INTRODUCTION

The mammalian target of rapamycin (mTOR) is a highly conserved serine/threonine kinase. It acts as a key regulator of protein synthesis and cell growth and is activated by positive signals from growth factors and nutrients (Wullschleger et al., 2006). mTOR exists as two distinct complexes, mTORC1 and mTORC2. mTORC1 forms a large multi-protein complex with regulatory-associated protein kinase (raptor), proline-rich Akt substrate (PRAS40), mLST8 and mTOR and is inhibited by rapamycin (Sabatini, 2006; Hara et al., 2002). mTORC1 regulates protein synthesis and cell growth by phosphorylating ribosomal S6 kinase (p70S6K) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1). Phosphorylation of p70S6K facilitates the recruitment and translation of a specific mRNA subset that contains a 5’ polypurine tract (Dufner and Thomas, 1999). Phosphorylation of 4E-BP1 releases eIF4E, allowing initiation of translation (Gingras et al., 2004). Some human diseases, including cancer and metabolic syndromes, are very closely related to aberrantly activated mTORC1 signaling (Dann et al., 2007; Guertin and Sabatini, 2007; Inoki et al., 2005).

AMP-activated protein kinase (AMPK) is a master metabolic regulating enzyme activated upon phosphorylation at threonine 172 by upstream kinases such as LKB1 under nutrient depletion or metabolic stress (Birnbaum, 2005; Hawley et al., 2005). Activation of AMPK negatively regulates mTOR activity, leading to repressed protein synthesis and cell growth (Mandal et al., 2005; Bokko et al., 2007). Gwinn et al. (2008) reported that AMPK directly inhibits mTORC1 activity by phosphorylation of raptor at Ser792 (Bissler et al., 2008). The mechanism of prostate cancer promotion by monocyte chemoattractant protein-1 (CCL2) is negative regulation of AMPK and enhanced phosphorylation of raptor at Ser792 resulting in hyperactivation of mTORC1 and cell proliferation (Roca et al., 2009).

Orotic acid (OA) is an intermediate of pyrimidine synthesis that is readily synthesized in mammals. Major sources of exogenous exposure of human to OA are via cow’s milk and dietary supplements (Hurlbert and Potter, 1954; Richards et al., 1997). Administration of large amounts of OA induces fatty liver and promotes carcinogenesis. Although somewhat inconclusive, report-
ed mechanisms of OA-induced fatty liver include impairment of fatty acid oxidation, stimulation of lipogenesis, and reduction in lipid transport from the liver (Miyazawa et al., 1982; Hebbachi et al., 1997). Studies using numerous animal models have shown that OA exerts tumour-promoting activity in several organs, including the liver (Rao et al., 1986; Kokkinakis and Albores-Saavedra, 1994; Laconi et al., 1993; Laurier et al., 1984; Lin and Tung, 1965). Furthermore, Colombano et al. (1982) reported that OA functions as a growth stimulator in carcinogen-altered hepatocytes. However, the molecular mechanisms by which OA induces cell proliferation and tumor promotion are poorly understood. We investigated the effect of OA on the proliferation of hepatocellular carcinoma cells and the role of the mTORC1 signaling pathway. We demonstrated that OA induces cell proliferation and inhibits starvation-induced apoptosis via negative regulation of AMPK and activation of mTORC1.

MATERIALS AND METHODS

Materials, cell culture and transient transfection
Anti-p70S6K, anti-phospho-p70S6K (Thr389), anti-GAPDH, anti-phospho-raptor (Ser792), anti-raptor, anti-AMPKa, anti-phospho-AMPKa (Thr172) were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). OA monohydrate and aminoimidazole carboxamide ribonucleotide (AICAR) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Rapamycin was prepared from Selleck Chemicals LLC (Houston, TX, USA). SK-Hep1 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; Gibco-BRL, Rockville, MD, USA), supplemented with 10% fetal bovine serum, 3.7 mg/ml sodium bicarbonate, 100 units/ml penicillin and streptomycin. Cells were incubated in a 37°C incubator in an atmosphere of 5% CO₂ in air. The expression constructs constitutively active AMPK (CA-AMPK) was provide by Dr. S.G. Kim (Seoul National University, Korea) originally provided by Dr. J. Ha (Kyung Hee University, Korea). The expression plasmids of pEGFP-LKB1 were obtained from Dr. J. Ha (Kyung Hee University, Korea). Cells were transfected with appropriate plasmids constructs, CA-AMPK and pEGFP-LKB1, by using the LipofectAMINE 2000 (Invitrogen, Carlsbad, CA, USA) as described previously (Jung et al., 2011).

Cell proliferation assay
Cell proliferation rate was determined with the CellTiter96 aqueous nonradioactive cell proliferation kit (Promega, Southampton, UK), which is based on the MTS (a tetrazolium compound) colorimetric dye reduction that allows the determination of the number of viable cells. The MTS assay was carried out according to the manufacturer’s instructions.

Western blotting analysis
Whole cell lysates were prepared and examined by western blotting as established procedures. Bands were visualized using a chemiluminescence detection system (ChemidocTM XRS system, Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturer’s instructions. Protein contents were determined using the method for DC protein assay (Bio-Rad lab, Inc.) using BSA as a standard.

Quantification of apoptosis
Apoptosis was quantified by Annexin V-FITC/PI assay (Santa Cruz Biotechnology, Santa Cruz, CA, USA) following manufacturer’s instructions. Fluorescence was measured with FACS Calibur™ and analyzed by CellQuest software (BD Biosciences, San Jose, CA, USA).

Cell cycle analysis
Cells were trypsinized and washed with 1 × PBS and kept in 70% ethanol for fixation. Fixed cells were washed with PBS and incubated with propidium iodide (PI) solution for 15 min. Stained cells were analyzed by FACS Calibur, and the number of the cells in each stage was calculated with the ModFit LT ™ cell cycle analysis program according to the manufacturer’s instructions.

Statistical analysis
All the values are presented as the means ± S.E. of at least three independent experiments. Significant differences were obtained using a one-way ANOVA with turkey’s test (Fig. 1A, 2B and 3B). A difference was considered significant at p < 0.05.

RESULTS
OA increases cell proliferation and decreases starvation-induced apoptosis in SK-Hep1 hepatoma cells
OA is a tumor promoter for experimental liver carcinogenesis initiated by several DNA reactive carcinogens (Laurier et al., 1984; Rao et al., 1983). We investigated whether OA induces proliferation of SK-Hep1 human hepatocellular carcinoma cells. The cells were cultured under serum starvation in a 96-well plate, and the viable cells were determined by MTS assay in 24-hr increments up to 72 hr in control and OA-treated cells. As shown in Fig. 1A, OA increased the proliferation of SK-Hep1 cells.
Fig. 1. OA increases cell proliferation and decreases starvation-induced apoptosis in SK-Hep1 hepatoma cells. (A) Serum-starved SK-Hep1 cells were treated with or without OA for 24-48 hr at the indicated doses, and proliferation rates were monitored by MTS assay. Each bar represents the mean ± S.E. of three independent experiments. *p < 0.05 and **p < 0.01 compared with corresponding control; a, p < 0.05; b, p < 0.01 and c, p < 0.001 compared with corresponding 0 hr control, one-way ANOVA with turkey’s test. (B) For the apoptosis assay, SK-Hep1 cells were stimulated with OA in serum-deprived or standard media for 48 hr and stained with Annexin V-FITC followed by FACS analysis. Representative histograms showing Annexin V-positive cells are shown. The mean ± S.E. of the results from three independent experiment are summarized in the upper part of each diagram.
in a dose- and time-dependent manner. Treatment of the cells with 100 μM OA for 24-72 hr increased the cell proliferation by 10-40%. We then analyzed the effect of OA on apoptosis induced by serum deprivation. Starvation for 48 hr increased the number of Annexin V-positive cells by more than fourfold. However, OA (100 μM, 48 hr) decreased the number of starvation-induced apoptotic cells by 35% (13.65 ± 0.45 vs. 8.60 ± 0.31) (Fig. 1B). Together, OA exerts proliferative effects on SK-Hep1 hepatoma cells, manifested by the increase in viable cells and decrease in apoptotic cells.

**OA induces cell proliferation via mTORC1 activation**

We examined whether mTORC1 activation is required for OA effects on cell proliferation in SK-Hep1 cells. Activation of mTORC1 was monitored by the phosphorylation of p70S6K at Thr389. OA increased the phosphorylation of p70S6K compared to a serum-deprived control in a dose-dependent manner, indicating the activation of mTORC1 (Fig. 2A). mTOR is activated by two separate pathways, i.e., Akt activation by growth factors and AMPK inhibition by nutritional stress. AMPK is a key regulator of energy metabolism in cells. Recently, raptor was reported to be a direct substrate of AMPK, and the phosphorylation of raptor at Ser722 or Ser792 by AMPK was shown to be required for suppression of mTORC1 activity by energy stress (Gwinn et al., 2008). Immunoblot analysis showed that treatment of OA inhibited the phosphorylation of AMPK at Thr172, an essential phosphorylation site for its activation. The dose-dependent inhibition of AMPK phosphorylation and increase in p70S6K phosphorylation by OA correlates very well. Consistent with the inhibition of AMPK phosphorylation, cells treated with OA showed a significant decrease in raptor phosphorylation at Ser792, again indicating the activation of mTORC1 (Fig. 2A).

Rapamycin completely reversed the effects of OA on mTORC1 activation and cell proliferation. Rapamycin upregulated the phospho-raptor at Ser792 and down-regulated the phospho-p70S6K at Thr389 in OA-treated cells (Fig. 2A). Moreover, OA-induced cell proliferation was significantly inhibited by rapamycin (Fig. 2B). These results suggest that OA increases SK-Hep1 cell proliferation mediated by mTORC1 activation.

**OA-induced cell proliferation is attenuated by AMPK activation**

Next, the role of AMPK regulation in OA-induced mTORC1 activation and cell survival was investigated. HeLa cells express undetectable levels of endogenous LKB1 and show attenuated responses to AMPK signals (Corradetti et al., 2004). To determine whether LKB1 could mediate the OA effects on AMPK phosphorylation, we compared the results of LKB1-deficient cells to those of LKB1-expressing cells in their response to OA using LKB1-deficient as well as LKB1 reconstituted HeLa cells. Compared to the wild type, HeLa cells overexpressed with LKB1 showed evident AMPK-mTOR signals (Fig. 3A). However, OA treatment to these cells reversed the effects of LKB1, as observed by decreased raptor phosphorylation and increased p70S6K1 phosphorylation. These results are in line with our data, wherein we observed proteasomal degradation of LKB1 and subsequent attenuation of AMPK phosphorylation by OA in LKB1-overexpressed HeLa cells (Jung et al., 2011).

To investigate the role of AMPK in OA-induced mTORC1 activation, we transfected SK-Hep1 cells with constitutively active AMPK and monitored p70S6K and raptor phosphorylation. As shown in Fig. 3A (right panel), neither the inhibition of raptor phosphorylation at Ser792 nor the increase in p70S6K phosphorylation at Thr389 by OA was observed when CA-AMPK was transfected in the cells. To test the effects of AMPK activation on OA-induced cell survival, we used AICAR as an AMPK activator at a concentration of 500 μM, and cell viability was measured at 24 and 48 hr in control and OA-stimulated cells. Fig. 3B clearly shows that the basal level of cell viability was reduced substantially by AICAR treatment. Moreover, OA-induced cell proliferation was also suppressed by AICAR. These results indicate that OA increases SK-Hep1 cell survival by AMPK-dependant mTORC1 activation.

**OA interrupts the G1 arrest via mTORC1 activation**

G0/G1 phase cell-cycle arrest is induced under serum starvation conditions in many cell lines (Cooper, 2003; Yoshida and Beppu, 1988). SK-Hep1 cells showed the same properties in terms of starvation-induced G0/G1 arrest. Cells were cultured under serum deprivation and the cell cycle was determined by cytomteric analysis at the indicated time increment up to 48 hr. The percentage of cells in G0/G1 phase increased twofold, while that in S phase decreased by 70%. To determine whether cell-cycle perturbation is accompanied by OA-induced cell proliferation, cell-cycle distribution was measured in the cells cultured under the serum-deprived conditions in the absence or presence of OA. OA treatment induced the cell cycle across S and G2/M phase and decreased the G0/G1 phase compared to controls. In line with data shown in Fig. 2B, OA-induced proportions of S-phase
cells decreased significantly following co-treatment with rapamycin (Fig. 4).

**DISCUSSION**

OA, an intermediate in pyrimidine metabolism, is a tumor promoter for liver and other organs (Kokkinakis and Albores-Saavedra, 1994; Laconi et al., 1993; Laurier et al., 1984; Lin and Tung, 1965), at least in part, through stimulating the growth of cells initiated by carcinogens (Columbano et al., 1982). While many studies have demonstrated the properties of OA as a tumor promoter, few have provided evidence and understanding of the molecular mechanisms of OA-induced cell proliferation. Recently we reported that OA induces fatty liver mediated by mTOR activation (Jung et al., 2011). Based on these results, we hypothesized that OA-induced mTOR activation could contribute to the cell proliferation. This study is the first to demonstrate that OA increases the proliferation and decreases the starvation-induced apoptosis...
of SK-Hep1 hepatocellular carcinoma cells via mTORC1 activation mediated by negative regulation of AMPK.

mTOR is a effector of cell growth and proliferation in response to the growth factors and nutrient status. The major downstream targets of mTOR include components of the translation machinery that are responsible for ribosome recruitment to mRNA such as 4E-BP1 and p70S6K (Hay and Sonenberg, 2004). Moreover, mTOR inhibitor rapamycin inhibits proliferation and induces apoptosis, although the latter is not observed in general. mTOR and some of the targets of the mTOR kinase are activated in cancer (Easton and Houghton, 2006). Therefore selective inhibitor of mTOR kinase improved overall survival among patients with metastatic renal-cell carcinoma and a poor prognosis (Hudes et al., 2007). Grzelkowska demonstrated that OA stimulates proliferation of L1210 leukemic cells and diminishes the necrotic effect of TGFβ-1 (Grzelkowska et al., 1995). A notable finding of their study is the induction of ornithine decarboxylase by OA. Ornithine decarboxylase is the first and the rate-limiting enzyme in polyamine biosynthesis. It is induced during carcinogenesis by a variety of oncogenic stimuli and is known to be expressed by the activation of mTORC1 (Manzella et al., 1991). Although their data did not address the relationship with AMPK or mTOR, we can suggest it as a potential mechanism of OA action on TGFβ-1 based on our current observations.

Recently, we reported that OA-induced hepatic steato-
sis is associated with the AMPK/mTOR/SREBP pathway (Jung et al., 2011). These findings prompted us to study the role of mTOR in OA-induced cell proliferation. Here, we demonstrate that OA induces cell proliferation and survival via the AMPK-mTOR pathway. OA functions as a negative regulator of AMPK, which was evaluated by the decreased phosphorylation of its major regulatory site Thr172 in SK-Hep1 cells. We verified the requirement of mTORC1 activation in OA-induced cell proliferation by performing two independent experiments. Down-regulation of raptor phosphorylation and upregulation of p70S6K phosphorylation by OA was completely rescued by co-treatment with rapamycin. Moreover, rapamycin significantly inhibited OA-induced proliferation of SK-Hep1 cells. Rapamycin binds to mTOR adjacent to its kinase domain as a complex with the immunophilin FKBP12 and inhibits mTOR activity by blocking the assembly of mTORC1, but not mTORC2 (Rosner et al., 2009). mTORC1-mediated phosphorylation of S6K and 4EBP1 has been established as contributing to cell growth and cell-cycle progression. Rapamycin-resistant S6K mutants rescue these rapamycin-induced decreases in cell size (Fingar et al., 2002), whereas overexpression of an mTOR-insensitive 4EBP1 or interfering RNA-mediated reduction of p70S6K expression inhibits G1 phase progression (Fingar et al., 2004). Although not completely defined, the mechanisms underlying mTOR-mediated proliferation inhibition may involve reduced protein synthesis (Hay and Sonenberg, 2004).

OA induced S-phase progression with increased G2/M and decreased G1 populations in starvation-induced cell-cycle arrest, which was completely inhibited by rapamycin. The ability of rapamycin to block the cell cycle at G1/S phase is ascribed to the control of p27 expression by mTORC1. In rapamycin-resistant cell lines, which had intact p70S6K regulatory responses but exhibited constitutively low p27 levels even after mitogen deprivation, rapamycin no longer increased the p27 levels. Moreover, p27 null fibroblasts and T lymphocytes derived from p27−/− mice exhibited significant resistance to growth inhibition by rapamycin (Luo et al., 1996).

Pichiri-Coni et al. (1990) reported that OA inhibited proliferation of primary hepatocytes cultured in the presence of FBS and growth factors as monitored by labeling.

**Fig. 4.** OA interrupts the G1 arrest via mTORC1 activation. SK-Hep1 cells were incubated with 100 μM OA in the absence or presence of 50 nM rapamycin for 36 hr, and cell cycle analysis was performed using FACS cytometry. The percentage of cells in each phase was calculated using the ModfitLT™ analysis program.
index, mitotic index and total DNA content. According to our results, however, OA induced SK-Hep1 cell proliferation when the cells were cultured under serum deprivation. Eukaryotic cell cycle is arrested in the G1 phase when the environmental conditions make cell division impossible or when the cell needs time for DNA repair in the presence of DNA damage. Therefore, cell cycle progression by OA in the nutrient poor condition can be regarded as a mechanism of tumor promoting effect. Similar results were reported by Columbano et al. (1982) that OA is a growth stimulus for carcinogen altered hepatocytes in rats.

In summary, our study provides critical information required for an understanding of the molecular mechanisms of OA-induced cell proliferation and survival. OA induces cell proliferation and inhibits starvation-induced apoptosis through negative regulation of AMPK, and activation of mTORC1 as evidenced by decreased phosphorylation of raptor at Ser792 and increased phosphorylation of p70S6K at Thr389. The central role of mTORC1 in OA functioning in cell-cycle progression and proliferation was corroborated by data demonstrating that rapamycin significantly rescued all of these processes.

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Proliferating effect of orotic acid through mTORC1 activation


