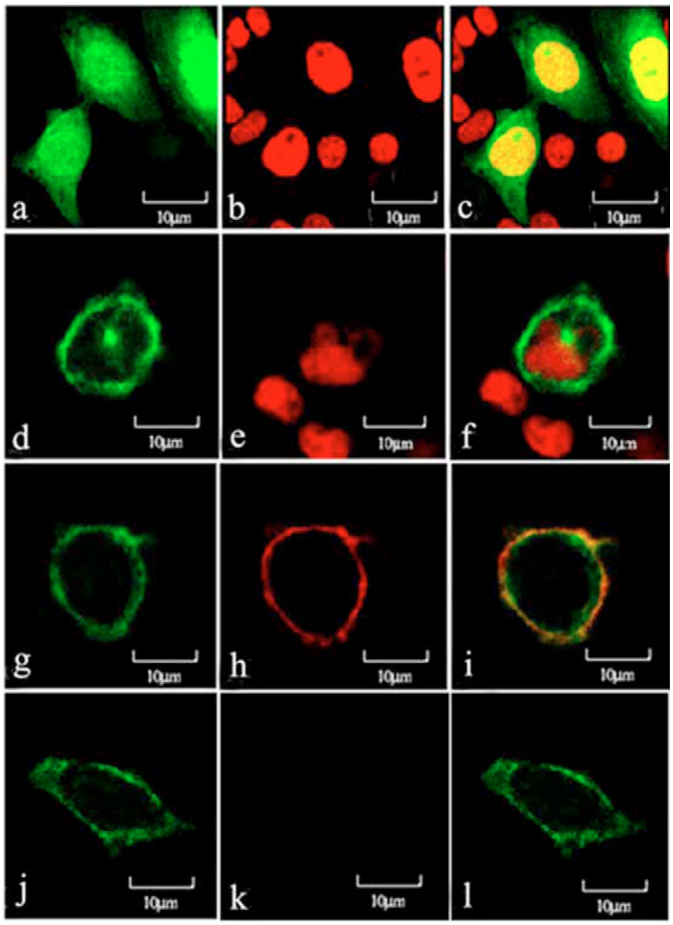

Fig. 1. Localization of the EGFP-YWK-II protein on the cell membrane visualized by laser confocal scanning microscopy. (a-c) CHO cells transiently transfected with pEGFP-N1 were fixed, permeabilized and stained with propidium iodide (PI). (a) EGFP-overexpressing CHO cells (green). (b) CHO cells stained with PI to delineate the nucleus (red). (c) superimposed image of a and b. (d-f) CHO cells transiently transfected with pEGFP-N1-YWK-II were fixed, permeabilized and stained with PI. (d) EGFP-YWK-II-overexpressing CHO cells (green). (e) CHO cells stained with PI to delineate the nucleus (red). (f) Superimposed image of d and e. (g-i) CHO cells were transiently transfected with pEGFP-N1-YWK-II, fixed and not permeabilized. (g) EGFP-YWK-II-overexpressing CHO cells (green). (h) EGFP-YWK-II protein overexpressed on the cell membrane with rabbit YWK-II antiserum as the primary antibody and TRITC-conjugated goat anti-mouse IgG as the second antibody (red). (i) Superimposed image of g and h. (j-l) CHO cells were transiently transfected with pEGFP-N1-YWK-II, fixed and not permeabilized. (j) EGFP-YWK-II-overexpressing CHO cells (green). (k) EGFP-YWK-II-overexpressing cells using rabbit preimmune serum as the primary antibody and TRITC-conjugated goat anti-mouse IgG as the secondary antibody showing negative staining. (l) Superimposed image of j and k.

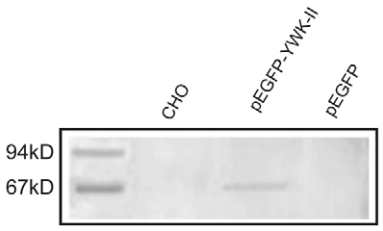

Fig. 2. Confirmation of YWK-II protein expression in the CHO cell line by western blotting. Electrophoresis pattern of lysates of CHO cell, CHO cells stably transfected with pEGFP-N1-YWK-II (pEGFP-YWK-II) or pEGFP-N1 (pEGFP), stained with rabbit YWK-II antibody. The total protein loaded on the gel was 20 μg. Only the 67 kDa protein in lysate obtained from stable CHO cells transfected with pEGFP-N1-YWK-II was detected by YWK-II antibody (molecular mass of recombinant EGFP-YWK-II protein, ~67 kDa).

Coupling of YWK-II protein to G_o in mediating MIS-activated ERK signaling pathways

To study the possible role of YWK-II protein in the ERK signaling pathway induced by MIS, its recombinant active form, rMIS, was added to culture medium at final concentrations of 0, 0.035 and 0.35 nM, and the amount of phosphorylated ERK1/2 and total ERK1/2 detected by western blotting. In YWK-II-overexpressing CHO cells, the levels of ERK1/2 phosphorylation were significantly higher than that of non-transfected or EGFP-transfected CHO controls at all concentrations of rMIS used (P<0.01, Fig. 3A). The amplified rMIS-induced ERK1/2 activation by overexpression of YWK-II protein suggests that the ERK signaling pathway induced by MIS may be mediated by YWK-II protein, consistent with the notion that YWK-II protein may be a receptor for MIS in cell survival. Further studies confirmed that the effect of rMIS on ERK1/2 activation in YWK-II-overexpressing CHO cells was concentration dependent (Fig. 3B). The MIS activated ERK1/2 signaling pathway was also enhanced in YWK-II-transfected COS-7 cells, similar to that observed in CHO cells, confirming that YWK-II protein is important for mediating the effect of MIS (data not shown).

Pertussis toxin (PTX), a known inhibitor of G_i and G_o, was added to the culture of YWK-II-overexpressing CHO cells and incubated for 24 hours. As shown in Fig. 3C, ERK1/2 phosphorylation induced by rMIS was inhibited by pretreatment of the cells with PTX (1 μg/ml), indicating that the MIS-induced and YWK-II protein-mediated ERK activation is coupled to either G_i or G_o protein (P<0.01).

To distinguish whether G_i or G_o is involved in the MIS-induced ERK activation, cDNAs encoding the C-terminal 11 amino acids of human G_α subunits of G_i ($G_{\alpha i1/2}$) or G_o ($G_{\alpha o1}$, $G_{\alpha o2}$), overexpression of which is expected to interfere with the cellular responses of the corresponding proteins, were cloned into pcDNA3.1(+) and transfected into the YWK-II-overexpressing CHO cells. The three types of cells were treated with rMIS and it was found that the rMIS-induced ERK1/2 phosphorylation was inhibited to a similar extent, in cells transfected with pcDNA3.1(+)-$G_{\alpha o1}$ or pcDNA3.1(+)-$G_{\alpha o2}$, but not in cells transfected with pcDNA3.1(+)-$G_{\alpha i1/2}$ (P<0.05, Fig. 3D). The results confirmed the involvement of G_o protein and excluded the involvement of G_i protein in the MIS-induced and YWK-II protein-medicated ERK signaling pathway.

The activation of G proteins induces the separation of G_α from $G_{\beta\gamma}$, whereby they may act independently or cooperatively. To provide further support for the coupling of YWK-II protein to G protein, cDNA encoding the C-terminal region of βARK1, an inhibitor of $G_{\beta\gamma}$, was cloned into pCMV to construct pCMV-βARK1-C, and transfected into YWK-II-overexpressing CHO cells. The rMIS-induced increase in ERK1/2 phosphorylation in YWK-II-overexpressing CHO cells was significantly reduced upon transfection with pCMV-βARK1-C (P<0.05; Fig. 3E).

One of the mediators of the G-protein-coupled ERK signaling pathway is Ras (Grewal et al., 1999). To examine this, the dominant negative and dominant positive mutants of $p21^{ras}$, pCMV-RasN17 and pCMV-RasV12, respectively, were transfected separately into YWK-II-overexpressing CHO cells. The rMIS-induced ERK1/2 phosphorylation was significantly reduced and enhanced following transfection with pCMV-RasN17 and pCMV-RasV12, respectively (P<0.05; Fig. 3F).

It has been reported that protein kinase C (PKC) is involved in G_i/G_o/PLC-coupled ERK activation (Kim et al., 2003). In the present study, when YWK-II-overexpressing CHO cells were pretreated with the PKC inhibitor GF109203X (3.5 μM) for 2 hours, the rMIS-induced ERK1/2 phosphorylation was significantly reduced (P<0.01, Fig. 3G), indicating the involvement of PKC. In addition, GF109203X (3.5 μM) could produce further reduction in rMIS-induced ERK1/2 activation in YWK-II-overexpressing CHO cells transfected with pCMV-RasN17 (P<0.01, Fig. 3H), suggesting that PKC and Ras may be involved in separate ERK activation pathways.

Enhanced rMIS-induced cell survival in YWK-II-overexpressing CHO cells

To confirm a role of YWK-II protein in cell survival, an MTT assay was conducted to examine whether overexpression of YWK-II protein could enhance cell viability under serum-starved condition, a condition known to induce apoptosis. Aliquots of rMIS were added to the serum-starved EGFP-YWK-II-overexpressing, as well as the EGFP-transfected and non-transfected control CHO cells, to the final concentrations of 0, 0.035, 0.35, 3.5 and 35 nM. Values of each control untreated group were set at 100%, permitting comparison with the data obtained with the various test groups. The levels of cell viability in response to all concentrations of rMIS in the YWK-II-overexpressing cells were significantly higher than those exhibited by the other two controls (*P<0.01, Fig. 4A), indicating the involvement of YWK-II protein in mediating the effect of MIS on cell survival.

To demonstrate that the MIS-enhanced cell viability was due to anti-apoptotic activity, the levels of the tumor suppressor p53 [a marker of apoptosis (Lowe et al., 1993)], in response to rMIS were examined and compared in the YWK-II-overexpressing cells and the EGFP-transfected and non-transfected CHO controls. As shown in Fig. 4B, the level of p53 in the YWK-II-overexpressing cells was greatly reduced as compared with the EGFP-transfected control, suggesting reduced apoptotic activity in the presence of YWK-II protein. To further confirm the involvement of YWK-II protein in mediating the anti-apoptotic effect of MIS, a specific antibody against YWK-II protein was used in conjunction with rMIS in western blot

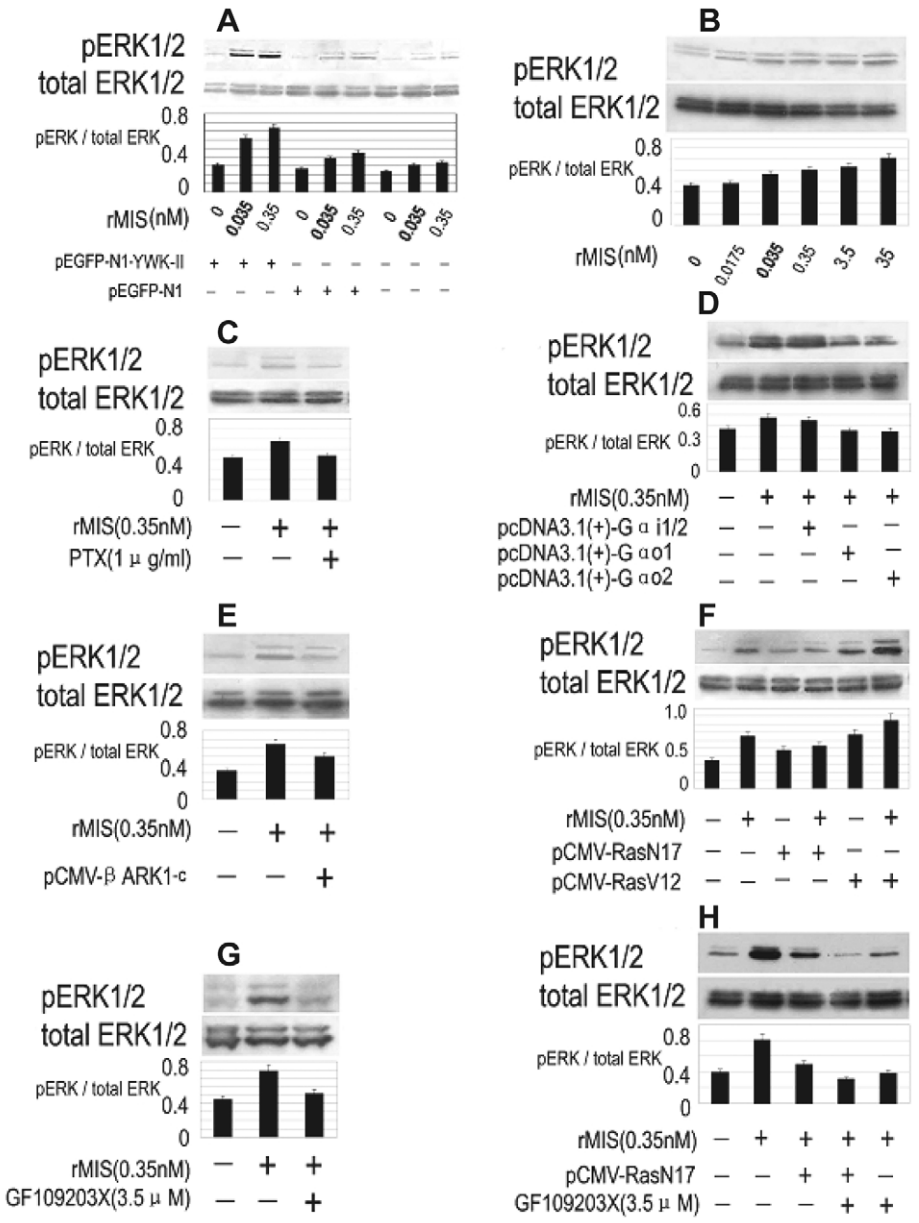

Fig. 3. Demonstration of involvement of YWK-II protein in MIS-induced ERK1/2 phosphorylation coupled to G_o, $G_{o\beta\gamma}$, Ras and PKC. Cells were lysed and assayed for p-ERK1/2 and total ERK1/2 by western blotting. The averaged ratio values of triplicate experiments are presented with corresponding western blot results. Unless stated otherwise, the cells were starved for 16 hours prior to stimulation with 0.35 nM rMIS at 37°C for 5 minutes. Statistical analysis was performed using Student's *t*-test (two-tailed comparison of means for paired samples). (A) Enhanced rMIS-induced ERK1/2 phosphorylation in YWK-II-overexpressing CHO cells (pEGFP-N1-YWK-II) as compared to that in EGFP-overexpressing CHO cells (pEGFP-N1) and non-transfected CHO cells. Different concentrations of rMIS used: 0, 0.035 and 0.35 nM (P<0.01). (B) Concentration-dependent effect of rMIS on ERK1/2 phosphorylation in EGFP-YWK-II-overexpressing CHO cells. Different concentrations of rMIS used: 0, 0.0175, 0.035, 0.35, 3.5 and 35 nM. (C) Effect of PTX on rMIS-induced ERK1/2 phosphorylation in EGFP-YWK-II-overexpressing CHO cells. Cells were starved for 16 hours and pretreated with PTX (1 μg/ml) or left untreated for 24 hours prior to stimulation with rMIS (P<0.01). (D) Effects of C-terminal regions of $G_{i1/2}$, $G_{\alpha o1}$ and $G_{\alpha o2}$ on rMIS-induced ERK1/2 phosphorylation. pcDNA3.1(+)-$G_{\alpha i1/2}$, pcDNA3.1(+)-$G_{\alpha o1}$ and pcDNA3.1(+)-$G_{\alpha o2}$ were transfected into EGFP-YWK-II-overexpressing CHO cells for 24 hours (P<0.05). (E) Effect of βARK1 on rMIS-induced ERK1/2 phosphorylation. The EGFP-YWK-II-overexpressing CHO cells were transfected with pCMV-βARK1-C for 24 hours (P<0.05). (F) Effects of pCMV-RasN17 and pCMV-RasV12 on rMIS-induced ERK1/2 phosphorylation. EGFP-YWK-II-overexpressing CHO cells were transfected with pCMV-RasN17 and pCMV-RasV12 for 24 hours (P<0.05). (G) Effect of GF109203X on rMIS-induced ERK1/2 phosphorylation in EGFP-YWK-II-overexpressing CHO cells. Cells were starved for 16 hours and pretreated with GF109203X (3.5 μM) or left untreated for 2 hours prior to stimulation with rMIS (P<0.01). (H) Effect of pCMV-RasN17 and GF109203X on rMIS-induced ERK1/2 phosphorylation. EGFP-YWK-II-overexpressing CHO cells were transfected with pCMV-RasN17 for 24 hours. The cells were starved for 16 hours and treated with or without GF109203X (3.5 μM) for 2 hours prior to stimulation with rMIS (P<0.01).

analysis for caspase-3, a central effector in the apoptosis cascade (Porter and Janicke, 1999). As shown in Fig. 4C, rMIS increased the level of pro-caspase-3 in YWK-II-overexpressing cells, suggesting its anti-apoptotic action. The rMIS-enhanced pro-caspase-3 level could be reduced by YWK-II antibody, but not the control IgG, lending further support for YWK-II protein as a receptor for MIS in cell survival.

Involvement of YWK-II protein in MIS-induced ERK activation and cell survival in mouse sperm

Having demonstrated the involvement of YWK-II protein in mediating the cell survival enhancing effect of rMIS in the YWK-II-overexpressing CHO model, we wanted to examine the involvement of endogenously expressed YWK-II protein in mediating the MIS-induced cell survival and related signaling pathways in sperm. The possible expression of MIS type II receptors in sperm or CHO cells was excluded by RT-PCR experiments (data not shown). As shown in Fig. 5A, for mouse spermatozoa collected from the caudal epididymis and suspended in sperm washing medium for 10 and 30 minutes, the addition of rMIS (0.075 μM) significantly increased the sperm viability at 30 minutes (*P<0.05); however, YWK-II

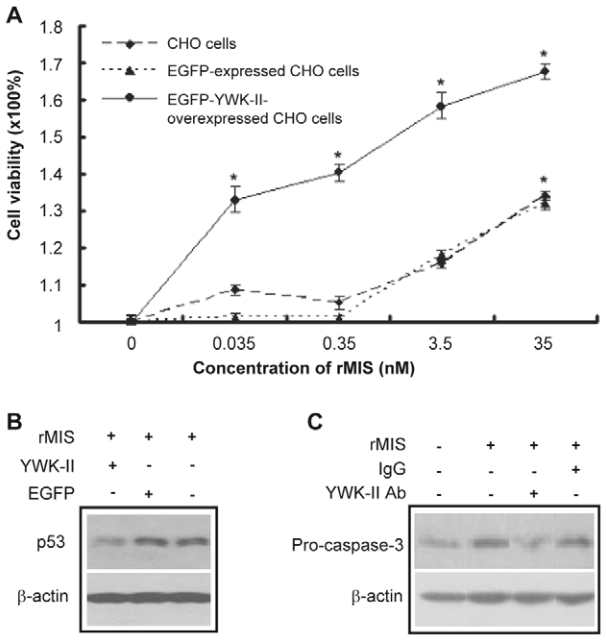

Fig. 4. Effect of rMIS on cell viability and apoptotic activity in EGPF-YWK-II-overexpressing CHO cells. (A) Effect of rMIS and YWK-II proteins on cell viability. Cells transfected with EPGFP-YWK-II protein or EGFP and non-treated CHO cells were treated with different concentrations of rMIS for 3 days and assayed using the MTT method. The values obtained with the control untreated groups were set at 100% (**P*<0.01). (B) Effect of rMIS on p53 levels in CHO cells. The established EGFP-YWK-II-overexpressing (YWK-II) and EGFP-transfected (EGFP) CHO cells and non-transfected CHO controls were cultured to confluence and incubated in serum-free medium for 24 hours prior to stimulation with 0.35 nM rMIS for 5 minutes. (C) Effect of rMIS on caspase-3 levels in YWK-II-overexpressing CHO cells. The cells were treated with 0.35 nM rMIS in the presence of YWK- II antibody (YWK-II Ab, 150 ng) or control IgG (150 ng) followed by western blot analysis of pro-caspase-3, with β-actin as the loading control.

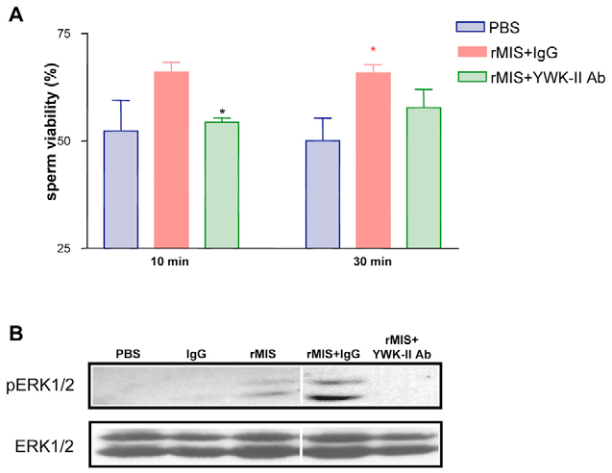

Fig. 5. Effect of rMIS and YWK-II antibody on mouse sperm viability and ERK activation. (A) Sperm viability in response to treatment with rMIS and YWK-II antibody. Sperm were preincubated with control IgG or YWK-II antibody (Ab) for 15 minutes prior to addition of equal volume of PBS or rMIS (0.075 μM) followed by further incubation for 10 minutes or 30 minutes. Sperm were mixed with 0.04% Trypan Blue for evaluation of viability. The data are the mean percentage of viable sperm from three experiments (**P*<0.05). (B) Effect of YWK-II antibody on rMIS-induced ERK1/2 activation in mouse sperm. Sperm were preincubated with control IgG or YWK-II antibody for 15 minutes prior to addition of rMIS (0.075 μM) followed by further incubation for 30 minutes. The proteins were extracted for western blot analysis with total ERK1/2 as a loading control.

antibody reversed the rMIS-enhanced sperm viability. An irrelevant control antibody, IgG, was also included in the rMIS-treated group as a control for YWK-II antibody. Western blotting of the sperm samples (Fig. 5B) showed that rMIS activated ERK1/2 (in the presence of control IgG), which could be completely abolished by YWK-II antibody.

In vivo induction of apoptosis and sperm count decrease by injection of YWK-II antibody into mouse testis

Since MIS is produced in the testis and YWK-II protein is expressed in the sperm, they may play a role in sperm survival. To examine the role of YWK-II protein in MIS-induced cell survival in vivo, YWK-II antibody (40 μg/ml) was injected into the seminiferous tubule of mouse testis, with control antibody IgG injected into the opposite testis in each experiment animal to neutralize the endogenous YWK-II protein and examine its effect on apoptosis and sperm count. Sperm were collected from the epididymis 48 hours after antibody injection, and as shown in Fig. 6A, the sperm count from the YWK-II antibody-treated testes was significantly lower than that from the testes treated with control IgG (**P*<0.05, *n*=9). The YWK-II antibody-induced decrease in sperm count could be due to apoptosis of germ cells in the testis. To test this, we examined the testicular level of p53, which is known to be highly expressed in the testis, with reported translocation into the nucleus upon stress inducing apoptosis (Yin et al., 1997). Therefore, we checked whether YWK-II antibody injection could induce entry of p53 into the nucleus. Western blots showed increased expression of p53 in nuclear extract of the YWK-II antibody-treated, but not the control, testes (Fig. 6B), indicating anti-apoptotic action of the MIS/YWK-II protein signal system. We further examined caspase-3 activation to confirm apoptosis in the testis upon YWK-II antibody injection. Western blotting indeed revealed increased caspase-3 cleavage (activation) in testicular nuclei after injection of YWK-II antibody (Fig. 6B).

Discussion

In this study, we have tested the hypothesis that YWK-II protein may act as a G_o-coupled receptor for MIS in mediating its cytoprotective or cell-survival promoting effect. Apart from the previously demonstrated binding of YWK-II protein to both rMIS and G_o protein (Huang et al., 2000; Tian et al., 2001), the present study has provided further evidence of the involvement of YWK-II protein in MIS-activated and G_o-coupled ERK signaling pathways as well as in mediating the effect of MIS on cell survival in a CHO model and mouse sperm in vitro and in vivo.

We first demonstrated the ability of YWK-II protein to

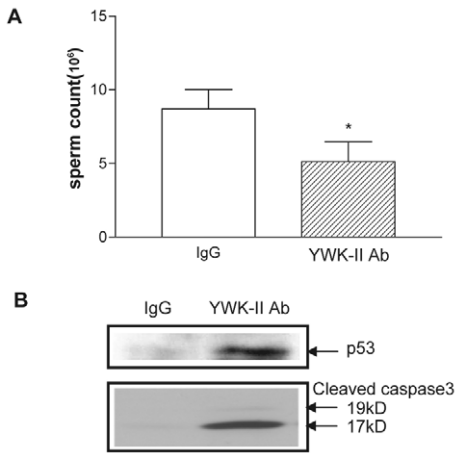

Fig. 6. In vivo effect of YWK-II antibody on sperm count and apoptotic activity of mouse testis. (A) Adult BALB/c mice were injected with YWK-II antibody (Ab; 40 μg/ml) or control IgG as described in the Materials an Methods and cauda sperm were recovered 48 hours later and sperm count was analyzed by computer-assisted sperm analysis. The data are the mean number of sperm per testis from nine mice (*P<0.05, n=9). (B) In vivo effect of YWK-II antibody on expression level of nuclear p53 and cleaved caspase-3 in mouse testis, demonstrated by western blot analysis.

transduce the extracellular signal of MIS into intracellular signals leading to activation of ERK, which is well known for its crucial role in cell growth and survival, in a CHO cell model. The ovary-derived CHO cell is similar to the sperm in that it produces G_o protein (Vanhauwe et al., 2002) while lacking the MIS type II receptor (Gouedard et al., 2000; Salhi et al., 2004). Thus a stable CHO cell line overexpressing YWK-II protein has been established to investigate new receptor pathway for MIS. The exogenously expressed YWK-II protein is found localized to the cell membrane, and therefore, the stable YWK-II-overexpressing cell line can be used as an ideal model to study ligand-receptor interaction between MIS and YWK-II protein, as well as the related signaling pathway. In fact, activation of ERK1/2 is most pronounced when rMIS is added to CHO cells overexpressing YWK-II protein as compared to non-transfected or EGFP-overexpressing CHO cells. These findings indicate that the overexpressed and membrane-bound YWK-II protein is able to mediate the function of MIS and activate the ERK signaling pathway.

Our previous study demonstrated that YWK-II protein interacts with G_o (Huang et al., 2000), and others have shown that this G protein is connected to the ERK cascade (Kim et al., 2003). Therefore, YWK-II protein, as a receptor for MIS, may be coupled to G_o protein to activate ERK. This notion is supported by the present results showing inhibition of MIS-induced ERK activation in YWK-II-overexpressing CHO cells by PTX, a toxin that acts by ADP ribosylation of the Cys^{351} residue in the C terminus of G_i and G_o proteins, thereby neutralizing their ability to interact with receptors and preventing the activation of the downstream signaling pathways. The coupling of MIS-induced and YWK-II protein-mediated ERK activation to G_o, but not G_i, is further supported by the observed inhibition of the rMIS-induced ERK activation by transfection of YWK-II-overexpressing cells with two isoforms of the C terminus of $G_{\alpha o}$ subunits, $G_{\alpha o1}$ and $G_{\alpha o2}$, but not that of the $G_{\alpha i}$ subunit $G_{\alpha i1/2}$. These C termini have been implicated in mediating receptor-G protein interaction, receptor selectivity and interaction with downstream signals, and thus, overexpression of the C-terminus peptides would tend to block these cellular responses. These results show that ERK1/2 phosphorylation induced by MIS and YWK-II protein is mediated by G_o instead of G_i. The coupling of YWK-II protein-mediated ERK activation to G protein is also demonstrated by the present results showing that ERK1/2 phosphorylation induced by rMIS in YWK-II-overexpressing CHO cells is inhibited by transfection with a peptide, βARK1, that can bind to the C-terminal region of $G_{\beta\gamma}$ and neutralize its activity (Koch et al., 1994). The activation of G proteins induces the separation of G_α from $G_{\beta\gamma}$, whereby they may act independently or cooperatively. Activated $G_{\beta\gamma}$ is believed to be connected with phospholipase C (PLC) activity (Vanhauwe et al., 2002; Camps et al., 1992) and to the Ras signaling pathway. Both pathways may occur in parallel to increase the level of ERK1/2 phosphorylation. In the present study, transfection of YWK-II-overexpressing CHO cells with the dominant negative and positive Ras mutants resulted in decreased and enhanced rMIS-induced ERK1/2 phosphorylation, respectively, suggesting that the YWK-II protein-mediated MIS signaling system also involves Ras, a key player in the ERK cascade.

The present study has further demonstrated that the YWK-II protein-mediated MIS signaling system involves PKC, which occupies a central cytoprotective (i.e. anti-apoptotic) role in the intracellular signaling networks governing cell survival, including the ERK/MAPK pathway (Jarvis and Grant, 1999). The PKC inhibitor GF109203X partially inhibited phosphorylation of ERK1/2 induced by MIS. PKC is known to be activated either by $G_{\beta\gamma}$ through the activation of PLC, or by Ras through the activation of phosphoinositide 3-kinase (Lambert et al., 2002) and PLC (Kelley et al., 2001). Interestingly, the present study shows that transfection with the Ras negative mutant and co-treatment with the PKC inhibitor has an additive inhibitory effect on MIS-induced ERK1/2 phosphorylation, indicating that YWK-II protein mediates the intracellular signaling pathway involving the parallel action of Ras and PKC. The present finding that the MIS/YWK-II protein signaling system involves a number of key signaling molecules known to connect to G_o protein and the ERK cascade further supports YWK-II protein as a G_o-protein-coupled receptor for MIS, particularly in cell survival.

Apart from the demonstrated coupling of YWK-II protein to the ERK cascade, which is known to govern cell growth and survival, the evidence supporting the role of YWK-II protein in mediating the effect of MIS on cell survival comes from experiments in which overexpression of YWK-II protein or interference with YWK-II protein function by its antibody affected viability of CHO cells or mouse sperm, respectively. This effect is further demonstrated to be due to altered apoptotic activities by changed levels of p53 and caspase-3, which are important markers for apoptosis. The presently observed anti-apoptotic or cytoprotective effect of MIS mediated by YWK-II protein is at variance with the studies of Segev et al. (Segev et al., 2000; Segev et al., 2002; Segev et al., 2001), demonstrating that MIS induced apoptosis by

activating an NF-κB-mediated pathway through the MIS type II receptor. These contrasting findings suggest that YWK-II protein may be a novel receptor for MIS, occurring in cells lacking the MIS type II receptor, including CHO cells and sperm. The present findings of YWK-II protein being a novel receptor for MIS in cell survival, together with the fact that YWK-II protein differs from both MIS type II and type I receptors in distribution and structure, suggest that MIS may have multiple actions depending upon the nature of the receptors present in different cells.

The physiological role of YWK-II protein in mediating the effect of MIS on cell survival is further demonstrated in the present study by interfering with YWK-II protein function in vivo by injection of its antibody into the seminiferous tubule of the mouse testis. This manipulation results in reduction in sperm count and altered levels of p53 and caspase-3 in the antibody-treated testes, in particular, the injection of YWK-II antibody results in p53 and caspase-3 activation and accumulation in the testicular nucleus, consistent with a role of YWK-II protein in mediating the effect of MIS in sperm survival. Although MIS has been reported to bind to the sperm head (Fallat et al., 1998), be able to facilitate sperm motility and sustain their viability in vitro (Tian et al., 2001), the underlying mechanism remains unclear since MIS type II receptor is not found in sperm. The presently demonstrated YWK-II protein-mediated signal transduction pathway induced by MIS leading to ERK activation may be the basis for its ability to sustain viability and longevity of sperm, with YWK-II protein as the MIS receptor in the sperm for their survival.

In conclusion, the present study has demonstrated that YWK-II protein acts as a G_o-protein-coupled receptor for mediating the effect of MIS on ERK1/2 activation and cell survival. The present findings may have broad implications in a range of physiological and pathophysiological processes including neurodegenerative diseases, such as Alzheimer's disease, since YWK-II protein is known to have a wide tissue distribution including the brain.

Materials and Methods

Raising YWK-II polyclonal antibody

The extracellular region (226 amino acids) of the YWK-II protein, contiguous to the transmembrane region, was amplified by PCR and cloned into pET30a. The recombinant protein was isolated and purified according to the method previously described (Tian et al., 2001). A rabbit polyclonal antibody specific for YWK-II protein, tested by western blot, was raised according to a standard protocol.

Cell culture and transfection

Chinese hamster ovarian (CHO) cells were maintained and cultured in F12 medium (Gibco) supplemented with 10% fetal bovine serum (HyClone). The cells were seeded on glass coverslips or culture plates and transfected using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). Each sample of transfected cells was treated according to the protocol for the respective assay.

Plasmids construction

Two oligonucleotides were synthesized, namely, the 5′-end of the mouse IgGκ signal peptide gene sequence: 5′-GTCGACACCATGGAGACAGACACACTCCT-GCTATGGGTACTGCTGCTC-3′ and the complementary sequence of the 3′-end: 5′-GAATTCGTCACCAGTGGAACCTGGAACCCAGAGCAGCAGTACC-CA-3′. These oligonucleotides contained overlapping segments constructed in vitro. The primers used to amplify the partial sequence of the YWK-II gene were: 5′-GAATTCATGGTTAAAGCTTTAGAG-3′ and 5′-GGATCCCGAATCTGCATC-TGCTCCAG-3′. These two fragments were cloned into the vector pEGFP-N1 (Clontech). The partial sequence of YWK-II gene was situated distal to the mouse IgGκ gene sequence. The recombinant vector was designated pEGFP-N1-YWK-II, and the encoded recombinant protein designated EGFP-YWK-II protein.

The C terminus interference plasmids of G_α were constructed as described by Gilchrist et al. (Gilchrist et al., 1999). In brief, the cDNAs encoding the terminal 11 amino acids of human G_α subunits corresponding to $G_{\alpha i1/2}$, $G_{\alpha o1}$ and $G_{\alpha o2}$ were synthesized. The 5′- and 3′-ends contained the cleavage sites for *Bam*HI and *Xho*I. The three cDNAs were annealed in 1× PCR buffer and cloned into pcDNA3.1(+) (Invitrogen), designated pcDNA3.1(+)-$G_{\alpha i1/2}$, pcDNA3.1(+)-$G_{\alpha o1}$ and pcDNA3.1(+)-$G_{\alpha o2}$, respectively.

Total RNA was extracted from CHO cells using the Trizol Reagent Isolation kit (Gibco). The first strand of cDNA was synthesized following the protocol of the Superscript first-strand synthesis system for RT-PCR (Invitrogen). The following primers used to amplify the cDNA were segments of β-adrenergic receptor kinase 1 (βARK1): 5′-GAATTCGCCGCCACCATGGGAATCAAGTTACTGGAC-3′ and 5′-GGATCCTCAGAGGCCGTTGGCACT-3′. They were cloned into pCMV and designated pCMV-βARK1-C.

Construction of stable cell lines expressing EGFP and EGFP-YWK-II protein

CHO cells were transfected with pEGFP-N1 and pEGFP-N1-YWK-II by incubating for 48 hours. Geneticin (G418, 500 μg/ml; Gibco) was added to the culture. Antibiotic-resistant clones emitting green fluorescence were picked after about 2~3 weeks in culture and expanded into several cell lines. Stable transfected cells were identified by fluorescent staining and by western blot using rabbit YWK-II antiserum and rabbit anti-GFP antibody (Clontech). These two stable cell lines were named EGFP-overexpressing CHO cells and EGFP-YWK-II-overexpressing CHO cells, respectively.

Immunofluorescence localization of EGFP-YWK-II protein

CHO cells transiently transfected with pEGFP-N1-YWK-II for 24~48 hours were washed three times with PBS and fixed by treatment with 4% paraformaldehyde in PBS for 10 minutes. The cells were blocked with 3% bovine serum albumin (BSA) at 37°C for 30 minutes. After each subsequent step, the samples were washed with PBS three times. The cells were incubated with the primary antibody (rabbit YWK-II antiserum at 1:100 dilution) at 37°C for 30 minutes. As controls, preimmune rabbit serum was substituted as the primary antibody at 1:100 dilution. Cells were incubated with the secondary antibody (TRITC-conjugated goat anti-rabbit IgG at 1:100 dilution; Santa Cruz) at 37°C for 30 minutes. After washing with PBS, the samples were washed twice with deionized water. Coverslips were mounted upside-down on the slides with 90% glycerol/DABCO containing 2% triethylenediamine (Sigma) and examined under a Leica TCS NT laser confocal microscope. All of these procedures were performed at ambient temperature.

Thiazolyl Blue tetrazolium bromide (MTT) assay

Intact CHO cells, EGFP-transfected and EGFP-YWK-II-overexpressing CHO cells, were seeded onto 96-well plates at 5×10^3 cells/well. The cells were serum-starved for 24 hours. Aliquots of recombinant active MIS (306-376; rMIS) were added to the medium at the following concentrations: 0, 0.035, 0.35, 3.5 and 35 nM. On the third day, 20 μl MTT (5 mg/ml; Sigma) were added to 200 μl of culture medium and the cells were incubated at 37°C for 4 hours. The medium was discarded and 100 μl DMSO was added to lyse the cells. Absorbance of the solution was measured at OD_{570}. The experiments were repeated three times.

Detection of the signal transduction pathway

The cells were initially serum-starved by incubation in F12 medium containing 1 mg/ml bovine serum albumin (BSA) for at least 16 hours and treated subsequently with rMIS (Tian et al., 2001). CHO, EGFP-overexpressing or EGFP-YWK-II-overexpressing cells, were incubated with rMIS at different concentrations at 37°C for 5 minutes. Pertussis toxin (PTX; 1 μg/ml; Calbiochem) and the nontoxic protein kinase C (PKC) inhibitor bisindolylmaleimide GF109203X (3.5 μM; Calbiochem) were added to the EGFP-YWK-II-overexpressing CHO cells for 24 hours and 2 hours, respectively, followed by 0.35 nM rMIS and incubated at 37°C for 5 minutes. pcDNA3.1(+)-$G_{\alpha i1/2}$, pcDNA3.1(+)-$G_{\alpha o1}$, pcDNA3.1(+)-$G_{\alpha o2}$, pCMV-βARK1-C, pCMV-RasN17 (Clontech) and pCMV-RasV12 (Clontech) were transfected into EGFP-YWK-II-overexpressing CHO cells by incubating for 24 hours and the cells were then treated with 0.35 nM rMIS for 5 minutes.

At the end of treatment, cells were washed with cold PBS. Whole-cell protein extracts were prepared by suspending the cells in a lysis buffer containing 1% NP-40, 150 mM NaCl, 10 mM Tris-HCl (pH 8.0), 10 mM NaF, 1 mM Na_2VO_3, 2 mM EDTA (pH 8.0), 1 mM DTT, 1 mM PMSF, 1 μg/ml aprotinin and 1 μg/ml leupeptin and placed on ice. The phosphorylated ERK1/2 (p-ERK1/2) and total ERK1/2 were detected by western blot. The phosphorylated ERK1/2 was immunostained with mouse anti-phospho-p44/42 ERK (Thr 202/Tyr 204) antibody (Santa Cruz) as the primary antibody, followed by HRP-conjugated anti-mouse antibody (1:5000; Santa Cruz), and the signals detected using chemiluminescence (ECL, Amersham Biosciences, UK). Subsequently, the PVDF membrane was stripped off using the strip buffer (147 mM NaCl and 10 mM Tris-HCl, pH 2.3) and probed with rabbit anti-ERK1/2 antibodies (Santa Cruz) to determine the total amount of ERK1/2 in the cell samples as control. Other primary antibodies used included p53 (Oncogen), caspase-3 (Cell Signaling Technology) and cleaved caspase-3 (Cell Signaling

Technology). The data presented were values obtained from a representative run of at least three independent experiments.

Analysis of sperm viability

To investigate possible effects upon sperm viability, the cauda epididymis was placed in 1 ml of prewarmed (37°C) Biggers-Whitten-Wittingham (BWW) medium (100 mM NaCl, 2.8 mM KCl, 0.5 mM NaH_2PO_4, 2.0 mM $CaCl_2$, 0.5 mM $MgCl_2$, 25 mM $NaHCO_3$, 10 mM HEPES, 5.0 mM glucose, 1.0 mM sodium pyruvate, 20 mM sodium lactate, 4 mg/ml BSA). The tissue was partially macerated with a sterile scalpel and incubated at 37°C for 3 minutes to allow the sperm to swim free of the surrounding tissue. After having been washed with sperm washing medium (Irving Scientific, Santa Anna, CA), the sperm were exposed to different treatments and further incubated for 10 or 30 minutes in sperm washing medium before being analyzed for viability. One hundred microliters of sperm were mixed with 0.04% Trypan Blue and live and dead cells were counted.

In vivo immunodepletion by seminiferous tubule injection

BALB/c mice of 6- to 8-week old were anesthetized by ketamine. Testes were lifted from the abdomen cavity and the efferent duct isolated. YWK-II antibody (40 μg/ml) or rabbit IgG (Santa Cruz) in 0.04% Trypan Blue was injected into the seminiferous tubule via the efferent duct. The testes were placed back in the abdominal cavity and the wound was sealed with surgical instruments. After 48 hours, animals were sacrificed and sperm were recovered from the cauda epididymis for counting, and testis nuclear proteins were extracted for western blot. An HTM-IVOS system (version 10.8, Hamilton-Thorn Research, Beverley, MA) was used to count total sperm recovered from cauda epididymis. Testis nuclear proteins were extracted as described previously (Kashiwabara et al., 2000).

Statistics

For two groups of data, two-tail *t*-tests were used. For three or more groups, data were analyzed by one-way ANOVA and Dunnett's post-hoc test. A probability $P<0.05$ was considered to be statistically significant.

This work was supported by grants from the National Important Research Plan (2006CB0F1002) and National Basic Research Program of China (973 Program-2006CB504001-2006CB504002), the Ministry of Science and Technology of China, the Creative Research Group (No. 30421003), the National Natural Science Foundation of China, the research fund for the Doctoral Program of High Education (20040023020), the Strategic Investment and Li Ka Shing Institute of Health Sciences of The Chinese University of Hong Kong. H.C.C. received a Croucher Senior Research Fellowship. The experimental study was performed in compliance with the laws of the People's Republic of China.

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