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Novel mechanism of reducing tumourigenesis: Upregulation of the DNA repair enzyme OGG1 by rapamycin-mediated AMPK activation and mTOR inhibition

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ABSTRACT

Inhibition of mTOR by rapamycin is an important approach in cancer therapy. In early clinical trials, tuberous sclerosis complex (TSC)-related kidney tumours were found to regress following rapamycin treatment. Since loss of function of the DNA repair OGG1 enzyme has a major role in multistep carcinogenesis of the kidney and other organs, we investigated the effect of rapamycin on OGG1 regulation. Treatment of HK2 cells, mouse *Tsc*-deficient cells and human *VHL*-deficient cells (786-O) with rapamycin resulted in decrease in p70S6K phosphorylation at Thr^{389}, and increase in the expression of NF-YA and OGG1 proteins. In addition, rapamycin increased OGG1 promoter activity in cells transfected with OGG1 promoter construct. Furthermore, rapamycin increased the phosphorylation at Thr^{172} of the energy sensor AMPK. Downregulation of AMPK phosphorylation by high glucose (HG) increases the phosphorylation of p70S6K and decreases the protein expression of NF-YA and OGG1. Pretreatment of the cells with rapamycin before exposure to HG reversed the effects of HG. However, downregulation of AMPK by dominant negative (DN)-AMPK in $Tsc2^{+/-}$ cells abolished AMPK and decreased OGG1 expression. In contrast, transfection of $Tsc2^{+/-}$ cells with DN-S6K abolished p70S6K phosphorylation and increased OGG1 expression, a response enhanced by rapamycin. Treatment of $Tsc2^{+/-}$ mice with rapamycin resulted in activation of AMPK, downregulation of phospho-p70S6K and enhanced OGG1 expression. Our data show that inhibition of mTOR can activate AMPK and lead to upregulation of DNA repair enzyme OGG1. These data comprise the first report to provide one mechanism whereby rapamycin might prevent or inhibit the formation and progression of certain cancers.

1. Introduction

A deficiency in the DNA repair enzyme 8-oxoG-DNA glycosylase (OGG1) has important functional consequences, particularly the ability of cells to repair DNA.[1,2] *OGG1* deficiency in yeast, as well as formamidopyrimidine-DNA glycosylase (FPG) deficiency in bacteria, results in spontaneous mutator phenotypes.[3] Increasing impairment in DNA repair contributes to the genomic instability, and consequently increases the risk of cancer.[4] The steady-state level of the DNA damage

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product, 8-oxodG, which reflects the balance between its continuous generation and removal, is significantly higher in *Ogg1*$^{-/-}$ mice compared to wild-type animals.[5] Furthermore, the development of spontaneous adenoma and carcinoma is five times higher in *Ogg1* knockout mice compared to wild-type mice at the age of 1.5 years, and is associated with the accumulation of 8-oxodG in genomic DNA.[6–8] The *OGG1* gene is highly polymorphic among humans and is somatically mutated in some cancer cells.[9] Loss of heterozygosity of the *OGG1* allele was found in 85% of 99 human kidney clear cell carcinomas, identifying the loss of *OGG1* function as a potentially important event in multistep carcinogenesis in the kidney.[10] Nuclear factor-YA (NF-YA) has been identified as a transcription factor that binds and regulates the *OGG1* promoter.[2] Decreased NF-YA expression is associated with decreased OGG1 protein levels in renal angiomyolipomas of patients with tuberous sclerosis complex (TSC) disease.[11] In addition, both NF-YA and OGG1 protein expressions are lost in renal cell carcinomas of Eker rats, which carry a mutation in *Tsc2* indicating that NF-YA is an important regulator of OGG1 expression and function.[12]

The mammalian target of rapamycin (mTOR), a serine/threonine kinase belonging to the phosphatidylinositol kinase-related kinase family, plays a central role in regulating cell growth, proliferation and survival, in part by the regulation of translation initiation.[13–15] In addition, mTOR signalling pathways are constitutively activated in many types of human cancer.[13,16] Mutations of the tuberous sclerosis complex that lies downstream of Akt in the mTOR signalling pathway also occur in human cancers and contribute to mTOR activation.[17] Recently, rapamycin has emerged as an important therapeutic agent for the treatment of some cancers.[13,14] The potential of mTOR inhibitors in the treatment of various types of cancer has been actively studied both pre-clinically and clinically. In the United States, several phase II and phase III trials are in different clinics to test the effects of mTOR inhibitor on various cancers, including renal cell carcinoma; prostate, breast, pancreatic and small cell lung cancers; recurrent brain tumours; recurrent mantle-cell lymphoma and melanoma.[14,18–20]

A recent animal study has shown that rapamycin inhibits mTOR activity, induces apoptosis of epithelial cells and reverses Akt-dependent prostate intraepithelial neoplasia.[19] In the Eker rat, short-term inhibition of mTOR by rapamycin was associated with a significant tumour response, including induction of apoptosis and reduction in cell proliferation.[20] Treatment of Eker rats (*Tsc2*$^{+/-}$) with rapamycin elicited a significant biochemical and histological tumour response in keeping with the hypothesis that mTOR is a relevant target for therapeutic intervention in TSC patients.[20] The tumour response to rapamycin treatment was accompanied by downregulation of ribosomal S6 kinase activity, reduction in cell size and induction of apoptosis.[21] The intrinsic sensitivity of mTOR inhibition to rapamycin varies among different cancer cell lines by several orders of magnitude ranging from 1 to 5000 nmol/L (IC_{50}), indicating that some cancer cell lines may be resistant to mTOR inhibition.[22]

Activation of mTOR by phosphorylation of S6K is associated with a decrease in OGG1 and results in accumulation of oxidised DNA in kidney tumour tissue from TSC patients.[11] While rapamycin effectively downregulated the mTOR activity and decreased kidney tumour size, the mechanism by which rapamycin can inhibit the formation and progression of tumour is not fully understood. In the present study, we investigated the potential role of rapamycin in the regulation of the DNA repair enzyme OGG1 in different types of normal and cancer cells, and in *Tsc2*$^{+/-}$ mice to understand the mechanism by which rapamycin inhibits tumour progression.

2. Materials and methods

2.1. Cell culture

Tsc2$^{-/-}$, *Tsc2*$^{+/-}$ and *Tsc2*$^{+/+}$ mouse embryonic fibroblast (MEF) cells were generously provided by Dr. D.J. Kwiatkowski (Harvard Medical School, MA). The cells were grown in DMEM supplemented with 10% foetal bovine serum (FBS). Human kidney proximal tubular epithelial cells (HK2) and 786-O human renal carcinoma cells were obtained from American Type Culture Collection (Manassas, VA) and maintained in DMEM with 10% FBS. All cell lines were grown at 37 °C in a humidified atmosphere of 5% CO_2.

2.2. Isolation and culture of proximal tubular cells

Renal primary proximal tubular cells were isolated from *Tsc2*$^{+/-}$ mice and cultured as previously described.[23]

2.3. Downregulation of AMPK and S6K

Tuberin-deficient cells (*Tsc2*$^{+/-}$) grown to 60–70% confluency in complete medium in six-well plates were transfected with a recombinant plasmid expressing DN-AMPK or DN-S6K. The plasmid containing AMPK carrying K45R mutation of the α_1-subunit (pCAGGS) was kindly provided by Dr. N. Fujii, Joslin Clinic, Boston, MA. While the plasmid expressing DN-S6K was kindly provided by Dr. Jianping Ye Pennington, Biomedical Research Center, Baton Rouge, LA. Cells were employed for transient transfection using lipofectamine and Lipo-plus reagent (Invitrogen). Twenty-four hours after transfection, cells were treated with either AICAR (2 mM) or rapamycin (20 nM) for 24 h. Forty-eight hours after transfection, cells were harvested for Western blot analysis.

2.4. AICAR and rapamycin treatment

HK2, 786-O and *Tsc2*$^{-/-}$, *Tsc2*$^{+/-}$ and *Tsc2*$^{+/+}$ cells were grown to 80–90% confluency in 60 mm Petri dish. Cells were then treated with different concentrations of rapamycin (0, 20, 40, 60 and 100 nM) for 24 h. *Tsc2*$^{-/-}$, *Tsc2*$^{+/-}$ and 786-O cells were treated with different concentrations of 5-aminoimidazole-4-carboxamide (AICA)-riboside (AICAR) (0, 2, 4, 6, 8 and 10 mM) for 24 h. The cells were lysed in a lysis buffer as described previously.[25] Cell lyastes were used for Western blot analysis.

2.5. Immunostaining of OGG1

Wild-type *Tsc2*$^{+/+}$ cells were grown in two-well chamber slides and treated with 100 nM rapamycin for 24 h. A double

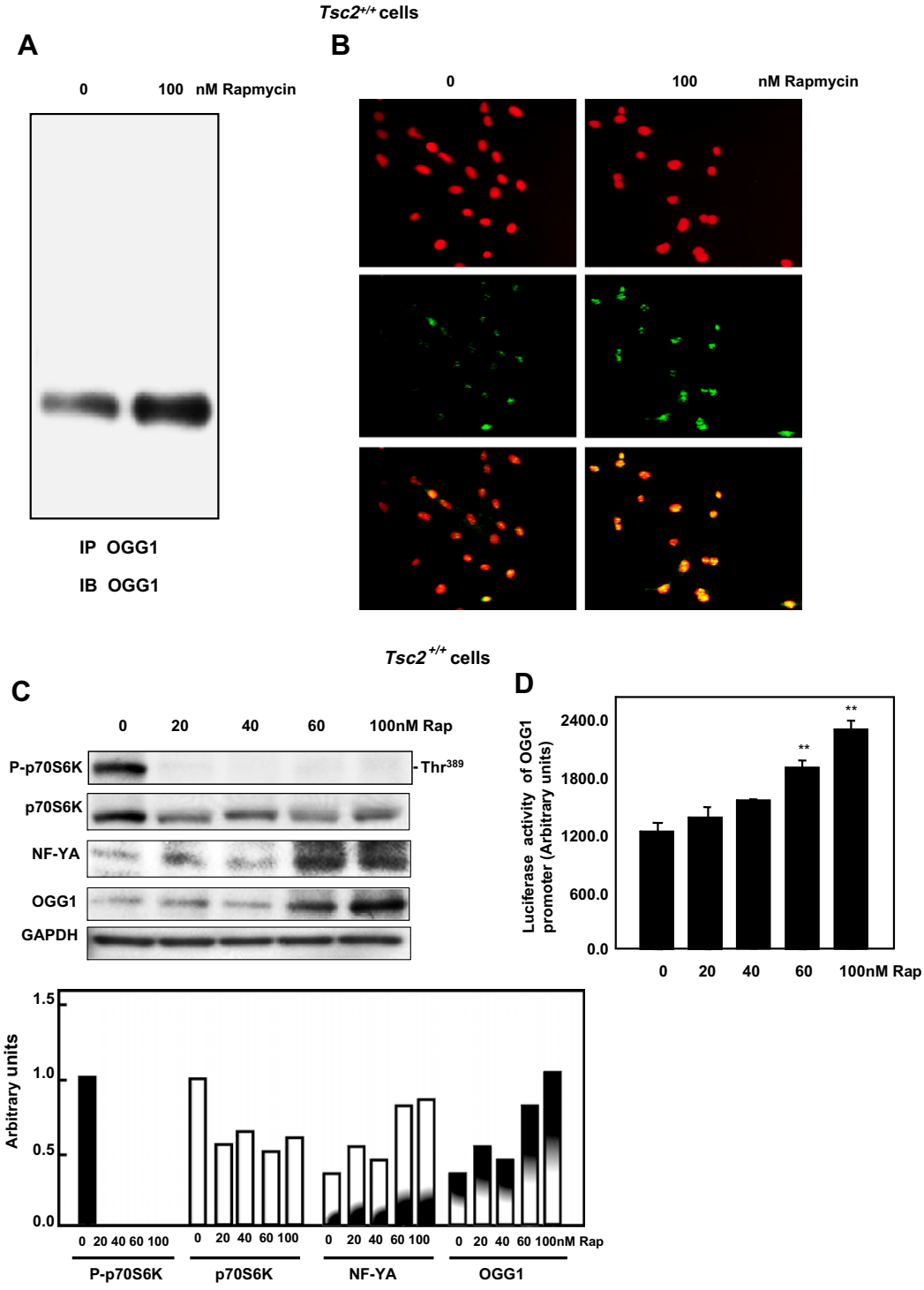

Fig. 1 – Rapamycin inhibits mTOR activity to increase the protein expression and promoter activity of OGG1 in normal and cancer cells. (A–D) MEF wild-type *Tsc* cells treated with 100 nM of rapamycin show (A) increases in OGG1 protein expression by immunoprecipitation and immunoblotting (B) increases in nuclear staining of OGG1 as detected by FITC-conjugated secondary antibody. Western blots show inhibition of p70S6K phosphorylation at Thr^{389} and increased NF-YA and OGG1 protein expression in (C) *Tsc2*$^{+/+}$, (E) *Tsc2*$^{+/-}$ and (G) *Tsc2*$^{-/-}$ MEF cells, (I) HK2 cells and (K) 786-O cells treated with different concentrations of rapamycin (0–100 nM). GAPDH was used as a loading control. Histograms in the bottom panels represent the average of protein expression of two independent experiments normalised to GAPDH. A reporter plasmid containing the *OGG1* promoter driving expression of the luciferase reporter gene was transfected into all cells using LipofectAMINE and Plus Reagent™. Twenty-four hours after transfection, cells were treated with different concentrations of rapamycin (0–100 nM) for 24 h then collected and washed to prepare cell lysates. Luciferase activity was determined and normalised to protein amount. Rapamycin enhances the promoter activity of OGG1 to different extents in (D) *Tsc2*$^{+/+}$, (F) *Tsc2*$^{+/-}$ and (H) *Tsc2*$^{-/-}$ MEF cells, (J) HK2 cells and (L) 786-O cells. Data represent mean ± SE of three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$.

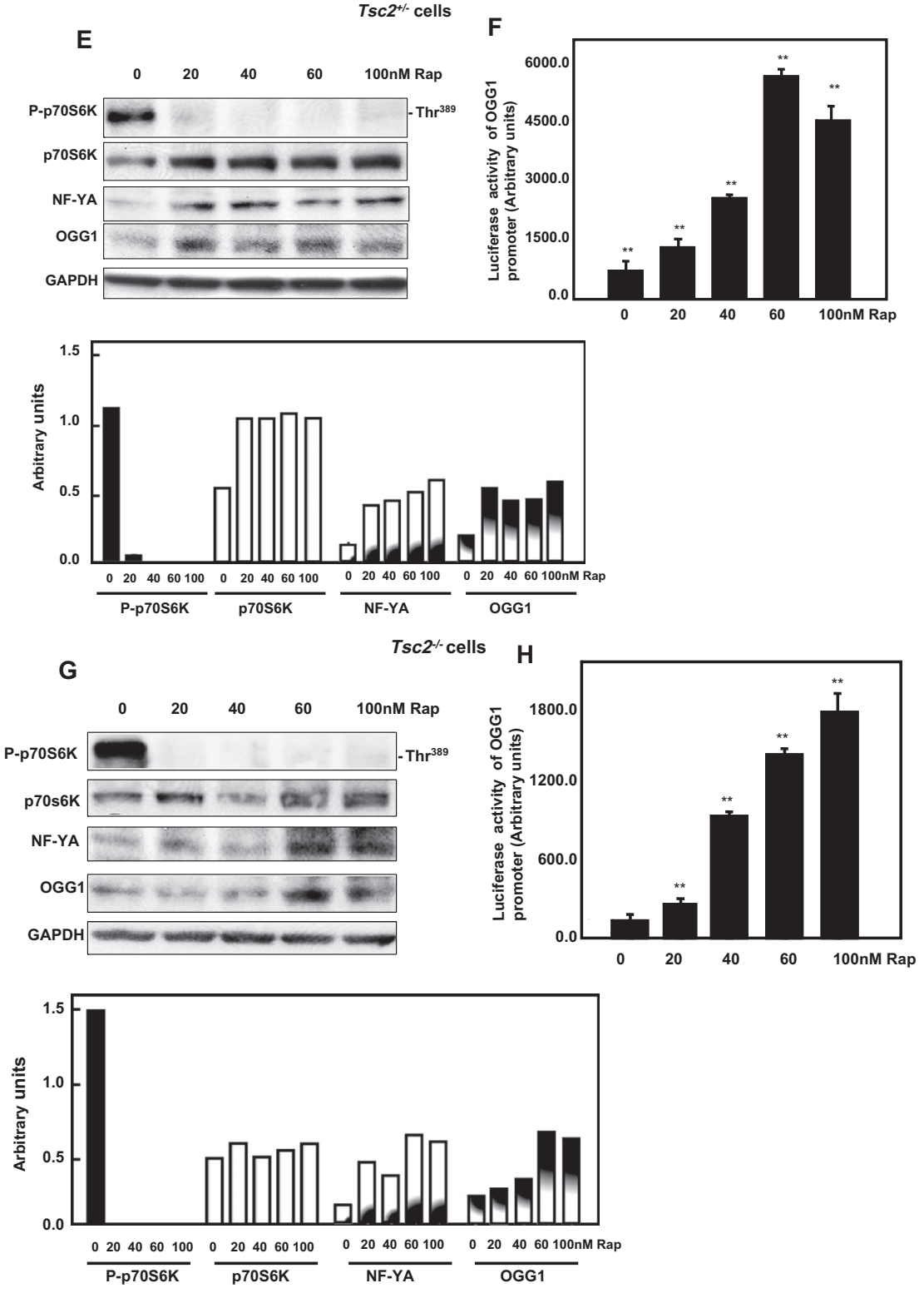

Fig. 1 *(continued)*

fluorescent labelling method for OGG1 and nucleus was used as previously described[26] with minor modifications. The cells were incubated with non-immune donkey IgG to block non-specific binding, then incubated with rabbit anti-OGG1 primary antibodies (dil 1:100 v/v) followed by FITC-conjugated donkey anti-rabbit IgG (Chemicon International, Inc., Temecula, CA, USA) as secondary antibody (dil 1:1000 v/v). All incubations of primary and secondary antibodies were for 30 min with three 5-min washes with phosphate-buffered saline (PBS) containing 0.1% (w/v) bovine serum albumin (BSA) between steps. Controls consisted of PBS/BSA in place of primary antibody followed by detection procedures as outlined

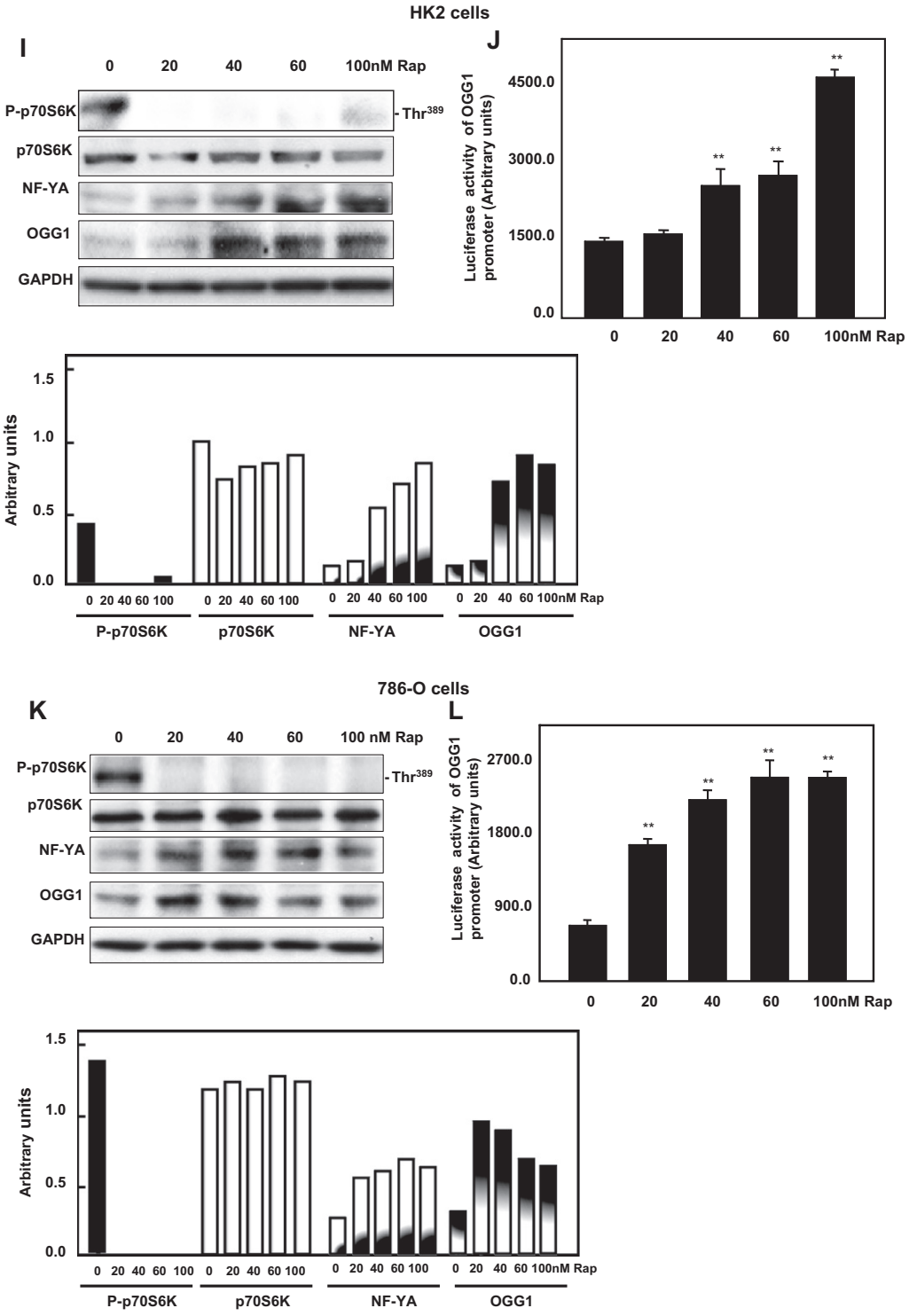

Fig. 1 *(continued)*

above. FITC green signals for OGG1 were detected using a filter with excitation at 488 nm. Propidium iodide (PI) red signals for nuclear DNA were detected using a filter with excitation at 535 nm. FITC and PI were detected using an Olympus FV-500 Laser Scanning Confocal microscopy. To demonstrate staining specificity, control cells were stained without primary antibody.

2.6. *Protein extraction and immunoblot analysis*

Protein concentration of the cell lysates was determined with the Bradford reagent[26] using bovine serum albumin as a standard. Western blot analysis was performed as previously described.[25] Rabbit polyclonal antibody raised against human OGG1 protein was generously provided by Dr. S. Mitra

(University of Texas Medical Branch at Galveston, Texas). NF-YA, tuberin, p-p70S6K and p70S6K antibodies were purchased from Santa Cruz Biotechnology. P-AMPK, AMPK, p-ACC and ACC antibodies were purchased from Cell Signaling Technology. Mouse ß-actin antibody was purchased from Oncogene Research Products. All primary antibodies were diluted to 1:1000 (v/v) and secondary antibodies to 1:5000 (v/v). Rapamycin (Sirolimus) and AICAR were purchased from Calbiochem. Expression of each protein was quantified by densitometry using NIH Image 1.62 software.

2.7. *Transcriptional activity of OGG1 promoter*

A luciferase reporter plasmid containing the OGG1 promoter (courtesy of Dr. P. Radicella)[27] was used to determine *OGG1* gene transcription activity. The cells were grown in six-well plates to 60–70% confluence, then washed with PBS and the media replaced with 800 μl of OPTI-MEM I (Invitrogen, CA). Pre-complex of the DNA with Plus Reagent™ in Opti-MEM was mixed and incubated at RT for 15 min. LipofectAMINE was added to the complex of DNA and Plus reagent™ and incubated for 15 min at room temperature. DNA and Plus reagent™-LipofectAMINE/plus reagent/DNA complexes were added to each well and incubated at 37 °C with 5% CO_2. After incubation for 3–4 h, 1 ml of fresh media with 20% (v/v) serum was added to a final concentration of 10% (v/v). Twenty-four hours after transfection, the cells were treated with different concentrations of rapamycin or AICAR, then incubated for 24 h. The cells were harvested for luciferase assay. Luciferase activity was determined using the Luciferase Reporter Assay System in a luminometer (Promega, WI) and normalised to protein concentration.

2.8. *Animals*

Two-month-old male TSC2-deficient ($TSC2^{+/-}$) mice were purchased from Jackson Laboratory. The animals were allowed food and water *ad libitum* prior to and during the experiments. The mice were divided into two groups of four mice each. Group 1 mice (controls) were injected with an equal amount of DMSO. Group 2 mice were injected i.p. with 2 mg/kg body weight rapamycin in DMSO 5 d/week for 4 weeks. Injections were carried out under isofluorane inhalation anaesthesia (Abbott, Abbott Park, IL). Animals were euthanised at 4 weeks and the kidneys were removed rapidly for dissection and biochemical analysis.

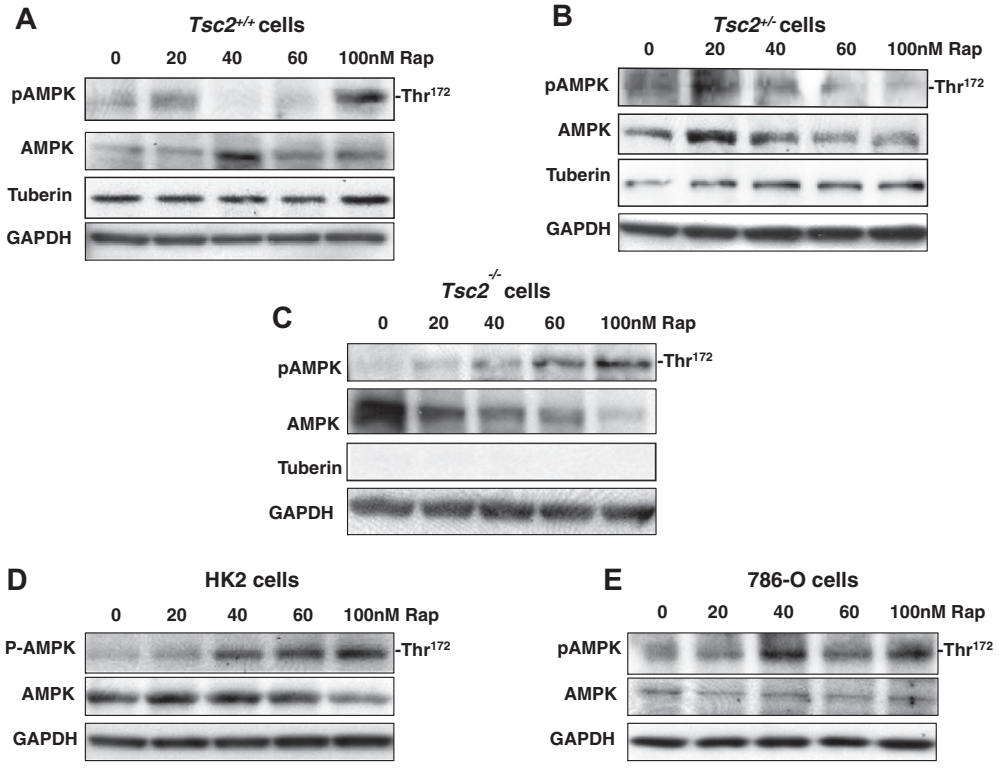

Fig. 2 – Rampamycin activates AMPK in normal and cancer cells. MEF ($Tsc2^{+/+}$, $Tsc2^{+/-}$ and $Tsc2^{-/-}$), HK2 and 786-O cells treated with different concentrations of rapamycin for 24 h. Western blot was performed using the cell lysates. The blots were incubated with antibodies against phospho-Thr^{172} of AMPK and total AMPK. Blots from MEF cells were incubated also with tuberin to confirm the cell type. Treatment with rapamycin increases the phosphorylation of AMPK in all cells. Phosphorylation levels of AMPK were different in wild-type versus cancer cells treated and higher concentrations of rapamycin show maximum AMPK phosphorylation in all normal and cancer cells. GAPDH was used as a loading control. Data representative of two independent experiments.

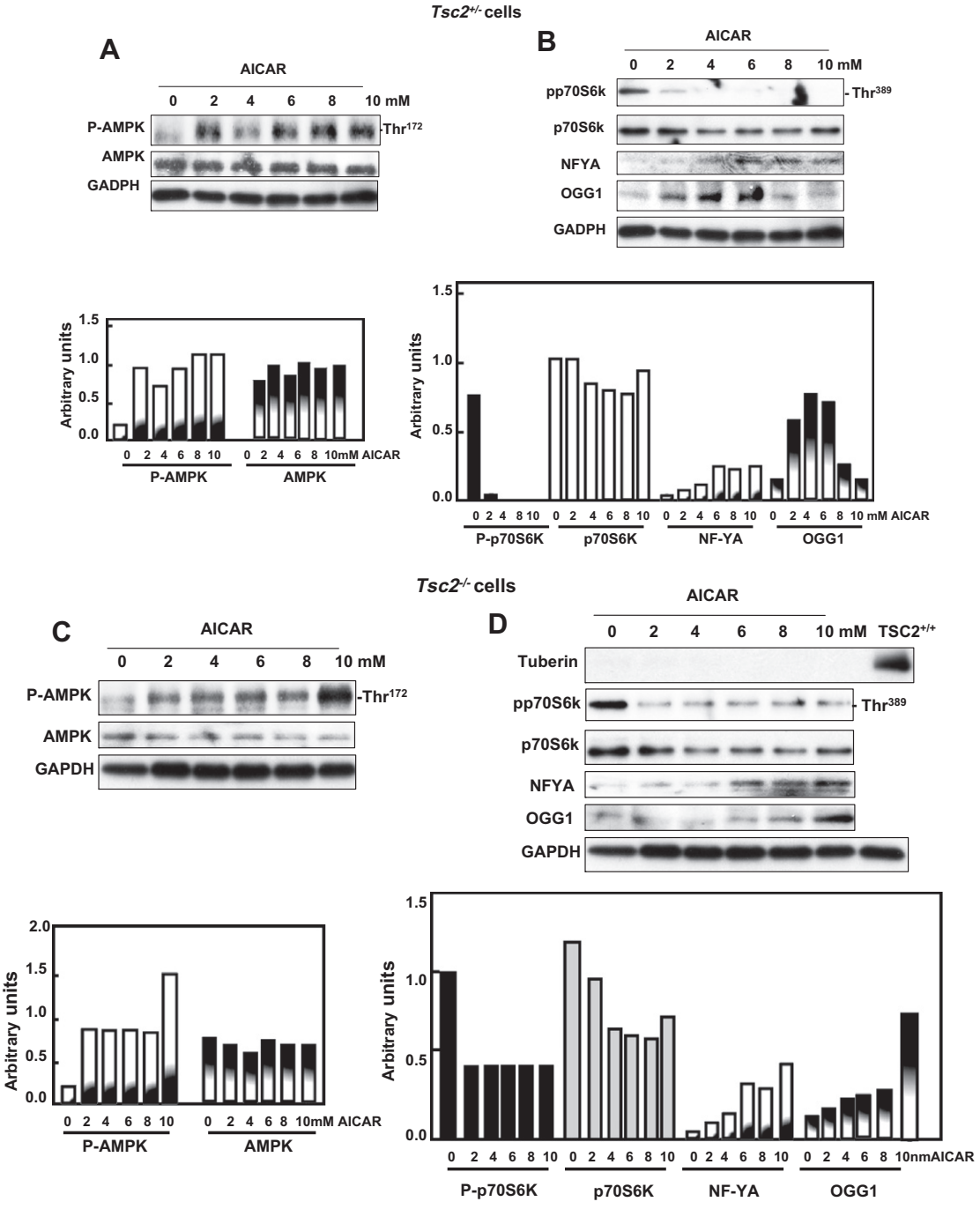

Fig. 3 – AICAR activates AMPK and inhibits mTOR to increase the promoter activity and protein expression of OGG1. *Tsc2*$^{+/-}$, *Tsc2*$^{-/-}$ and 786-O cells were treated with different concentrations of AICAR (0–10 mM) for 24 h. Western blot analysis was performed in cell lysates using p-AMPK, total AMPK, phospho-p70S6K, total p70S6K, NF-YA and OGG1 antibodies. Lower concentration (2 mM) of AICAR enhances AMPK phosphorylation at Thr^{172} and significantly decreases p70S6K phosphorylation at Thr^{389} in TSC2$^{+/-}$, TSC2$^{-/-}$ and 786-O cells. Increased AICAR concentration was associated with increase in AMPK phosphorylation and decreased p70S6K phosphorylation results in increase in NF-YA and OGG1 protein expression. While 2–6 mM of AICAR shows a higher expression of OGG1 in TSC2$^{+/-}$ cells, higher concentration of AICAR (10 mM) was required to reach the maximum expression of OGG1 in TSC2$^{-/-}$ cells. Also in VHL-deficient cells, higher concentration of AICAR (4–8 mM) showed the maximum increase in OGG1 protein expression. Histograms in the bottom panels represent the average of protein expression of two independent experiments normalised to GAPDH. (G) *Tsc2*$^{+/-}$, (H) *Tsc2*$^{-/-}$ and (I) 786-O cells treated with AICAR after infection with a reporter plasmid containing the *OGG1* promoter driving expression of the luciferase reporter gene show increase in OGG1 promoter activity and reach a maximum at a higher dose of AICAR. Note that maximum increase in OGG1 promoter activity in 786-O cells was significant at 2 mM and reach 6-fold by 4 mM of AICAR. Data represent mean ± SE of three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$.

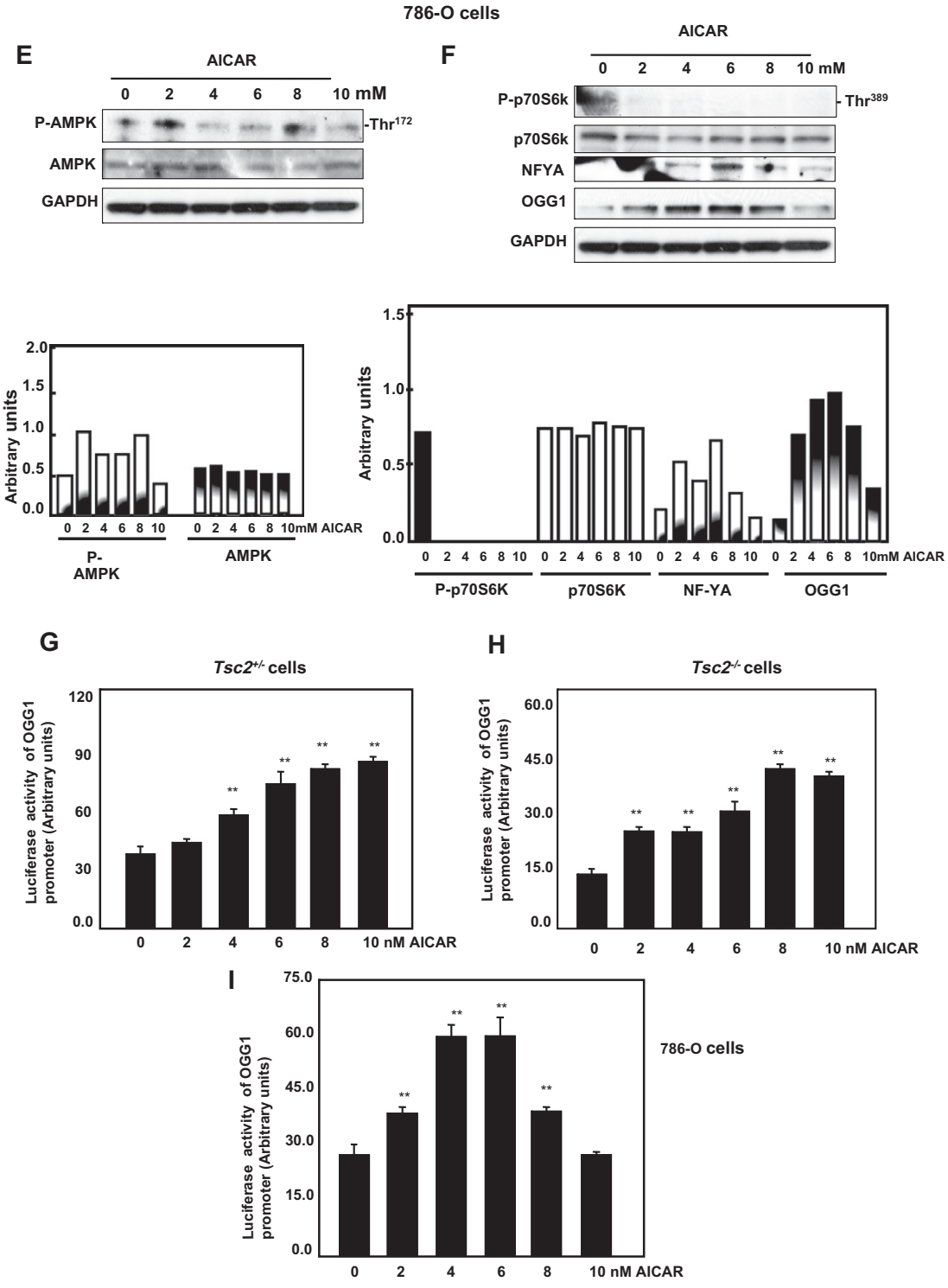

Fig. 3 *(continued)*

2.9. Statistics

Data are presented as mean ± standard error. Statistical differences were determined using ANOVA followed by Student Dunnett's (Exp. versus Control) test using one trial analysis. P-values less than 0.01 and 0.05 were considered statistically significant.

3. Results

3.1. Inhibition of mTOR increases OGG1 protein expression and enhances OGG1 promoter activity

We first explored the role of rapamycin in the regulation of DNA repair enzyme OGG1 in several normal and cancer cell

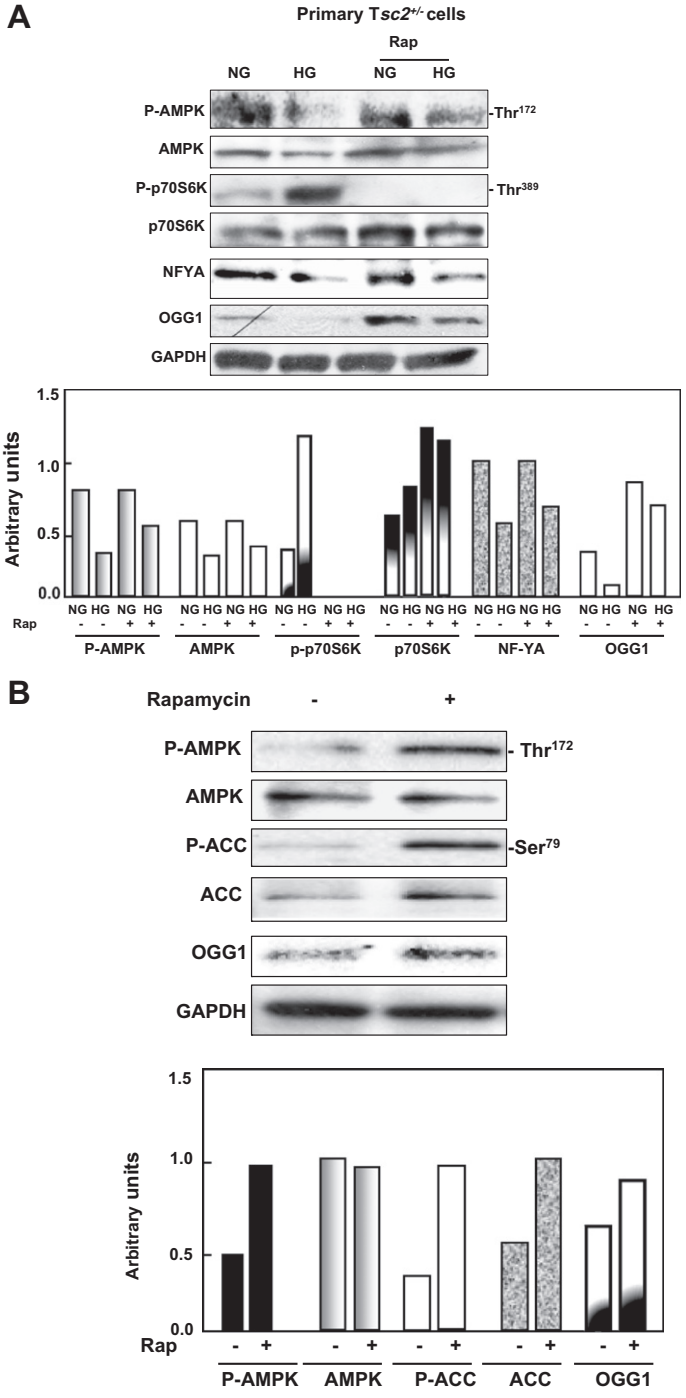

Fig. 4 – Rapamycin activates AMPK and increases OGG1 protein expression in primary proximal tubular *Tsc2*$^{+/-}$ mouse cells (A) Primary proximal tubular mouse cells treated with high glucose show significant decrease in AMPK phosphorylation and increase in p70S6K phosphorylation that are associated with decrease in NF-YA and OGG1 protein expression. Cells pretreated cells with rapamycin before exposed to HG show increase in p-AMPK, reduction in p70S6K phosphorylation and significant increase in NF-YA and OGG1 expression suggesting that rapamycin may activate AMPK to enhance the protein expression of OGG1. (B) Primary proximal tubular mouse cells treated with 20 nM of rapamycin for 24 h show a significant increase in AMPK phosphorylation at Thr^{172} and ACC phosphorylation at Ser^{79}. Histograms in the bottom panels represent the average of protein expression of two independent experiments normalised to GAPDH.

types. To investigate whether rapamycin can increase OGG1 protein expression, *Tsc2*$^{+/+}$ cells were treated with or without rapamycin and OGG1 was immunoprecipitated and immunoblotted with OGG1 antibody. A significant increase in OGG1 protein expression was observed in *Tsc2*$^{+/+}$ cells treated with rapamycin compared to non-treated cells (Fig. 1A). To test

whether rapamycin can increase nuclear OGG1, double staining of OGG1 and nucleus was performed in $Tsc2^{+/+}$ cells treated with rapamycin. Rapamycin increases nuclear staining of OGG1 suggesting that rapamycin enhances protein expression of OGG1 in the nucleus (Fig. 1B).

To determine the maximum concentration of rapamycin for mTOR inhibition and activation of OGG1 in normal and cancer cells, the cells were treated with different concentrations of rapamycin (0–100 nM) for 24 h. Inactivation of one of the major downstream signalling components of mTOR, phosphop70S6 kinase at Thr^{389}, was examined by Western blot analysis. Wild-type ($Tsc2^{+/+}$) and heterozygote ($Tsc2^{+/-}$) cells treated with low concentration of rapamycin (20 nM) showed complete inhibition of p70S6K phosphorylation and significant increase in the expression of NF-YA and OGG1 protein as well as OGG1 promoter activity (Fig. 1C–F). The maximum increase in OGG1 promoter activity was observed in cells treated with 60 and 100 mM rapamycin in both cell types (Fig. 1D and F). In homozygous $Tsc2^{-/-}$ cells, the relatively higher expression of p70S6K phosphorylation was also abolished by 20 nM of rapamycin (Fig. 1G), whereas the higher concentrations of rapamycin (60 and 100 nm) produced maximum increases in NF-YA and OGG1 protein expression as well as OGG1 promoter activity (Fig. 1G and H).

Since MEF cells are fibroblastic cells and have a different phenotype from renal cell carcinoma cells, which originate from proximal tubular cells, we next tested the effect of rapamycin on human proximal tubular HK2 cells. Treatment of HK2 cells with 20 nM rapamycin abolished p70S6K phosphorylation, while 40–100 nM rapamycin was required for an increased NF-YA and OGG1 protein expression (Fig. 1I and J). The maximum increase in OGG1 promoter activity was observed with 100 nM of rapamycin (Fig. 1J). These data suggest that HK2 and MEF cells have approximately the same response to rapamycin and require high concentrations to obtain the maximum increase in the protein expression and promoter activity of OGG1.

To determine the optimum concentration of rapamycin for the induction of OGG1 in cancer cells deficient in VHL, 786-O cells were treated with different concentrations of rapamycin

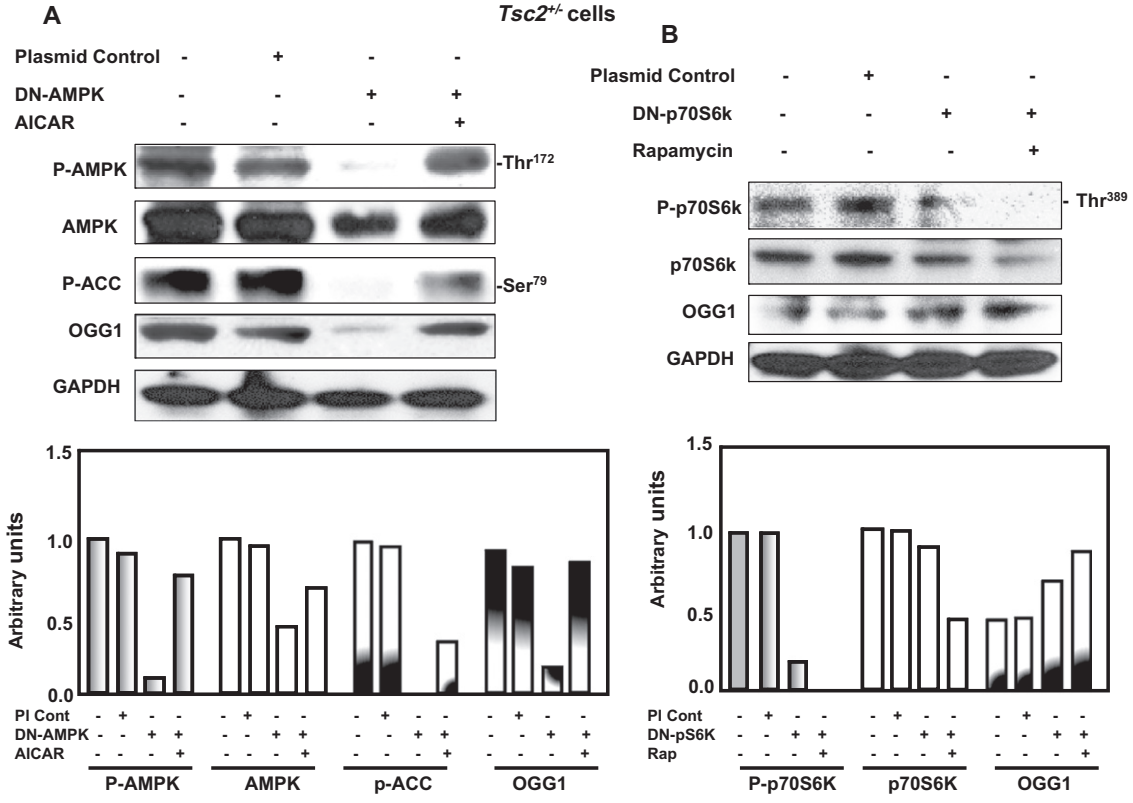

Fig. 5 – AMPK and S6K regulate the protein expression of OGG1. (A) $Tsc2^{+/-}$ cells were infected with a recombinant plasmid expressing DN-AMPK or control plasmid for 48 h. Cells grown in another plate were treated with 2 mM AICAR after transfection with DN-AMPK. Western blot was performed on cell lysates using P-AMPK, total AMPK, p-ACC, total ACC and OGG1antibodies. Cells transfected with DN-AMPK show complete abolition of ACC phosphorylation and significant decrease in AMPK phosphorylation and OGG1 protein expression, while treatment of the transfected cells with 2 mM of AICAR reversed these changes. (B) $Tsc2^{+/-}$ cells were transfected with a recombinant plasmid expressing DN-S6K or control plasmid for 48 h. Cells grown in another plate were treated with 20 nM rapamycin after the transfection with DN-S6K. Western blot was performed in cell lysates using P-p70S6K, total p70S6K and OGG1. Cells transfected with DN-S6K or empty plasmid and treated with rapamycin show complete abolition of p70S6K phosphorylation and significant increase in OGG1 protein expression. GAPDH was used as a loading control. Histograms in the bottom panels represent the average of protein expression of two independent experiments normalised to GAPDH.

for 24 h. Cells treated with 20 nm of rapamycin showed complete downregulation of p70S6K phosphorylation and a significant increase in NF-YA and OGG1 protein expression as well as in OGG1 promoter activity (Fig. 1K and L). VHL-deficient cells were more susceptible to lower doses of rapamycin in increasing the protein expression and promoter activity of OGG1 suggesting that cancer cells can respond very well in this context to rapamycin treatment.

AMPK has been recognised as an important upstream signalling intermediate intimately involved in the downregulation of the mTOR pathway.[28] To demonstrate whether rapamycin-induced OGG1 expression is dependent on blocking the mTOR pathway, or is mediated by upstream target of mTOR, we treated the normal and cancer cells with different concentrations (0–100 nM) of rapamycin and measured AMPK phosphorylation by Western blot using Thr^{172} phospho-AMPK-specific antibodies. Maximum phosphorylation of AMPK was observed with 100 nM of rapamycin in MEF wild-type ($Tsc2^{+/+}$) and HK2 cells (Fig. 2A and D), which required for the maximum increase in OGG1 promoter activity. Increase in phosphorylation of AMPK was also detected in $Tsc2^{+/-}$ and $Tsc2^{-/-}$ treated with rapamycin and reached a maximum with 20 and 100 nM, respectively (Fig. 2B and C). In cancer cells deficient in VHL, maximum increase in AMPK phosphorylation was observed with 40 nM of rapamycin, which correlates with 3-fold increase in the promoter activity of OGG1 (Fig. 2E). These data suggest that rapamycin functions as an activator of AMPK as well as an inhibitor of mTOR to increase the protein expression and promoter activity of OGG1.

To determine whether activation of AMPK by AICAR enhances the protein expression and promoter activity of OGG1, tuberin-deficient and VHL-deficient cells were treated with different concentrations of AICAR (0–10 mM) for 24 h. Tuberin-deficient cells ($Tsc2^{+/-}$ and $Tsc2^{-/-}$) showed a significant increase in AMPK phosphorylation at Thr^{172} in response AICAR treatment (Fig. 3A and C). The increase in AMPK activity was dose dependent and reached a maximum effect when the cells were treated with 10 mM AICAR (Fig. 3A and C). The increase in AMPK phosphorylation was associated with the abolition of p70S6K phosphorylation at low concentrations of AICAR (2 mM) (Fig. 3B and D). In addition, increased AMPK phosphorylation was associated with an increase in the protein expression of NF-YA and OGG1 (Fig. 3B and D) as well as the promoter activity of OGG1 in both cell lines (Fig. 3G and H). A significant increase in AMPK phosphorylation was also detected in VHL-deficient cells treated with 2 mM of AICAR (Fig. 3E). The increase in AMPK activation was also associated with an increased NF-YA and OGG1 protein expression (Fig. 3F). Moreover, cells treated with 2–6 mM AICAR showed a 3–6-fold increase in OGG1 promoter activity (Fig. 3I) suggesting that AMPK may play an important role in blocking mTOR and activating OGG1 promoter.

Renal primary proximal tubular cells are ideal cells in which to determine the effect of rapamycin on tumourigenesis, since these cells are transformed in the renal cell carcinoma. To test whether downregulation of AMPK phosphorylation can be restored by rapamycin, we used high glucose (HG) concentration as an agonist in primary proximal tubular cells isolated from kidney cortex of $Tsc2^{+/-}$ mice. Cells

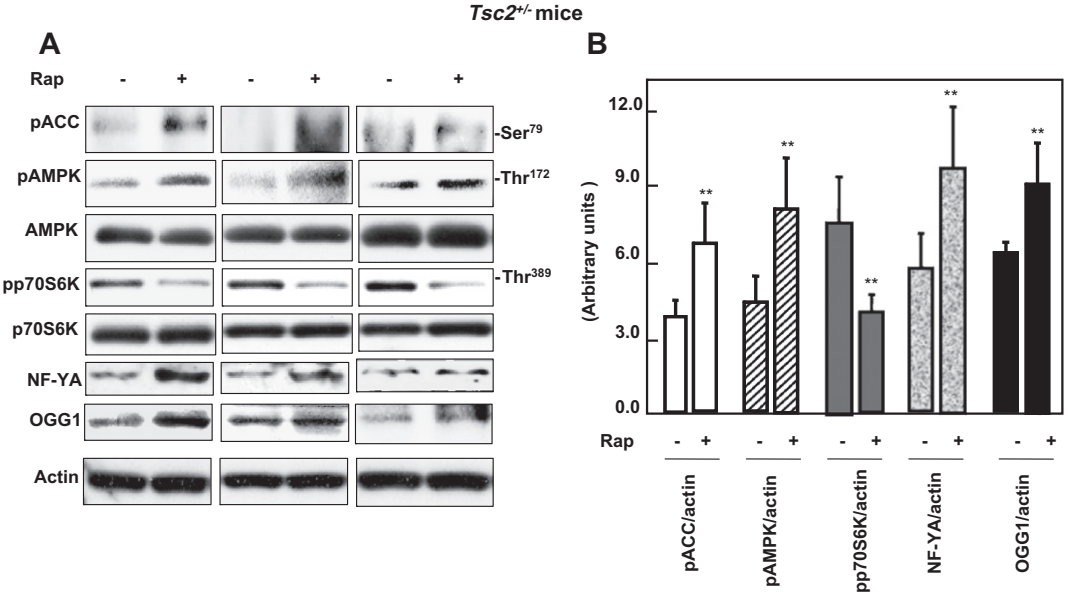

Fig. 6 – Rapamycin enhances AMPK activation and inhibits mTOR activity to increase OGG1 protein expression in kidney cortex of mice. (A) Western blot analysis was performed using kidney cortex homogenate of mice treated or non-treated with rapamycin. Rapamycin-treated mice show increase in ACC phosphorylation at Ser^{79} and phosphorylation of AMPK at Thr^{172} and decrease in phosphorylation of p70S6K at Thr^{389}. Activation of AMPK and inhibition of mTOR activity resulted in increased protein expression of NF-YA and OGG1 in rapamycin-treated mice. Actin was used as a loading control. Data representative of three individual mice. (B) Histograms represent means ± SE of three animals. Significant difference from control animals is indicated by $^{**}P < 0.01$.

treated with HG for 24 h showed a significant decrease in AMPK phosphorylation that was associated with a significant increase in p70S6K phosphorylation (Fig. 4A). Decrease in AMPK phosphorylation and increase in mTOR activity resulted in a significant decrease in NF-YA and abolished OGG1 protein expression (Fig. 4A). Cells treated with 20 nM rapamycin before being exposed to HG showed an increase in AMPK phosphorylation and a reduction in mTOR activity (Fig. 4A). Activation of AMPK and inhibition of mTOR by rapamycin resulted in increased protein expression of NF-YA and OGG1 (Fig. 4A). Note that rapamycin significantly increases AMPK phosphorylation and subsequently increases OGG1 expression in both cells grown in normal and high glucose, suggesting that rapamycin can function as an AMPK activator to increase OGG1 function under both low and high energy conditions.

AMPK acts as an intracellular energy sensor, stimulated by increased intracellular AMP/ATP ratios. AMPK is also activated by the adenosine analogue, AICAR, in many cells. AMPK has also emerged as an important mediator of glucose metabolism, particularly glucose uptake.[29] Activated AMPK phosphorylates and inactivates metabolic enzymes involved in reducing cellular ATP consumption during metabolic stress.[28,29] To confirm that AMPK activation by rapamycin results in typical AMPK-mediated downstream responses, we monitored ACC, a primary target of activated AMPK[28] in *Tsc2*$^{+/-}$ primary proximal tubular cells treated with 20 nM of rapamycin for 24 h. Using an antibody that recognises ACC phosphorylated at Ser^{79}, we found that rapamycin induced robust phosphorylation of ACC (Fig. 4B). The increase in AMPK and ACC phosphorylation was associated with increase in OGG1 expression indicating that rapamycin activates downstream targets of AMPK.

To confirm the role of AMPK and p70S6K in the regulation of OGG1, we transfected *Tsc2*$^{+/-}$ MEF cells with dominant negative (DN) AMPK or DN-p70S6K, and measured AMPK, ACC and p70S6K phosphorylation and OGG1 protein expression in response to rapamycin. In cells transfected with DN-AMPK, phosphorylation of ACC at Ser^{79} was abolished and resulted in a significant decrease in AMPK phosphorylation at Thr^{172} (Fig. 5A). Inhibition of AMPK by DN-AMPK significantly decreased the protein expression of OGG1, while treatment with AICAR reversed these changes strongly suggesting that AMPK is a major kinase involved in the regulation of OGG1 (Fig. 5A). In contrast, cells transfected with DN-p70S6K, p70S6K phosphorylation at Thr^{389} was significantly decreased and resulted in increase in OGG1 protein expression (Fig. 5B). In addition, treatment of the transfected cells with rapamycin resulted in increases in the protein expression of OGG1 confirming the role of p70S6K in regulating of OGG1 (Fig. 5B).

To confirm the results of the *in vitro* studies that rapamycin works as an AMPK activator to block mTOR and activate OGG1, *Tsc2*$^{+/-}$ mice were treated with rapamycin (2 mg/kg BW), or with vehicle (DMSO) for 4 weeks. Homogenates of kidney cortex were examined for ACC phosphorylation, AMPK phosphorylation, mTOR activity as well as for OGG1 protein expression. Kidney tissues from mice treated with rapamycin showed an increase in ACC phosphorylation compared to controls (Fig. 6A and B). Increased phosphorylation of ACC at Ser^{79} was accompanied by an increase in AMPK phosphorylation at Thr^{172} and inhibition of mTOR activity (measured by p70S6K phosphorylation). Furthermore, increased NF-YA and OGG1 protein expression was also seen in the kidney cortex of rapamycin-treated mice as a result of increase in AMPK phosphorylation and mTOR inactivation (Fig. 6A and B). Collectively, these data obtained *in vitro* and *in vivo* strongly suggest that rapamycin acts as an activator of AMPK (measured by ACC phosphorylation) and an inhibitor of mTOR (measured by p70S6K phosphorylation) to upregulate the DNA repair enzyme OGG1.

4. Discussion

This is the first evidence that the inhibition of mTOR by rapamycin enhances AMPK phosphorylation and leads to increased protein and promoter activity of the DNA repair enzyme OGG1. Our *in vitro* and *in vivo* data showed that rapamycin inhibits mTOR activity and increases the expression of OGG1. We also show that rapamycin regulates the protein expression and promoter activity of OGG1 through an increase in the protein expression of the transcription factor, NF-YA. Although lower concentrations of rapamycin appeared to inhibit mTOR activation in all cell types, higher concentrations were required to increase the protein and promoter activity of OGG1, suggesting that high concentrations of rapamycin may be required to regulate other signals in addition to blocking mTOR activity. In support of this hypothesis, we show that rapamycin increases phosphorylation of AMPK, an upstream signalling intermediate in the mTOR pathway in different cell types. Moreover, we show that rapamycin induces AMPK activity as evidenced by increasing ACC phosphorylation. Activation of AMPK by rapamycin results in the inhibition of mTOR activity and increases the protein expression and promoter activity of OGG1 in proximal tubular cells. In addition, downregulation of AMPK activity by high glucose activates mTOR and decreases NF-YA and OGG1 protein expression, indicating that AMPK is the upstream kinase of OGG1. Moreover, we show that activation of AMPK by AICAR resulted in the inhibition of mTOR and increases the promoter activity and protein expression of OGG1 in tuberin-deficient and VHL-deficient cells. Since AICAR increased the protein expression and the promoter activity of OGG1 in *Tsc2*-null cells, it opens the possibility that induction of OGG1 by AMPK is through a pathway independent of *Tsc2*. Thus AMPK may inhibit mTOR without activation of *Tsc2* and augment OGG1 expression, or, AMPK may induce OGG1 expression through unknown mTOR-independent mechanism. Using DN-AMPK we demonstrate that AMPK is a major upstream kinase involved in the upregulation of OGG1 expression. In addition, we found that downregulation of p70S6K by DN-p70S6K abolished p70S6K phosphorylation and resulted in increased OGG1 protein expression, confirming the role of mTOR in the regulation of OGG1. Finally, we show that treatment of *Tsc2*$^{+/-}$ mice with rapamycin resulted in increased phosphorylation of AMPK and ACC, NF-YA and OGG1 protein expression and decreased phosphorylation of p70S6K. Collectively, these data, obtained *in vitro* and *in vivo*, demonstrate that rapamycin is an activator of AMPK that subsequently inhibits mTOR activity and enhances OGG1 protein expression and promoter activity.

Tuberin encoded by TSC2 is an upstream target of mTOR. Loss of tuberin function leads to activation of mTOR pathway, which promotes activation of p70S6 kinase.[20] Deficiency of tuberin in null and heterozygous cells is associated with decrease in NF-YA expression and protein and promoter activities of OGG1.[24] However, decreased OGG1 and NF-YA expression was detected in tuberin-deficient tumour angiomyolipomas kidney of TSC patients and was associated with significant increase in mTOR activity.[11] In addition, loss of tuberin is associated with loss of OGG1 expression in kidney tumour tissue from Eker rat ($TSC2^{-/-}$) and resulted in increase of mTOR activity suggesting that loss of tuberin is biologically relevant in affecting OGG1.[30]

Targeting mTOR is emerging as an important approach in cancer therapeutics.[13] Early clinical trials show that TSC and VHL-related kidney tumours regress in response to treatment with the mTOR inhibitor, rapamycin.[21,31,32] Treatment of patients with the tuberous sclerosis complex or sporadic lymphangioleiomyomatosis with rapamycin resulted in reduction in angiomyolipoma volume by nearly 50%.[33] Although rapamycin can effectively inhibit mTOR activity and decrease kidney tumour size, the mechanisms involved are not fully understood. However, it is accepted that a deficiency in DNA repair can lead to accumulated multiple gene mutations and result in tumours development.[34] Many of these mutations can occur as a result of irreparable or incompletely repaired genomic DNA, due to deficiencies in DNA repair enzymes such as OGG1. Loss of OGG1 has major consequences in multistep carcinogenesis in the kidney.[10,35] Decreased OGG1 expression and activity leads to a mutator phenotype,[35,36] with faulty repair of oxidised DNA lesions, and an accumulation of the oxidised DNA product (8-oxodG).[11] Our data show that rapamycin increased protein expression and promoter activity of OGG1 in cancer cells. The increase in OGG1 promoter activity was associated with an increase in expression of the transcription factor NF-YA in cancer cells treated with rapamycin indicates that the mTOR plays an important role in transcription activity of OGG1, perhaps by augmentry NF-YA expression. Decreased NF-YA expression is associated with decreased OGG1 expression in renal angiomyolipomas of TSC patients.[11] In addition, loss of NF-YA and OGG1 protein expression was also found in the renal cell carcinomas of the Eker rat, suggesting that NF-YA is an important regulator of *OGG1*.[12]

AMPK is a well-known physiological cellular energy sensor that is activated by phosphorylation at Thr^{172} in response to changes in cellular ATP levels. Our data show that rapamycin promoted a significant increase in AMPK activity in cells treated with HG further supporting the importance of rapamycin as an activator of AMPK. Using a phospho-ACC-specific antibody, we found that rapamycin treatment led to a robust phosphorylation and activation of AMPK and ACC to a degree similar to that observed with AICAR stimulation. Our data also confirmed that AMPK is an upstream kinase in the regulation of OGG1 since the cells over expressing DN-AMPK showed a significant inhibition of ACC phosphorylation and of AMPK activity, and a significant decrease in NF-YA and OGG1 protein expression. Taken together, these results indicate that rapamycin acts as an activator of AMPK and an inhibitor of mTOR pathway to upregulate OGG1.

As a sensor of nutrients, AMPK has been recognised as an important upstream signalling intermediate intimately involved in the regulation of the mTOR pathway.[29] We found that rapamycin activates AMPK and leads to increased NF-YA and OGG1 protein expression in $Tsc2^{+/-}$ primary proximal tubular cells. In addition, the activation of AMPK by AICAR results in decreased p70S6K phosphorylation and increased NF-YA and OGG1 protein expression in MEF cells. In support of this model, rapamycin inhibits Thr^{389} phosphorylation of p70S6K and increases Thr^{172} phosphorylation of AMPK in $Tsc2^{+/-}$ primary proximal tubular cells treated with high glucose and results in increased NF-YA and OGG1 protein expression. Further, interference of p70S6K by DN-p70S6K abolished p70S6K phosphorylation at Thr^{389} and resulted in increased OGG1 protein expression, confirming the role of mTOR pathway in the regulation of OGG1. On the other hand, inhibition of AMPK activity by HG was restored by rapamycin treatment, indicating that rapamycin works as an activator of AMPK and an inhibitor of mTOR to upregulate of OGG1. Interference of AMPK by DN-AMPK abolished phophorylation of ACC at Ser^{79} and AMPK at Thr^{172} and resulted in decreased OGG1

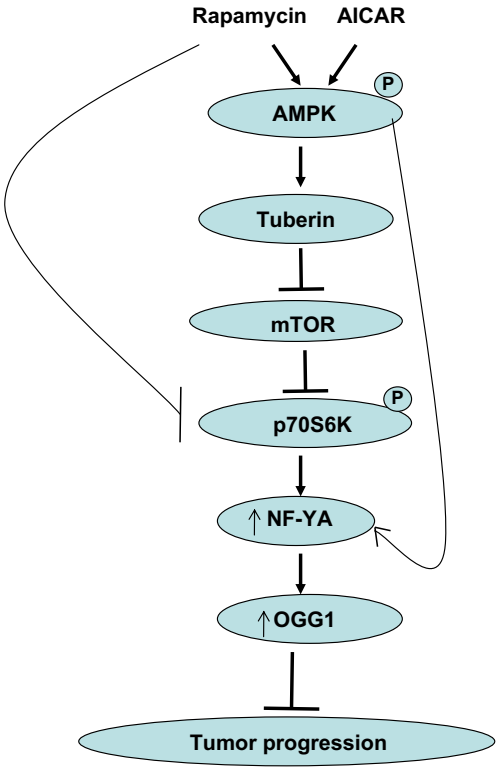

Fig. 7 – Proposed model for the role of rapamycin in activation of AMPK and enhances OGG1 function. Rapamycin treatment results in phosphorylation of AMPK. Activation of AMPK by AICAR and rapamycin may directly increase the NF-YA protein expression to increase the promoter activity and protein expression of OGG1. In addition, blocking mTOR activity by rapamycin and AICAR may increase NF-YA expression to increase the promoter activity of OGG1 that may inhibit the formation and progression of certain tumors.

protein expression, confirming that AMPK is a major upstream kinase of OGG1.

The response of spontaneously occurring renal tumours of the Eker rat to short-term rapamycin treatment supports the use of this agent in the clinical setting.[20] mTOR serves a critical role in the regulation of the translational machinery and, in doing so, affects cellular responses to growth, proliferation and differentiation, all of which are abnormally manifested in TSC lesions. In support of these data obtained in the rat, rapamycin treatment also inhibited mTOR activity in the cortical kidney tissue of mice. Our new observation in cultured cells was confirmed *in vivo* where we found that rapamycin activated ACC, the downstream target of AMPK and increased the AMPK phosphorylation at Thr^{172} resulting in increased NF-YA and OGG1 protein expression. Collectively these data indicate that rapamycin works to enhance the upstream inhibitor signal of mTOR such as AMPK to upregulate OGG1.

In summary, these data describe a novel role for rapamycin in the regulation of AMPK that subsequently enhances OGG1 in cancer cells. We show for the first time that rapamycin activates AMPK that indirectly leads to inhibition of mTOR and increases the promoter activity and protein expression of DNA repair enzyme OGG. Inhibition of AMPK by DN-AMPK or by HG resulted in the downregulation of OGG1, while activation of AMPK pharmacologically by rapamycin and AICAR resulted in increased promoter activity and protein expression of OGG1. In addition, the inhibition of mTOR activity by DN-p70S6K or pharmacologically by rapamycin resulted in increased promoter activity and protein expression of OGG1 (Fig. 7). Collectively these data provide one mechanism for enhancing DNA repair in cancer cells treated with rapamycin. This mechanism may explain the role of rapamycin in inhibiting the tumour progression.

Conflict of interest statement

None declared.

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